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THE EFFECTS OF AIR CELL INJECTION OF PERFLUOROOCTANE SULFONATE PRIOR TO INCUBATION ON THE DEVELOPMENT OF THE WHITE LEGHORN CHICKEN (*GALLUS DOMESTICUS*) EMBRYO

presented by

Elizabeth Denisse Molina

has been accepted towards fulfillment
of the requirements for the

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Major Professor's Signature

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Date

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By

Elizabeth Denisse Molina

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ABSTRACT

THE EFFECTS OF AIR CELL INJECTION OF PERFLUOROOCTANE SULFONATE PRIOR TO INCUBATION ON THE DEVELOPMENT OF THE WHITE LEGHORN CHICKEN (*GALLUS DOMESTICUS*) EMBRYO

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Fifty White Leghorn chicken (*Gallus domesticus*) eggs per group were injected with 0.1, 1.0, 10.0 or 20.0 µg perfluorooctane sulfonate (PFOS)/g egg prior to incubation to investigate the effects of PFOS on the developing embryo. At hatch, chicks were weighed and examined for gross developmental abnormalities and then transferred to a battery brooder where they were raised for seven days. Twenty chicks per treatment were randomly chosen for necropsy, weighed and killed by cervical dislocation. The brain, heart, kidneys and liver were removed and weighed. Five livers were immediately frozen on dry ice and stored at -80°C for subsequent determination of PFOS concentrations and five livers were preserved in a 10% formalin solution for subsequent histological examination. Hatchability was significantly reduced in all treatment groups in a dose-dependent manner. The calculated LD_{50} was 0.97 µg PFOS/g egg. PFOS did not affect post-hatch body or organ weights. Exposure to PFOS caused pathologic changes in the liver characterized by bile duct hyperplasia, periportal inflammation and hepatic cell necrosis at doses as low as 1.0 µg PFOS/g, wet wt egg. Hepatic PFOS concentrations increased in a dose-dependent manner. Based on reduced hatchability, the lowest observed adverse effect level (LOAEL) was 0.1 ug PFOS/g egg.

DEDICATION

To my family, Mami, Papi, Cesar y Fernando Molina and my husband Jesse Sweeney who supported me throughout this entire venture.

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INTRODUCTION

Sulfonated fluorochemicals were first manufactured in Decatur, AL by Minnesota Mining and Manufacturing Company (3M) in 1961. They have been widely used in a variety of products as surfactants, as carpet, textile and paper protectants and as fire fighting foams (USEPA, 2000a). The basic building block of sulfonated fluorochemicals is perfluorooctane sulfonyl fluoride (POSF) (Figure 1). POSF is used to create many different products, all of which may degrade to a compound called perfluorooctane sulfonate (PFOS) (Figure 2), which cannot be metabolized any further (USEPA, 2000a). Time frames for POSF-derived product degradation are variable, in that some fluorochemical-containing polymers are stable for long periods of time and some degrade very quickly. Thus, the rate at which POSF-derived products degrade to PFOS remains uncertain (USEPA, 2000b).

PFOS is widespread in the environment. Its presence has been reported in water, soil, fish, birds and mammals including mink (*Mustela vison)*, river otters (*Lutra canadensis*), polar bears (*Ursus maritimus)*, seals (*Phoca hispida*) and humans (Table 1). Its stability, persistence, ubiquitous presence and potential for bioaccumulation makes PFOS a chemical of concern. As a result, the United States Environmental Protection Agency (USEPA) mandated that PFOS and all related perfluorinated compounds be phased out of the market within a three year period beginning in 2000 and the mayor manufacturer of PFOS (3M) agreed (USEPA, 2000a).

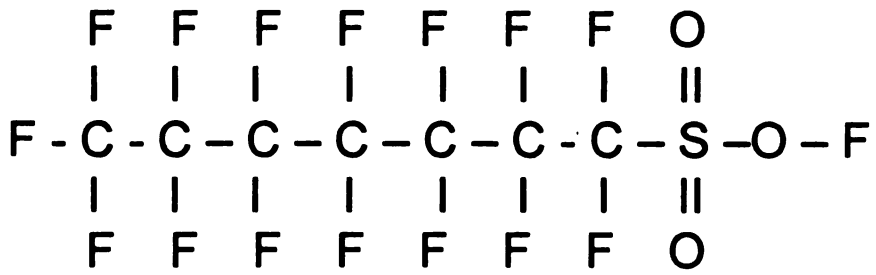

Figure 1.The structure of perfluorooctanesulfonyl fluoride (POSF) which is the basic building block of perfluorooctyl sulfonates.

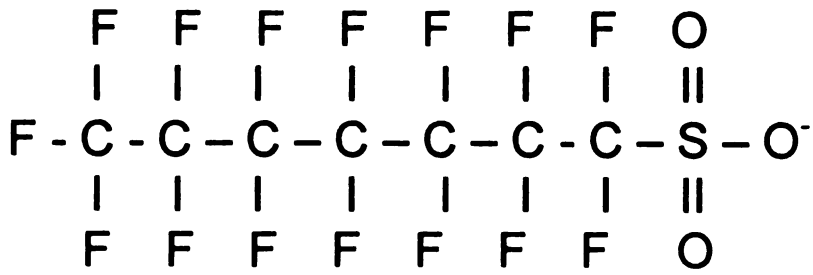

Figure 2. The structure of perfluorooctane sulfonate (PFOS) which is the ultimate degradation product of POSF-based compounds

Table 1. Concentrations of perfluorooctane sulfonate in liver (µg/g, wet wt), blood plasma (µg/ml), eggs (µg/ml) and muscle (ng/g, wet wt) of humans, aquatic mammals, fish-eating water birds, waterfowl and fish throughout the world.

Species	Location	Tissue	PFOS Concentration[a]	Reference[b]
Humans		Liver	<4.5-57.0 ng/g	2
		Plasma	<6.1-58.3 ng/g	2
Aquatic mammals				
Ringed seal	Baltic Sea	Liver	0.13-1.10 (0.490)	4
Polar bear	Alaska	Liver	0.18-0.68 (0.35)	6
Mink	Illinois	Liver	0.093-5.14 (1.61)	5
River otter	Oregon	Liver	0.097-0.994 (0.579)	5
Ringed seal	Canadian Arctic	Plasma	<0.003-0.012	6
Ringed seal	Baltic Sea	Plasma	0.016-0.23 (0.110)	6
Gray seal	Canadian Arctic	Plasma	0.011-0.049 (0.028)	6
Fish-eating water birds				
Brandt's cormorant	San Diego, CA	Liver	0.046-1.78 (0.907)	7
Sea gull	Tokyo Bay, Japan	Liver	0.230	1
Common cormorant	Tokyo Bay, Japan	Liver	0.17-0.65 (0.39)	1
Bald eagle	Midwestern US	Plasma	0.001-2.57 (0.36)	6
Laysan albatross	North Pacific Ocean	Plasma	0.006-0.034 (0.014)	6
Double crested cormorant	Lake Winnipeg, Canada	Egg	0.13-0.32 (0.21)	6
Double crested cormorant	Great Lakes	Egg	0.57-1.800 (1.3)	8
Caspian tern	Great Lakes	Egg	1.9-3.4 (2.6)	8
Waterfowl				
Mallard	Tokyo Bay, Japan	Liver	0.493	3
Pintail duck	Tokyo Bay, Japan	Liver	0.239-0.497 (0.368)	3
Fish				
Chinook salmon	Michigan waters	Liver	0.033-0.170 (0.110)	6
Yellow fin tuna	North Pacific Ocean	Liver	>0.007	6
Blue gill	Lake Biwa, Japan	Liver	0.254-0.310 (0.282)	3
Ornate jobfish	Okinawa (Kin Bay), Japan	Liver	0.593-7.9 (3.25)	3
Lake whitefish	Michigan waters	Egg	0.15-0.38 (0.26)	6
Carp	Saginaw Bay, MI	Muscle	0.06-0.3 (0.12)	6

