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ASSESSING THE STABILITY, BIOLOGICAL TRANSFER AND DEVELOPMENTAL IMPACT OF ENVIRONMENTALLY RELEVANT NITROSAMINES USING A CHICKEN EGG MODEL

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by

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ASSESSING THE STABILITY, BIOLOGICAL TRANSFER AND DEVELOPMENTAL IMPACT OF ENVIRONMENTALLY RELEVANT NITROSAMINES USING A CHICKEN EGG MODEL

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Nitrosamines comprise a large group of potentially toxic compounds occurring in the environment as by-products of various manufacturing, agricultural and natural processes. Nitrosamines are produced from reaction of nitrite with a suitable secondary amine in an acidic matrix; these acidic conditions can occur in environmental media and in the mammalian gastrointestinal (GI) tract. This research focused on the stability, transfer, and impacts of the environmentally relevant nitrosamines, *N*-nitrosodimethylamine (NDMA), *N*-nitrosmorpholine (NMOR), and *N*-nitrosoatrazine (NNAT) (formed from reaction of nitrite with dimethylamine, morpholine, and atrazine), using the chicken egg and embryo model systems. Chicken eggs were used to determine nitrosamine transfer between a hydrophilic medium (egg white or albumin) and a lipophilic medium (yolk) via a biomembrane (vitelline membrane). Results from these studies with unfertilized chicken eggs showed that the selected nitrosamines transferred from the egg white to yolk where they were relatively stable. NNAT has a relatively higher affinity for the lipophilic yolk fraction, which suggests that it may have a greater potential to bioconcentrate than NDMA and NMOR. An understanding of the transfer behavior of nitrosamines can be used to assess bioavailability and fate, as well as potential environmental and biological

impacts. An observed decrease in total nitrosamine in the yolk with time may indicate denitrosation, releasing nitrous acid, which can decompose to nitrite and nitric oxide (NO), an important biological messenger during embryonic and fetal development. Thus teratogenicity of these compounds was assessed using chicken embryos. Major defects observed after exposure to these selected compounds included ectopic heart, gastroschisis, caudal regression, craniofacial hypoplasia, and neural tube defects. A significant relationship was observed between malformed embryos and NNAT (0.46 µg). Additionally nitrotyrosine concentrations (a marker of NO-mediated stress) in NNAT treated, malformed embryos were greater than those observed in treated, normal-appearing embryos. Results indicate that NNAT may be teratogenic and that nitrotyrosine, a marker of NO-dependent oxidative stress, maybe reflective of one biochemical pathway through which nitrosamines exert teratogenic effects.

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INTRODUCTION

Nitrosamines comprise a large class of mutagenic, teratogenic and carcinogenic chemicals found in the environment as by-products of various manufacturing, agricultural and natural processes (Magee and Barnes 1967, Lijinksy and Epstein 1970, Loeppky et al. 1994, McKnight et al.1999). Nitrosamines generally affect the GI tract, associated organs, and the brain (Mirvish 1995). Exposure to nitrosamines is estimated to be approximately 1.10 µmol/day (Tricker 1997, Lijinksy 1999). Nitrosamines are found in certain foods that contain nitrite or are exposed to nitrogen oxides (Walker 1990). Humans are also exposed to a wide range of nitrogen-containing compounds and nitrosating agents which can react *in vivo* under the acidic conditions of the gastric environment to form nitrosamines (Mirvish 1975, 1977, Lijinsky and Taylor 1977). *N*-nitroso compounds (NOCs) formed endogenously in the maternal stomach may be transmitted via the placenta to the fetus (Cowdin et al. 2003). Studies demonstrate that exposure to NOCs may be associated with birth defects such as neural tube defects and cleft palate, neonatal deaths and stillborns in rodents but the mechanisms are not yet understood (WHO, Takeuchi 1984, Carozza et al. 1995).

Partitioning between liquid and solid phases, membrane penetration, entry in the organs of the host, and subsequent biochemical effects determine chemical toxicity. The transfer behavior of nitrosamines can be studied using model systems such as the chicken egg. Aside from its use as a model of embryological development, chicken eggs can be used to study the partitioning of nitrosamines between hydrophilic or lipophilic biological compartments or phases.