[a] Data are expressed as the range with the mean in parentheses.
[b] 1 = Kannan *et al.*, (2003), 2 = Olsen *et al.* (2003c), , 3 = Taniyasu *et al.*, (2003), 4 = Kannan *et al.*, (2002a), 5 = Kannan *et al.*, (2002b), 6 = Giesy and Kannan, (2001), 7 = Kannan *et al.*, (2001a), 8 = Kannan, (personal communication)

PFOS has not been studied as thoroughly as other persistent organic pollutants such as organobromines and organochlorines. Epidemiological studies have indicated the presence of PFOS in blood plasma and livers of occupationally exposed humans as well as members of the general population (Olsen et al., 1999; USEPA, 1999; Hansen et al., 2001). No adverse effects have yet been associated with reported concentrations of PFOS in humans.

In mammals, PFOS caused hepatic changes including induction of hepatic enzymes, hepatic vacuolization, as well as hypertrophy and disruption of gap junction intercellular communication. Additional changes included gastrointestinal effects, weight loss, convulsions and death (Goldenthal et al., 1978b; Seacat et al. 2003). PFOS may also cause reproductive toxicity, characterized by neonatal mortality and developmental delays (Lau et al., 2003).

Only a few studies assessing the effects of PFOS in avian species have been conducted (Gallagher et al., 2000a; 2000b). Two dietary studies conducted in the mallard (*Anas platyrhynchos*) and the northern bobwhite (*Colinus virginianus*) reported LC_{50} values of 628 and 293 µg PFOS/g feed, respectively. Mortality of 90% of the mallards and 100% of the northern bobwhites occurred at the highest dietary concentration (1171 µg PFOS /g feed) before day seven of the trial.

During preliminary trials conducted in our laboratory, PFOS was detected in non-injected eggs collected at the Michigan State University's Poultry Science Research and Teaching Center (0.33 ug/g egg) and in those purchased from a local supermarket (0.34 to 0.70 ug/g egg). This finding suggests maternal

the effects of PFOS on the development of the avian embryo using an egg injection technique. Specific objectives of this study were to determine the dose of PFOS that resulted in 50% mortality (LD_{50}) and to evaluate the chicken as a suitable model to further study the effects of PFOS in wild avian species.

LITERATURE REVIEW

Fluorine

Fluorine (F) is responsible for the distinctive properties of all fluorochemicals. Fluorine is a yellow, poisonous, highly corrosive gas that does not exist in its elemental form due to its high reactivity. Fluorine reacts with most organic and inorganic compounds and all of the elements in the periodic table except for nitrogen, chlorine, oxygen and the inert gases. Because it is the most electronegative of all elements in the periodic table, F forms very strong bonds with electropositive elements (Banks and Tatlow, 1994).

One of the first uses of F can be traced to the 15th century. A naturally occurring F-containing mineral called fluorspar (CaF_2), also known as fluorite, was used to facilitate the smelting of ores. Fluorspar was later used to obtain hydrofluoric acid (HF). It is believed that HF may have been prepared in the 1700s by an unknown English glassworker who discovered that, by adding sulfuric acid to fluorite, he could obtain a corrosive vapor that could be used to etch glass. Due to F toxicity and high reactivity, many researchers were severely injured or died during their ill-fated attempts to isolate this element. Not until 1886 was elemental F isolated in France by Henri Moissan, who used electrolysis of

anhydrous HF and potassium fluoride (Weeks, 1945).

Today, fluorine continues to have many uses. Sodium fluoride is added to city water supplies and is used in toothpaste, in combination with stannous fluoride and sodium monofluorophosphate, to help prevent tooth decay. HF is used in etching semiconductor devices and in the cleaning and etching of glass, including most of the glass used in light bulbs. Hydrofluoric acid is also used in the synthesis of uranium tetrafluoride and uranium hexafluoride, both of which are used in the nuclear industry to separate isotopes of uranium (Liteplo, 2002).

Fluorocarbons

One of the most significant uses of F is in the production of fluorocarbons. When F, the most electronegative of the elements in the periodic table, is joined to carbon (C), the C-F bond that results is one of the strongest bonds in nature (~110 kcal). This bond requires a large amount of energy to form or break. As a result, biological synthesis of the C-F bond is rare, and those fluorocarbons that are synthesized chemically are very stable, extremely difficult to break down and, in most cases, are persistent in the environment (USEPA, 1999).

Fluorocarbons can be synthesized by electrochemical fluorination (ECF) through a process developed by Dr. Joseph Simmons at Pennsylvania State University in 1944. The ECF process entails substituting hydrogen atoms in hydrocarbon molecules dispersed in anhydrous hydrogen fluoride and passing an electric current through the solution causing the replacement of hydrogen atoms by fluorine (Alsmeyer et al., 1994).

One type of fluorocarbon is the chlorofluorocarbon (CFC).

One type of fluorocarbon is the chlorofluorocarbon (CFC). Chlorofluorocarbons are organic compounds that contain chlorine, fluorine and carbon. Frédéric Swarts first synthesized chlorofluoromethane in 1890. In 1928, dichlorodifluoromethane was identified as a safe refrigerant for domestic refrigerators built by Frigidaire, a General Motors (GM) subsidiary. General Motors approached DuPont to jointly manufacture and market this product, which was subsequently introduced under the trade name Freon™ (DuPont, 2003). DuPont introduced Freon 12 in 1931 followed by other Freon™ mixtures. The most common CFCs were dichlorodifluoromethane (CFC-12) and trichlorofluoromethane (CFC-11). CFCs were widely used in air conditioning refrigeration systems and in aerosol spray cans until 1994 when they were phased out by DuPont, because of damage caused to the ozone layer (Powell and Stevens, 1994).

Another fluorochemical of particular interest is polytetrafluoroethylene (PTFE), also known as Teflon™, a fluoropolymer originally discovered by Roy Plunkett at DuPont Laboratories in 1938. Plunkett was working with Freon-related gases when he inadvertently froze and compressed a sample of tetrafluoroethylene, which caused polymerization of the chemical resulting in PTFE. Large scale production of PTFE began in 1946 by DuPont for commercial use as a lining for frying pans and other utensils and as a soil and stain repellant for fabrics and textile products. Inert to virtually all chemicals, PTFE is considered the most slippery material in existence (Dupont, 2003; Banks and Tatlow, 1994). Fluoropolymers are unique among plastics because of their

be developed. However, perfluorooctanoic acid (PFOA), a component of Teflon™, is currently being reviewed due to a preliminary assessment by the USEPA that indicated potentially nationwide human exposure to low concentrations of PFOA. Based on animal studies, there is a potential risk of developmental and other adverse effects associated with PFOA in humans (USEPA, 2003).

Sulfonyl-based Fluorochemicals

In 1944, 3M acquired the rights for the ECF process patent. Sulfonyl-based fluorochemicals were then synthesized by ECF, utilizing 1-octanesulfonyl fluoride, HF, and an electric current to yield a mixture of perfluorinated fluorocarbons. The majority of the resulting products in this mixture have the same chain length and skeletal arrangement as the organic molecules used as feedstock. However, some fragmentation and rearrangement of the molecules occurs resulting in products of various chain lengths, branched molecules and cyclic compounds that are later used in a number of different products (USEPA, 1999).

Once fluorochemicals were manufactured, the challenge to create new products from sulfonyl-based fluorochemicals ensued. In 1953, Dr. Patsy Sherman, a 3M researcher, discovered the surfactant properties of these fluorochemicals by accident. A laboratory assistant in Sherman's lab spilled a small amount of this experimental chemical on Sherman's tennis shoes. Their inability to clean up the spill, both on the floor and on her shoe, with water, alcohol, or any other solvent, led to the realization that this compound could

protect fabrics from water and soiling. Three years later, Scotchguard™ Protector was launched in the marketplace followed by many other Scotchguard™ brand products (3M, 2003).

The characteristics of sulfonated fluorochemicals are a direct result of their chemical structure. The general structure of a sulfonated fluorochemical consists of a sulfonated moiety with an attached fluorinated tail. The sulfonated portion of the molecule makes the molecule highly soluble (hydrophilic) and the fluorinated, non-polar, insoluble portion repels both oil and water. The combination of a hydrophilic moiety with a hydrophobic and oleophobic portion gives fluorinated chemicals their desired surfactant properties. Moreover, their surfactant properties make them more effective than their non-fluorinated surfactants predecessors (hydrocarbons and silicone surfactants). This is due to the molecule's stability and the resistance of the C-F bond to breakdown, even in the most extreme chemical and thermal conditions (USEPA, 2000a).

Sulfonyl-based fluorochemicals have been used in various products that can be divided by function into three groups: surface treatments, paper protectors, and performance chemicals. In 2000, 3M produced approximately 6.6 million pounds of sulfonyl-based fluorochemicals for all uses in the US (USEPA, 2000a).

Surface treatment provides protection against oil, water and soil for carpets, upholstery, furniture and apparel fabric. This protectant may be applied by carpet manufacturers, by the consumer (Scotchguard™) or through aftermarket carpet treatments. Production volume for surface treatments was

approximately 2.4 million pounds, 36% of the total amount of sulfonyl-based fluorochemicals produced (USEPA, 2000a).

Paper protectors provide oil, grease and water resistance. They are widely used in food packaging applications including food wraps, bags, containers and plates. They are also utilized in non-food contact applications such as folding cartons and carbonless forms. Production volume for paper protectors was approximately 2.7 million pounds, 41% of the total production (USEPA, 2000a).

Performance chemicals are used in many specialized industrial applications including fire fighting foams, mining and oil well surfactants, photographic film, shampoos, dental cleaners, carpet spot cleaners, floor polishes and insecticides. Production volume for performance chemicals was approximately 1.5 million pounds, 23% of the total amount of sulfonyl-based fluorochemicals produced (USEPA, 2000a).

Surface treatments and paper protectants comprised the largest volume of sulfonyl-based chemical production and may present the greatest potential for human exposure. This is due to the great volume in which they were produced and their proximity to the human body through food, clothing and personal hygiene products (USEPA, 2000a).

Sulfonyl-based fluorochemicals derived from POSF degrade to perfluorooctane sulfonate (PFOS) (Figure 2), which cannot be metabolized any further. PFOS is an eight-carbon fluorinated molecule that has a sulfonate group on the terminal carbon and is part of the sulfonyl-based fluorochemical family (USEPA, 2000a).

PFOS in the Environment

Fully fluorinated (perfluorinated) molecules like PFOS are very stable and extremely difficult to break down and thus are persistent in the environment (USEPA, 1999). The degree of fluorination has an effect on both the bond length and strength. When fluorination is increased, the bond length is decreased making the bond tighter and stronger. As a result, perfluorinated compounds tend to be resistant to hydrolysis, photolysis and microbial and metabolical degradation by vertebrates (Giesy and Kannan, 2001).

The occurrence of organofluorines in the environment was reported as early as 1960. However, it was not until the 1990s that the technology became available to identify specific perfluorinated compounds in biological matrices and to quantify their presence with accuracy.

Epidemiological studies on occupationally exposed humans reported the presence of PFOS in blood plasma at concentrations below 6ppm; however, no adverse effects were associated with these concentrations of PFOS in humans (Olsen et al., 1999). PFOS was reported in the serum of the general population in concentrations lower than those found in occupationally exposed workers. Analyses of commercially available serum samples and blood bank pooled serum from the US revealed mean PFOS concentrations of approximately 28.4 ng/ml and 29.7 ng/ml, respectively (USEPA, 1999; Hansen et al., 2001).

The presence of PFOS in human livers was recently reported in a human donor study. The mean hepatic PFOS concentration was 18.8 ng/g (Olsen et al., 2003c). The PFOS liver to serum ratio was 1.3:1, indicating that the blood mainly

distributes PFOS as it concentrates in the liver (Hansen et al., 2001). This ratio can be useful in human risk assessment estimates as PFOS blood serum concentrations can estimate PFOS liver concentrations (Olsen et al., 2003c).

PFOS is easily absorbed through the digestive tract and there is potential for dermal absorption. Unlike organochlorines and organobromines such as polychlorinated byphenyls (PCBs) and polybrominated byphenyls (PBBs), PFOS does not accumulate in fatty tissue; conversely, it has high affinity for proteins. It is more likely to be found in blood plasma and muscle and tends to concentrate in the liver.

In February of 2000, researchers at Michigan State University reported the presence of PFOS in wildlife. This study revealed the extent of PFOS distribution. Various animal species throughout the world were sampled including polar bears, marine mammals, fish-eating water birds, fish and frogs. Tissue samples including bird and fish eggs, liver, blood plasma, kidneys and muscle were analyzed for the presence of PFOS. PFOS was found in most of the samples including those collected from remote locations such as the Artic (Giesy and Kannan, 2001).

Fish-eating water birds, including bald eagles from the Great Lakes region, had blood plasma concentrations of up to 2750 ng PFOS/ml. An average PFOS concentration of 330 ng/ml was reported for blood samples of nestling bald eagles less than 70 days old. The presence of relatively high concentrations in young birds may indicate maternal transfer of PFOS through egg proteins (Kannan et al., 2001a). In fact, eggs from Caspian terns and double crested

cormorants had concentrations ranging from 1900 to 3400 ng/ml and 570 to 1800 ng/ml, respectively (Kannan, K., personal communication).

PFOS was also found in fish-eating predators such as mink and river otters from the midwestern and east coast regions of the United States. Greater concentrations were found in mink collected from urbanized areas, with liver concentrations as high as 5140 ng/g. Ranch mink fed diets containing 40% Saginaw River fish, which contained an average of 160 ng PFOS/g, had average liver PFOS concentrations of 3250 ng/g. This finding suggested that PFOS may bioaccumulate, since PFOS concentrations detected in mink were considerably greater than that those found in fish (Kannan et al., 2002a).

Toxic Effects

PFOS is moderately toxic to rats, having an oral LD_{50} of 251 µg/g (Dean et al., 1978). Rusch and Reinhart (1979) reported that rats exposed to PFOS by inhalation for one hour to doses ranging from 0.0 to 24 mg PFOS/L exhibited labored breathing, decreased activity, lung and liver discoloration and death of all rats in the highest dose group.