The fertilized chicken egg and the developing embryo are useful models for the study of teratogenicity. The chick embryo provides an acceptable measure of embryotoxic potency (Jelinek et al. 1985) and chicken embryos are widely used to study development and developmental abnormalities (Rosenquist et al. 2001, 2007, 2010, Lie et al. 2010).

Denitrosation of nitrosamines in the endoplasmic reticulum may be a pathway of detoxification (Lee 1996, Williams 2004) that can lead to increases in nitric oxide which can cause cell injury. Hiramoto (2002) demonstrated that nitrosamines decomposed on contact with reactive oxygen species, accompanied by release of NO. Nitrotyrosine is a product of tyrosine nitration mediated by reactive nitrogen species such as peroxynitrite anion and nitrogen dioxide. Its concentration is a marker of NO-dependent oxidative stress and may reflect one pathway by which nitrosamines exert their teratogenic effects.

The three nitrosamines explored in this thesis are *N*-nitrosodimethylamine (NDMA), *N*-nitrosomorpholine (NMOR) and *N*-nitrosoatrazine (NNAT), representing environmentally significant nitrosamines. NDMA is a known hepatotoxin and carcinogen (Lijinsky et al. 1972, IARC). NMOR has also been found to be carcinogenic in many animal species (Preussmann and Tricker 1991, WHO). NNAT can be formed from atrazine, a widely used triazine herbicide that has been detected in groundwater (Spalding 2003).

The unfertilized chicken egg and developing embryo were used to determine:

(1) stability and transfer of the selected nitrosamines among biological compartments,

(2) teratogenic potential, and (3) impacts on nitrotyrosine concentrations.

LITERATURE REVIEW

Nitrosamine Formation

N-Nitroso compounds (NOC) consist of nitrosamines [RN(NO)R`] and nitrosamides [RN(NO)COR`]. *N*-Nitrosamines comprise cyclic nitrosamines (e.g., NMOR) and dialkylnitrosamines (e.g., NDMA) (Magee and Barnes 1967, Mirvish 1977). Nitrosamines first drew attention after an outbreak of acute hepatotoxicity in Norwegian sheep, which was linked to the presence of NDMA in nitrite-preserved fish meal (Magee and Barnes 1967). Magee and Barnes (1967) were the first to discover that NDMA was acutely hepatotoxic in a number of animal species. In murine models, nitrosamines have been found to induce tumors of the esophagus, nose, liver, kidneys, pancreas and other organs (Mirvish 1977).

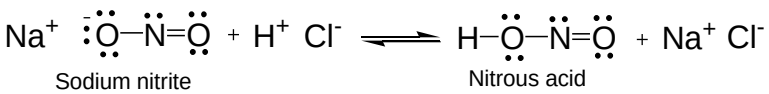
Nitrosamines have been found in foods that contain nitrite or are exposed to nitrogen oxides. These foods include fish, alcoholic beverages and cured meats (Walker 1990), especially bacon in which concentrations of 10-100 µmol/kg have been found (Lijinksy 1999). Cooking method, temperature and time influences the formation of nitrosamines in meat products (Lee et al. 2003). Humans are also exposed to a range of nitrogen-containing compounds and nitrosating agents which can react *in vivo* to form nitrosamines. Nitrosamines may originate from the reaction of nitrite and nitrosatable molecules under the acidic conditions of the gastric environment (Mirvish 1975, 1977, Lijinsky and Taylor 1977).