PFOS was mildly irritating to the eyes and non-irritating to the skin of rabbits (O'Malley and Ebbens, 1980). PFOS was negative for mutagenicity when tested in various strains of *Salmonella (*strains *TA-1535, TA-1537, TA-1538, TA-98* and *TA-100),* and *Sacharomyses cereviciae (*strain *D4)* (Jagannath and Brusic, 1978). PFOS did not cause chromosomal aberrations in mice when administered orally at doses as high as of 950 µg PFOS/g (Murli, 1996).

Various studies on sub-chronic effects of PFOS when administered orally

Various studies on sub-chronic effects of PFOS when administered orally have been conducted in rats and primates. Clinical signs observed in rats fed PFOS at concentrations from 0.0 to 3000 µg/g feed included emaciation, convulsions, reduced body and organ weights, hepatomegaly, gastrointestinal and immune system effects, hypoactivity and death. Serum chemistry changes included increases in creatinine phosphokinase and alkaline phosphatase activities and decreases in cholesterol and triglyceride concentrations. Plasma aspartate aminotransferase and alanine aminotransferase activities were increased. Histological changes in the liver included hepatocellular hypertrophy, vacuolation and necrosis (Goldenthal et al., 1978a).

Adverse effects seen in a 90-day, oral gavage, rhesus monkey study included weight loss, hypoactivity, emesis, diarrhea, prostration, hemorrhage, tremors and death at doses as low as 10 µg PFOS/g/day and death of all monkeys by day 20 of the trial (Goldenthal et al., 1978b). Serum chemistry analyses in a repeated-dose, cynomolgus monkey study (Seacat et al., 2002) indicated a reduction in serum cholesterol and high-density lipoprotein concentrations.

Two dietary studies have been conducted to assess the toxic effects of PFOS in avian species. Mallards and northern bobwhite quail were fed diets containing 0, 9.1, 18.3, 36.6, 73.2, 146, 293, 586 or 1171 µg PFOS/g feed for a period of five days. No treatment related mortality occurred in mallards in the lowest dose groups. However, there was 20% mortality in the 293 µg PFOS/g feed group, 30% mortality in the 586 µg /g feed group and 90% mortality in the

1171 µg /g feed group. Signs of toxicity included ruffled appearance, lethargy, lower limb weakness, decreased muscle mass, reduced weight gain, depression, convulsions and ultimately death. Toxicity signs were first observed on the second day of the five-day exposure period and mortality occurred as early as day four in the 1171 µg/g feed group. Gross examination findings in the 293 µg/g feed treatment group and higher included empty crops, loss of muscle mass, small and pale spleen, emaciation, and a generally thin body condition (Gallagher et al., 2000a).

Treatment-related mortality (11%) first occurred in the northern bobwhite at 146 µg/g feed. Mortality was 80% in the 233 µg/g feed group and 100% in the 586 µg/g feed and 1171 µg/g feed groups. Signs of toxicity included ruffled appearance, wing droop, reduced reaction to stimuli (sound and motion), loss of coordination, lethargy, depression, lower limb weakness, rigidity, prostration, convulsions and ultimately death. Toxicity signs were first observed on the second day of the five-day exposure period. There was 100% mortality as early as day three in the 1171 µg/g feed group and day seven in the 586 µg/g feed group. Body weight gain was reduced in the 146, 293 and 569 µg PFOS/g feed treatment groups and feed consumption decreased at 293 µg PFOS/g feed and higher. Goss examination findings for the northern bobwhite quail included loss of muscle mass, autolysis of tissues in the abdominal cavity, pale organs, prominent keel bone, emaciation and generally thin body condition (Gallagher et al., 2000b).

Mechanisms of Action

Related perfluorinated compounds have been found to interfere with gap junction intercellular communication (GJIC), mitochondrial biogenesis and induction of liver enzymes involved in fatty acid metabolism. These compounds are also known to be peroxisome proliferators. Recent studies using PFOS have confirmed these findings (Hu et al., 2002; Berthiaume and Wallace, 2002).

GJIC is described as the process in which plaque-like structures in the cell plasma membrane form tunnel-like structures that serve as a channel to aid synchronization of cell functions. These channel structures allow the exchange of cytosolic molecules like ions, second messengers and metabolites between adjacent cells (Yamasaki, 1996). GJIC plays a vital role in maintaining the homeostasis of tissues; therefore, disruption of GJIC results in abnormal cell growth, which is associated with tumor formation and promotion (Hu et al., 2002). Disruption of GJIC can also lead to neurological, cardiovascular, reproductive and endocrinological dysfunction (Trosko et al., 1998).

Perfluorinated compounds such as perfluorooctanoic acid, perfluorooctane sulfonamide, perfluorohexane sulfonic acid and perfluorodecanoic acid can inhibit GJIC in a dose-related manner (Upham et al., 1998). However, potency of inhibition is dependent on the length of the fluorinated carbon chain; compounds with less than five carbons or more than 16 have not shown inhibition of GJIC (Upham et al., 1998). PFOS, an eight-carbon compound, caused inhibition of GJIC in rat liver and dolphin kidney cell lines, and in the Sprague-dawley rat. Inhibition of GJIC was rapid, yet reversible, after removal of the compound. Inhibition was neither gender related nor tissue specific as reported by Hu *et al.*

(2002).

Many perfluorinated compounds like PFOA, N-ethyl perfluorooctane sulfonamide ethanol (N-EtFOSE) and PFOS are peroxisome proliferators. Although many perfluorinated compounds affect both mitochondrial biogenesis and peroxisomal proliferation, it was discovered that not all peroxisome proliferators have an effect on mitochondrial biogenesis. PFOS did not affect mitochondrial biogenesis in male rats when injected via single intraperitoneal injection (Berthiaume and Wallace, 2002).

Peroxisomes are organelles found mainly in hepatocytes and the kidney cell cytoplasm and are involved in the metabolism of long chain fatty acids (peroxisomal β -oxidation) (Baudhuin et al., 1965). Peroxisomes are rich in the enzymes peroxidase, catalase and d-aminoacid oxidase, which are involved in peroxide metabolism (Dorland, 1995).

When peroxisome proliferators, such as PFOA, clofibrate or various phthalate esters, are given to rodents, they cause an increase in the number and size of peroxisomes in the cell as well as an increase in liver size due to hyperplasia (Berthiaume and Wallace, 2002; Timbrell, 2000). This results in an increase in the rate of fatty acid metabolism, which may overwhelm the mechanisms necessary to metabolize hydrogen peroxide into water. This situation causes the formation of hydroxyl radicals and reactive oxygen species leading to oxidative stress in the cell. Reactive oxygen species will also cause DNA, protein, and cell damage. Cell proliferation due to hyperplasia along with cell damage can cause cells to progress into tumors. However, tumor occurrence

as a result of exposure to peroxisome proliferators does not appear to have the same effect in all species. Humans seem to be non-responsive to this phenomenon (Timbrell, 2000).

MATERIALS AND METHODS

Perfluorooctane sulfonate (heptadecafluorooctane sulfonic acid, potassium salt, MW= 538.22, 96% purity) was purchased from TCI, America (Portland, OR). PFOS was dissolved in dimethyl sulfoxide (DMSO). The choice of vehicle was based on the results of a preliminary study in our laboratory that evaluated DMSO, methanol, methylene chloride, acetone and saline in terms of PFOS solubility and hatchability of chicken eggs.

Doses and Design

Fifty White Leghorn chicken (*Gallus domesticus*) eggs were randomly assigned to each of six treatment groups; non-injected control, vehicle (DMSO) control and 0.1, 1.0, 10.0, and 20 µg PFOS/g egg. The injection volume was 0.1 µl/g egg. PFOS was injected into the air cell of the egg following the procedure of Powell *et al* (1996). Preliminary studies in our laboratory using methylene blue dye injected into the egg showed that PFOS is better distributed in the egg albumin. Doses of PFOS were chosen to bracket concentrations detected in the blood plasma and eggs of birds collected from the Great Lakes region.

Dosing solutions were prepared from stock solutions of 1,000, 10,000, 100,000 and 200,000 µg PFOS/ml by cold filtering (0.22 micron filter, Millipore Products Division, Bedford, MA) appropriate volumes of the PFOS stock solutions and DMSO into sterilized glass injection vials to give final concentrations of 1.0, 10.0, 100 and 200 µg PFOS/ml. All PFOS solutions were stored at room temperature.

Egg preparation

White Leghorn chicken eggs were obtained from the Michigan State University Poultry Science Teaching and Research Center fertile egg flock. Eggs were lightly sanded to remove excess dirt and stored in a cooler at 14 °C for 24 hr prior to injection. Eggs were candled to detect cracks in the eggshell and weighed to determine the injection volume. The air cell was outlined with a pencil and eggs were labeled with an individual identification number. Eggs were then placed in egg trays with the blunt end up.

Injection Procedure

Eggs were injected via the air cell on day 0 of incubation using a 10 ul syringe (Hamilton Company, Reno, Nevada). Immediately prior to injection, the egg surface was wiped with 70% ethanol and a small hole was made above the air cell with a pin wiped with 70% ethanol to insert the syringe into the air cell. The pin and the syringe needle were wiped with 70% ethanol between injections. After injection, the hole was sealed with melted paraffin and eggs were placed in the incubator (Petersime, Gettysburg, Ohio) with their blunt end up.

Incubation

The temperature of the incubator was maintained at 37.5-37.7 °C (99.5-99.75 °F), while the relative humidity was maintained at 60% (29.4-30.6 °C, wet bulb temperature). Eggs were automatically rotated every two hours. Eggs were candled weekly to assess embryo viability and to estimate the stage of development at which the embryo died. Eggs that did not develop were considered infertile and were excluded from the analysis.

On day 18 of incubation, eggs were placed in pedigree baskets and were

transferred to a hatcher (Natureform, Jacksonville, FL). Temperature and humidity of the hatcher were set at 37.5-37.7°C and 65% (31.1-37.7°F, wet bulb temperature), respectively, and were monitored daily.

Post-hatch

Hatching began on day-21 of incubation and was allowed to continue until day-24. Hatching dates of individual chicks were recorded. Hatchlings were identified with a Swiftak identification tag (Heartland Animal Health, Fairplay, MO), weighed and examined for gross deformations within 24hr of hatching and transported to a Petersime battery brooder in a containment room at the MSU Poultry Science Research and Teaching Center. Eggs that did not hatch by day 24 were opened to assess the stage of embryo death and the incidence of gross developmental abnormalities. Chicks were provided Purina Chick Starter (Purina Mills, St. Louis, MO) and water *ad libitum* for seven days. Chicks were introduced to their surroundings by dipping their beaks in feed and water before placing them in the brooder.

Necropsy

At seven days of age (post-hatch), 20 chicks per treatment were randomly chosen, weighed and killed by cervical dislocation. The brain, heart, kidneys and livers were removed and weighed. Five livers per treatment were frozen on dry ice before placement in an ultra-cold freezer (-78°C) for subsequent determination of PFOS concentration and five livers per treatment were placed in a 10% formalin solution for subsequent histopathological examination.

Contaminant Analysis

Hepatic concentrations of PFOS were determined as described by Kannan et al. (2001a), using high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS). For extraction of liver samples, a liver homogenate of 1 g liver to 5 ml of Milli-Q water was prepared. One ml of the homogenate was transferred to a polypropylene tube and 1 ml of 0.5 M tetra butyl ammonium hydrogen sulfate solution (pH adjusted to 10) and 2 ml of 0.25 M sodium carbonate buffer were added. After thorough mixing, 5 ml of methyl-tert-butyl ether (MTBE) were added, and the mixture was vigorously shaken for 20 min. The organic and aqueous layers were separated by extraction and 4 ml of MTBE were removed from the solution and transferred to a fresh 15 ml polypropylene tube. The solvent was evaporated under nitrogen before adding 1 ml of methanol. The sample was mixed for 30 sec and 1 ml was transferred into a 1.5 ml auto vial. Analyte separation was performed using a Waters 2695 Alliance high performance liquid chromatograph (HPLC) (Milford, MA). A total of 10 µl of extract was injected onto a 50 x 2 mm (5 µm) Keystone Betasil® (Runcorn, PA) C18 column with a 2 mM ammonium acetate/ methanol mobile phase starting at 10% methanol. The gradient was increased to 100% methanol at seven min at a flow rate of 200 µl/min before reverting to original conditions at 15 min. Column temperature was maintained at 25 °C. Although PFOS eluted at 9.8 min, a longer chromatographic run (20 min) was necessary to completely elute all extractables from the column. For quantitative determination, the HPLC system was interfaced to a Micromass ZQ atmospheric pressure ionization mass spectrometer (Beverly, MA) operated in the electrospray negative mode.

Instrumental parameters were optimized to transmit the $[M-K]^-$ ion. For all analyses, the capillary was held at 3 kV. Desolvation temperature and gas flow were held at 300°C and 300 l/hr respectively.

Statistical Analyses

All comparisons were made with respect to the vehicle control. PFOS effects on hatchability were evaluated using logistic regression. Body and organ weights (brain, heart, kidney and liver) were analyzed by one way analysis of variance (ANOVA) followed by Dunnett's test for comparisons with the vehicle control using SAS Software (SAS; Statistical Analysis Systems, Cary, NC). LD_{50} values were obtained by probit analysis (Version 1.5, USEPA). The level of statistical significance was $p < 0.05$ unless otherwise specified.

RESULTS

Hatchability was significantly ($p<0.0001$) reduced in all dose groups in a dose-dependent manner (Table 2). The LD_{50} was 0.97 µg PFOS/g egg (95% CL 0.014-5.584 ug PFOS / g egg). Post-hatch mortality through seven days of age was not affected by treatment.

There was a slight incidence of gross abnormalities that included the vehicle control group. One embryo in the control group had a deformed leg. One embryo in the 10 ug PFOS / g egg dose group had no skull or eyes and a cross bill, and at 20 ug PFOS / g egg, one case of celosomia or exposed viscera.

There were no significant changes in chick body weights at hatch and at seven days of age that were associated with PFOS exposure (Tables 3 and 4). Similarly, absolute and relative organ weights (liver, brain, kidney and heart) were not affected by injection of PFOS (Tables 5 and 6).

Morphological changes in the liver were assessed by gross examination and light microscopy. Livers in all groups were pale, which was attributed to absorption of the yolk through the first week of life. Lesions observed during histological examination included hepatic vacuolation, bile duct hyperplasia, periportal inflammation, multifocal hepatocellular necrosis and multiple subcapsular hepatocellular degeneration and one case of fatty cysts in the 1.0 µg/g dose group. Hepatic vacuolation was observed in all dose groups, but vacuolation tended to be more prevalent in the 1.0, 10.0 and 20.0 µg/g egg dose groups compared to the vehicle control and the 0.1 µg/g egg groups (Table 7). Two moderate lesions were observed in the 1.0 µg/g egg group, one lesion was

Table 2. Effect of perfluorooctane sulfonate injected into the air cell of White Leghorn chicken embryos prior to incubation on hatchability[1].