Mean nitrite levels were 0.1-2.6 and 26-54 µM for fasting gastric juice of pH < 5 and > 5, respectively (Xu and Reed 1993). An anion transport mechanism actively

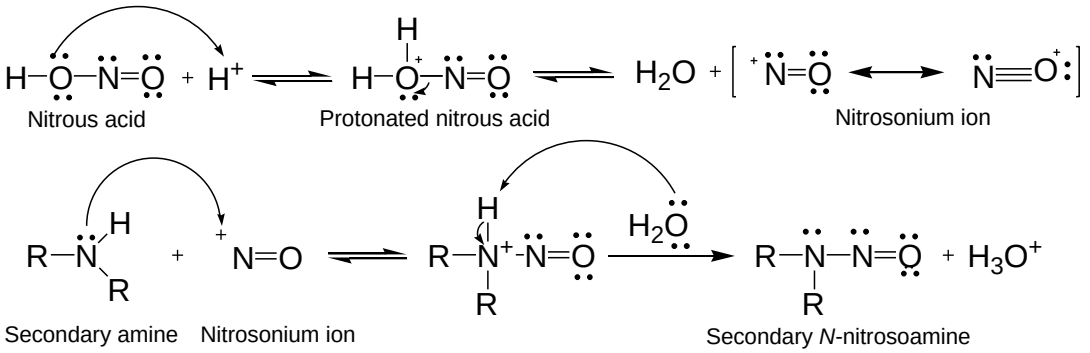
secretes 25% of the absorbed nitrate into the saliva. Oral bacteria reduce 5% of the ingested nitrate to nitrite (Spiegelhalder et al. 1976, McKnight et al. 1999). While nitrite itself can be sufficiently toxic, it also serves as a nitrosating agent (Spiegelhalder et al. 1976, Tenovuo.1986). The reduction of ingested or endogenous nitrate accounts for almost 80% of gastric nitrite in the normal acidic stomach. The remaining 20% of gastric nitrite arises from ingested nitrite found in processed foods (Mirvish 1977, 1983).

Nitrosatable molecules include secondary amines, tertiary amines, alkylureas and amino acids (Mirvish 1970, Lijinsky et al. 1972). Under the acidic conditions of the human stomach, nitrite is protonated to nitrous acid (HNO_2). HNO_2 can then spontaneously form dinitrogen trioxide (N_2O_3), nitric oxide (NO), and nitrogen dioxide (NO_2). NO^+ can also be donated by N_2O_3 to secondary and tertiary amines that can then form potentially carcinogenic nitrosamines *in vivo* (Leaf et al.1989). Under neutral conditions, NO can be formed from bacterial reduction of nitrite. NO in turn can react with molecular oxygen to form the nitrosating agents N_2O_3 and N_2O_4. Inducible nitric oxide synthase (iNOS) activity of inflammatory cells is also a source of NO. All of these mechanisms of endogenous nitrosation account for almost 40-75% of the total human exposure to nitrosatable compounds.

Secondary amines can be nitrosated to produce a nitrosamine. The formation of nitrosamines from secondary amines can be described by the following reactions (Eq. 1-3):



(1)



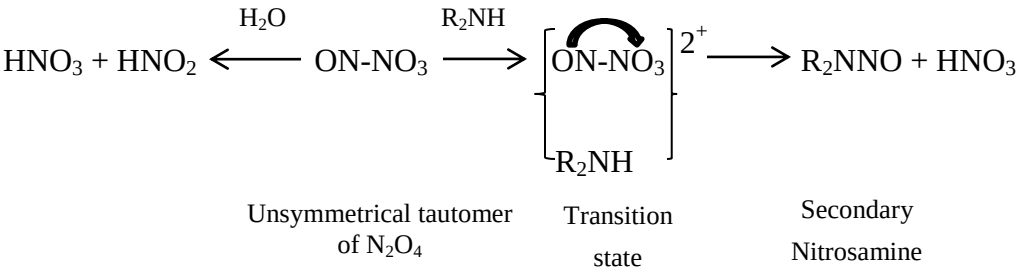
(2)

(3)