Dose (ug/g)	Number of hatchlings/ Number of fertile eggs	Hatchability (%)
Vehicle	42/49	85.7[a]
0.1	27/44	61.4[b]
1.0	23/48	47.9[b]
10.0	20/50	40.0[c]
20.0	17/46	37.0[c]

[1]Non-injected controls were excluded from the statistical analysis. Values with different superscripts are significantly different at $p<0.05$.Vehicle used was dimethyl sulfoxide.

Table 3. Effect of perfluorooctane sulfonate injected into the air cell of White Leghorn chicken embryos on body weights upon hatching[1].

Dose (ug/g)	n	Weight (g)
Vehicle	42	39.93 ± 0.52[a]
0.1	27	40.33 ± 0.64[a]
1.0	23	39.65 ± 0.70[a]
10.0	20	41.35 ± 0.75[a]
20.0	17	42.15 ± 0.81[a]

[1]Data presented as mean ± standard error of the mean. Values with different superscripts are significantly different at $p<0.05$ Vehicle used was dimethyl sulfoxide.

Table 4. Effect of perfluorooctane sulfonate injected into the air cell of White Leghorn chicken embryos on body weights at one week of age [1].

Dose (ug/g)	n	Weight (g)
Vehicle	37	59.59 ± 1.45[a]
0.1	27	56.72 ± 1.70[a]
1.0	21	55.38 ± 1.93[a]
10.0	19	58.50 ± 2.08[a]
20.0	16	57.09 ± 2.21[a]

[1]Data presented as mean ± standard error of the mean. Values with different superscripts are significantly different at $p<0.05$ Vehicle used was dimethyl sulfoxide.

Table 5. Effect of perfluorooctane sulfonate injected into the air cell of White Leghorn chicken embryos on absolute organ weights (g) at one week of age [1].

Dose (ug/g)	Liver	Brain	Kidney	Heart
Vehicle	2.24 ± 0.1 (18)	1.16 ± 0.03 (11)	0.84 ± 0.04 (13)	0.66 ± 0.03 (13)
0.1	2.05 ± 0.1 (16)	1.10 ± 0.03 (10)	0.75 ± 0.04 (11)	0.57 ± 0.03 (11)
1.0	2.00 ± 0.1 (15)	1.10 ± 0.03 (10)	0.74 ± 0.04 (10)	0.61 ± 0.03 (11)
10.0	2.19 ± 0.1 (15)	1.15 ± 0.03 (9)	0.84 ± 0.04 (10)	0.59 ± 0.03 (10)
20.0	2.28 ± 0.1 (15)	1.10 ± 0.03 (10)	0.78 ± 0.04 (10)	0.61 ± 0.04 (9)

[1]Data presented as mean ± standard error of the mean. Number in parentheses is sample size. Vehicle used was dimethyl sulfoxide.

Table 6. Effect of perfluorooctane sulfonate injected into the air cell of White Leghorn chicken embryos on relative organ weights (g) at one week of age [1].				
Dose (ug/g)	Liver	Brain	Kidney	Heart
Vehicle	3.78 ± 0.13(18)	1.89 ± 0.1 (11)	1.34 ± 0.05 (13)	1.05 ± 0.04 (13)
0.1	3.79 ± 0.12 (16)	1.99 ± 0.04 (10)	1.99 ± 0.04 (11)	1.04 ± 0.05 (11)
1.0	3.57 ± 0.14 (15)	1.99 ± 0.04 (10)	1.32 ± 0.04 (10)	1.09 ± 0.04 (11)
10.0	3.84 ± 0.13 (15)	1.96 ± 0.04 (9)	1.37 ± 0.05 (10)	1.05 ± 0.04(10)
20.0	3.96 ± 0.1 (15)	1.85 ± 0.04 (10)	1.30 ± 0.05 (10)	1.03 ± 0.04 (9)
[1]Data presented as mean ± standard error of the mean. Number in parentheses is sample size. Vehicle used was dimethyl sulfoxide.				

observed in the 10.0 µg/g egg group and two cases were observed in the 20.0 µg/g egg group. Bile duct hyperplasia was observed at PFOS concentrations greater than 1.0 µg/g egg. Multifocal hepatocellular degeneration and necrosis were observed in five liver samples, three in the 1.0 µg/g egg dose group and one each in the 10 and 20 µg PFOS/g egg groups (Table 7).

PFOS concentrations in livers significantly increased proportional to dose (Table 8). Concentrations in the 10.0 and 20.0 ug PFOS/g egg groups were significantly greater compared to concentrations in the vehicle control, 0.1 ug PFOS/g egg and 1.0 ug PFOS/g egg groups (Table 8).

Table 7. Histopathology of livers from seven-day-old White Leghorn chickens exposed to perfluorooctane sulfonate in ovo via air cell injection prior to incubation [1].

Treatment (ug/g egg)	Chick ID	Observations		Other
		Hepatocellular Vacuolation	Bile duct hyperplasia	
Vehicle	B854	1	0	
Vehicle	B857	1	0	
Vehicle	B861	1	0	
Vehicle	B882	1	0	
0.1	P831	1	0	
0.1	P840	1	0	
0.1	P845	1	0	
0.1	P864	1	0	
0.1	P869	1	0	
0.1	P871	1	0	1+ Periportal inflammation
1.0	PC399	1	0	1+ Periportal inflammation
1.0	PC872	2	1	Multiple subcapsular hepatocellular degeneration
1.0	PC873	2	1	Multifocal hepatocellular degeneration/necrosis
1.0	PC877	1	1	Multifocal hepatocellular necrosis; Fatty cyst
1.0	PC888	1	0	
10.0	Y397	1	0	
10.0	Y403	2	1	
10.0	Y404	1	2	Hepatocellular necrosis
10.0	Y406	1	1	
20.0	YC411	1	1	
20.0	YC412	1	1	
20.0	YC415	2	1	
20.0	YC416	2	1	Multifocal hepatocellular degeneration/necrosis
20.0	YC421	1	1	

[1]Vehicle used was dimethyl sulfoxide. Severity of lesion was graded using the following scale: 0 = normal; 1 = mild; 2 = moderate; 3 = severe.

Table 8.Hepatic perfluorooctane sulfonate concentrations of seven-day-old White Leghorn chickens exposed to perfluorooctane sulfonate in ovo via injection into the air cell [1].	
Dose (ug/g)	Concentration (μg/g, wet wt)
Vehicle	1.101 ± 0.5367[a]
0.1	1.411 ± 0.5367 [a]
1.0	1.762 ± 0.6000 [a]
10.0	3.165 ± 0.5367 [b]
20.0	4.752 ± 0.5367[b]
[1]Data presented as mean ± standard error of the mean. Values with different superscripts are significantly different at $p<0.05$. Vehicle used was dimethyl sulfoxide.	

Table 9. Concentrations of perfluorooctane sulfonate in chicken eggs from domestic commercial sources [1].		
Source	n	PFOS Concentrations (ug/g wet wt)
"Organic" eggs from local supermarket	4	0.703 ± 0.01^{a}
Eggs from local supermarket	4	0.344 ± 0.05^{b}
Eggs from MSU Poultry Science and Research Center	3	0.329 ± 0.07^{b}
[1]Data are presented as the mean ± standard error of the mean. Values with different superscripts are significantly different at $p<0.05$.		

DISCUSSION

This study was conducted to assess PFOS toxicity in the developing chicken embryo following introduction of the chemical into the air cell prior to incubation. The objectives of this study included determining an LD_{50} value as well as the suitability of the chicken embryo as an animal model for studying the adverse effects of PFOS in wild avian species.

Injection doses for this study (0.1-20.0 µg/g egg) were selected based on PFOS concentrations detected in eggs of wild avian species collected from the Great Lakes basin. The Great Lakes is a highly contaminated area because of the high volume of industries in the states surrounding the lakes. PFOS, like many other persistent organic pollutants, is present at higher concentrations in urbanized and industrialized areas like the Great Lakes basin. Fish and fish-eating water birds in these areas are now known to have relatively high concentrations of PFOS (Kannan et al., 2001a). Tissues of fish from the Great Lakes, including lake whitefish (*Coregonus clupeaformis*), brown trout (*Salmo trutta)*, chinook salmon (*Oncorhynchus tshawytscha)* and carp (*Cyprinus carpio)*, contained detectable PFOS concentrations in eggs, whole blood, blood plasma, liver and muscle (Giesy and Kannan,2002). Carp from the midwestern US had muscle PFOS concentrations up to 0.3 µg/g (Giesy and Kannan, 2001). The presence of PFOS in fish and its occurrence in fish-eating water birds suggests that fish could be a source of PFOS. Bald eagles *(Haliaeetus leucocephalus)* from the Great Lakes region had blood plasma concentrations as great as 2.75 µg PFOS/ml. (Giesy and Kannan, 2001). Other birds, such as double crested

cormorants (*Phalacrocorax auritus*), herring gulls (*Larus argentatus*) and ring billed gulls (*Larus delawarensis*) contained concentrations of PFOS in various tissues including blood plasma and liver. Moreover, nestling bald eagles had blood PFOS concentrations as great as 0.33 µg/ml. The presence of relatively high concentrations in young birds may indicate maternal transfer of PFOS through egg proteins (Kannan et al., 2001a). In fact, eggs from Caspian terns *(Sterna caspia)* and double crested cormorants were found to have PFOS concentrations ranging from 1.9 to 3.4 µg/ml and 0.57 to 1.8 µg/ml, respectively (Kannan, unpublished data).

PFOS was also found in fish-eating mammals such as mink (*Mustela vison)* and river otters (*Lutra canadensis*) from the midwestern and east coast regions of the United States. The greatest concentrations were found in mink collected from urbanized areas, with liver concentrations as high as 5.14 µg/g. Ranch mink fed diets containing 40% Saginaw River fish, which contained an average of 0.16 µg PFOS/g, had an average liver PFOS concentration of 3.25 µg/g. This finding suggested that PFOS may bioaccumulate, since PFOS concentrations detected in mink were considerably greater than those found in fish (Kannan et al., 2002b).

Hatchability and LD_{50}

In the present study, PFOS significantly lowered hatchability of chicken eggs in a dose-related manner when compared to the vehicle control group (Table 2). The LD_{50} for chicken eggs injected with PFOS via the air cell prior to incubation (Day 0) was 0.97 µg PFOS/g egg.

PFOS concentrations causing embryo mortality in this study were comparable to PFOS concentrations found in eggs collected from wild species. Egg samples from urbanized areas had higher concentrations of PFOS when compared to eggs from isolated regions. Double crested cormorants from the Great Lakes area contained PFOS concentrations from 0.57 to 1.8 µg/g and Caspian tern eggs from the same region contained PFOS at concentrations from 1.9 to 3.4 µg/g (Kannan, unpublished data). Double crested cormorant eggs from Canada had slightly lower PFOS concentrations, ranging from 0.02 to 0.32 µg/g (Giesy and Kannan, 2001). It is possible that hatching success of eggs in the wild may be compromised by PFOS if the sensitivity for these species in terms of mortality is similar to the chicken and air cell injection accurately represents whole egg concentrations achieved by dietary exposure of adults.

LD_{50} values have been derived for other species including the rat, the northern bobwhite quail (*Colinus virginianus)* and the mallard (*Anas platyrhynchos)*, all under different treatment regimes and conditions (age of animal, route of exposure and length of exposure). Pregnant Sprague-Dawley rats were given 1 to10 µg PFOS/g body weight daily by gavage beginning on gestation day (GD) 2 and continuing until GD 21. The LD_{50} for rat pups exposed *in utero* was 3 µg/g. Pregnant mice were dosed under similar conditions with 1 to 20 µg PFOS /g from the first day of gestation through GD 18. The LD_{50} for mice pups was 10 µg/g (Lau et al., 2003). In two avian dietary studies, young northern bobwhites and mallards were fed diets containing 0, 9.1, 18.3, 36.6, 73.2, 146, 293, 586 or 1171 µg PFOS/g feed for a period of five days. The LC_{50} derived for

the northern bobwhite quail was 293 µg/g feed and 628 µg/g feed for the mallards (Gallagher et al., 2000a; 2000b), which is equivalent to a total PFOS dose of 9 µg/g body weight and 748 µg/g body weight respectively. Even though there are various LD_{50} values reported, comparisons are irrelevant because of the unique conditions under which each one was determined. However, the results of the present study suggest that PFOS is very toxic to the chicken embryo when administered prior to embryo development.

There are two additional factors that may affect the viability and hatching success of the chicken embryo in addition to the toxicant concentration: the site of injection (yolk sac compared to air cell) and the time of injection (prior to incubation compared to day four of incubation). Injection of toxicants into the yolk sac causes considerably greater mortality to the chicken embryo when compared to air cell injection. Chicken embryos injected with 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) into the yolk sac had greater mortality than embryos injected with equal concentrations of TCDD via the air cell (LD_{50} values of 122 pg TCDD/g egg and 297 pg TCDD/g egg, respectively) (Henshel et al. 1997). In a preliminary study conducted in our laboratory, it was also observed that there was higher mortality when PFOS was injected into the yolk compared to the air cell. Air cell injection was selected based on the relatively high water solubility of PFOS. The egg albumen is composed primarily of water (88%) with the reminder being proteins and glycoproteins. The yolk, on the other hand, has greater lipid content and lesser water content than the albumin. It was anticipated that PFOS, and the vehicle DMSO, would be more evenly distributed in the water-rich

environment of the albumin.

The avian egg is exposed to toxins from the time it starts growing in the ovary until the yolk is completely absorbed four days post-hatch. In this study, the injection was conducted just prior to incubation to mimic this natural process of exposure via deposition of the compound into the egg by the female. The time of injection or state of development at which the substance is in contact with the embryo may adversely affect hatchability. TCDD was more toxic to embryos exposed to the compound on day four of incubation compared to days eight and 14 of incubation (Bruggemann et al. 2003).

Post-hatch survival was not affected by PFOS in the seven-day period the chicks remained in the battery brooder. In contrast, PFOS induced mortality in rat pups exposed *in utero* to 1-10 µg PFOS/g body weight and in mice pups exposed *in utero* to 1 - 20 µg PFOS/g body weight within the first 24 hr after birth (Lau et al., 2003). Although all animals were born alive, the neonates in the highest dosage groups (10 µg/g body weight for rat and 20 µg/g body weight for mouse) became pale, inactive, and moribund within 30 to 60 min, and all died soon afterward. In the 5 µg/g (rat) and 15 µg/g (mouse) groups, the neonates also became moribund, but survived for a longer period of time (8 to12 hr).

Teratogenicity

PFOS did not induce significant abnormalities in chicken embryos. The few abnormalities observed in this study fell within the normal range (6 to 7%) of spontaneous malformations for chicken embryos (Romanoff, 1972). Occasionally, malformed embryos have more than one malformation as was observed in this

trial. Three embryos had multiple abnormalities including deformed extremities (0.6%), anophthalmia or absence of one or both eyes (0.3%), exencephalia or exposed brain (0.3%), celosomia or exposed viscera (0.3%) and mandibular/maxillary malformation (0.3%). None of the embryos with abnormalities hatched with the exception of two chicks. One had curled toes and died before the end of trial and the other had a protruding eye and survived to the end of the trial.

Body and organ weights

PFOS did not affect body weights (Tables 3 and 4) or absolute and relative organ weights of chickens exposed *in ovo* (Table 5). However, previous studies in different species reported body weight loss or a decrease in body weight gain after repeated exposure to PFOS.

Primates, including rhesus and cynomolgus monkeys, had decreased body weights as a result of exposure to PFOS. Rhesus monkeys given 0-300 µg PFOS/g body weight/ day orally for 90 days had significant body weight loss, but organ weights were normal (Goldenthal et al., 1978b). In a subsequent 90-day rhesus monkey study, lower doses of PFOS (0.5 to 4.5 µg/g body weight/day) caused body weight loss, presumably as a result of anorexia, emesis and dehydration (Goldenthal et al., 1978b). Mean body weight gain was significantly reduced in cynomolgus monkeys administered PFOS at 0.75 µg/g body weight/day for six months (Seacat et al., 2002).

PFOS reduced body weights in a dose dependent manner in rats fed 0.5 to 20.0 µg PFOS/g feed/day for a period of four or 14 weeks. In addition, relative

liver weights were increased after 14 weeks of exposure in the group administered 20.0 µg/g feed/day (Seacat et al., 2003). In a two-generation developmental study, rat and mice pups exposed to PFOS prenatally (GD 2 to GD 21) had deficits in body weight gain at concentrations as low as 2 µg PFOS /g body weight/day (Lau et al., 2003).

During necropsy, we observed that three chicks in the 0.1 µg/g egg dose group had prominent keel bones. This was a common finding in the bobwhite and mallard dietary study and was associated with decreased food consumption in the higher treatment groups (293 to 1171 µg PFOS/g feed). In mallards, there was significant body weight loss in the 73.2 to 1171 µg/g treatment groups during the five-day exposure period. By day 22 of the post-exposure period, body weights appeared comparable in all dose groups. In bobwhite quail, significant body weight loss also occurred during the five-day exposure period at 146 to 586 µg PFOS/g feed (Gallagher et al., 2000a; 2000b). However, the emaciation and lethargic behavior seen in these studies were not observed in the present study at the doses and exposure period tested.

Histological analysis

PFOS caused morphological changes in the livers of chicken embryos at concentrations as little as 1.0 µg/g egg. Changes included hepatocellular vacuolation, bile duct hyperplasia, periportal inflammation, hepatocellular degeneration and necrosis.

Mild hepatocellular vacuolation was observed among all dose groups; however, moderate vacuolation was observed more frequently at doses of 1.0

µg/g or greater (Table 7). The appearance of hepatic vacuolation has been reported in previous PFOS studies. Hepatocellular vacuolation was observed in rats fed diets containing PFOS at 5 or 20 µg/g feed for a period of four or 14 wk and in cynomolgus monkeys administered PFOS at a dose of 0.75 µg/g body weight/day for six months (Seacat et al. 2002, 2003).

Vacuolation is the result of accumulation of lipids in hepatocytes, which occurs because of the absorption of the lipid-rich yolk sac that continues after hatching. Although it is not always injurious to the liver, it can be indicative of impairment such as blockage of triglyceride secretion into plasma or reduced synthesis of carrier lipoprotein (Osweiler, 1996). Previous studies have reported that PFOS may interfere with fatty acid binding to lipoprotein, contributing to lipid accumulation in the liver (Luebker et al., 2002).

Mild to moderate bile duct hyperplasia was observed in the highest dose groups (1.0, 10.0 and 20.0 µg/g egg). This lesion consists of an abnormal number of bile ducts occurring in the portal area accompanied by periportal inflammation. This lesion is considered to be a common lesion and it is unclear whether or not this can progress into benign or malignant tumors (Newberne, 1998).

Multifocal hepatocellular degeneration and necrosis were observed in five liver samples. Because three of the samples were from the 1.0 µg/g dose group, it was not clear if this was a treatment-related effect. Hepatocellular necrosis has only been reported in rhesus monkeys exposed to PFOS at concentrations as high as 300 to 3000 µg/g body weight.

Interestingly, some of the lesions observed in the present study have been observed in monkeys and rats exposed to PFOS for prolonged periods of time. In this study, chickens exposed before incubation experienced similar liver changes after a single exposure in a period of only 28 d. This may be due to the fact that fertile eggs were injected at a very early stage of development.

PFOS Concentrations in Eggs

PFOS concentrations in vehicle control chicks and those exposed *in ovo* to 0.1, 1.0, 10.0 or 20.0 µg PFOS /g egg ranged from 1.0 to 4.8 µg/g (Table 8). The highest concentration of PFOS measured in chick liver samples was 7.2 µg PFOS /g from a bird in the highest dose group (20.0 µg/g egg). The lowest concentrations were detected in the control group, yet, some individual liver samples in the vehicle control group had PFOS concentrations as high as 1.7 µg PFOS/g. PFOS concentrations in livers of wild avian species, mostly from urbanized areas (Table 1), had concentrations similar to those found in liver samples of embryos from the vehicle control, 0.1 and 1.0 µg PFOS/g egg treatment groups (Table 8). For example, Brandt's cormorants collected from San Diego, CA had hepatic PFOS concentrations ranging from 0.05 to 1.78 µg/g. Common cormorant liver samples collected from Tokyo Bay, Japan had PFOS concentrations that ranged from 0.17 to 0.65 µg/g and mallards and pintails had hepatic PFOS concentrations of 0.49 and 0.50 µg PFOS/g respectively (Table 1).

It is not known why control eggs contained measurable concentrations of PFOS. Samples of laying mash and chick starter used to feed the hens from which the eggs were derived and the hatchlings, respectively, were analyzed for

the presence of PFOS, and neither had detectable concentrations. A possible source of PFOS contamination is water, but this was not confirmed in this study. Eggs from a local supermarket had high concentrations of PFOS, ranging from 0.34 to 0.70 µg PFOS/g (Table 9). These concentrations are greater than those found in eggs from wild birds (Table 1).

CONCLUSION

In summary, the results of this study indicate that PFOS caused mortality of the chicken embryo after a single exposure prior to incubation. The LD_{50} for the chicken embryo was 0.97 µg PFOS/g egg. PFOS did not affect post-hatch body weights or organ weights. A single exposure to PFOS caused hepatic changes including bile duct hyperplasia, periportal inflammation and necrosis at doses as little as 1.0 µg/g egg. Hepatic PFOS concentrations increased in a dose-dependent manner.

Concentrations of PFOS at which toxic effects were observed in chicken embryos were similar to concentrations observed in wild bird eggs. PFOS caused significant embryo mortality in the 0.1 µg/g egg dose group, which had a mean hepatic PFOS concentration of 1.4 µg/g (range 0.7 to 2.2 µg/g). Similar concentrations have been found in the livers and eggs of wild fish-eating water birds. Even though previous studies have shown chickens to be more sensitive to some chemicals, such as polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) (Powell el al, 1996, 1997). It is difficult at this point to determine how the chicken responds to PFOS exposure compared to wild birds. If the chicken is more sensitive to PFSO compared to wild birds, then risk assessments based on Toxicity Reference Values (TRVs) derived from studies of the chicken would be protective of sensitive species.

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