The kinetics of the nitrosation reaction depends on the pH of the medium and the pKa of the amine. Formation of a nitrosating agent is regulated by pH. The nitrite ion is protonated to form HNO_2 (pKa 3.37) (Eq. 1). This reaction is favored under acidic conditions (which occur in the human stomach). Under these conditions, HNO_2 may protonate, lose water, and be converted to N_2O_3 or form other activating nitrosating species, including the nitrosonium ion, nitrosyl thiocyanate, or nitrosyl halide (Eq. 2). Secondary amines will react with the nitrosonium ion to form nitrosamines (Eq. 3). The mechanism for nitrosation involves nucleophilic attack on the nitrosonium ion to form the nitrosamine. The unprotonated form of the amine is the more reactive form, so reactivity is greater for weak bases (Mirvish 1975, Mergens 1982). Thus, nitrosation occurs readily with weakly basic secondary amines such as morpholine and relatively slowly with strongly basic secondary amines such as dimethylamine (DMA). The rate of the reaction depends on the concentrations of the non-ionized amine and HNO_2. At pH > 1, the principal nitrosating agent is N_2O_3. The rate of the reaction is proportional to the concentration of nitrous anhydride and the square of nitrous acid, thus the rate of the reaction increases tenfold for each one unit decrease in pH (Mirvish 1970, 1972).

Under suitable conditions, primary amines may be alkylated to form secondary amines which can then be nitrosated. Likewise, tertiary amines can be dealkylated to form secondary amines which can be nitrosated to form nitrosamines. Tertiary amines with dimethylamine functional groups have been identified as potent NDMA precursors (Shafer et al. 2010).

Nitrosamines can also be formed in an alkaline solution when the nitrosating agent is present as N_2O_3 or N_2O_4 gas (Challis et al. 1978). The mechanism by which this occurs is still not understood completely; however a study (Challis et al. 1978) shows that only the unprotonated amino-nitrogen of the selected compounds participates in the nitrosation reactions. The possible reaction which may be occurring can be described as follows (Eq. 4):



(4)

Nitrosamine formation also can occur in the lipid phase, when the reactive amines are soluble (Mergens 1982). Unsaturated fatty acids have been shown to enhance (nearly double in fatty acid ester solutions) nitrosation of dicyclohexylamine in a lipid medium, under aprotic conditions, upon exposure to low concentrations of NO_2 (Pryor 1981). The nitrosating species under these conditions is HONO formed by the NO_2-unsaturated ester reactions. Pryor (1981) reported that the rate of nitrosation of the amine nearly doubles in the fatty acid solutions. This suggests that the HNO_2 (nitrous acid) formed from the reaction between NO_2 and the unsaturated fatty acid may be participating in nitrosation of

the amine. Only the unprotonated (more reactive) amine species would be soluble in a lipophilic medium. The pH dependence of the reaction is therefore eliminated and the probability of a reaction occurring between an amine and a nitrosating agent is very high (Pryor 1981, Mergens 1982).

Aside from pH, the rate of nitrosation is influenced by the presence of thiocyanate ions, halide ions, and formaldehyde, which accelerate the reaction, and ascorbic acid and α-tocopherol that block the formation of *N*-nitroso compounds (Mirvish 1977). Ascorbic acid and α-tocopherol compete for the available nitrite (i.e. N_2O_3 and $H_2NO_2^+$) and are thus capable of inhibiting the formation of nitrosamines. Ascorbic acid reacts rapidly with nitrite under acidic conditions to reduce HNO_2 to NO and is itself oxidized to dehydroascorbic acid. α-Tocopherol reduces NO_2 to NO in organic solvents and lipids, and its emulsions in water reduce nitrite to NO (Mirvish 1986).

Selected Nitrosamines

The nitrosamines explored in this study are *N*-nitrosodimethylamine (NDMA), *N*-nitrosomorpholine (NMOR), and *N*-nitrosoatrazine (NNAT). The physicochemical properties of these nitrosamines and their parent compounds are reported in Tables I and II, respectively:

Table I. Physicochemical properties of selected nitrosamines

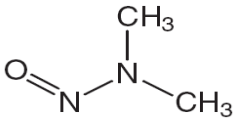
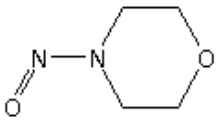
Structure and Name		Properties
	*N*-Nitrosodimethylamine (NDMA)	Volatile yellow oily liquid; Soluble in water, organic solvents and lipids; Photosensitive; Molecular weight = 74.1; Water solubility = 290 mg/mL; Density = 1.005 g/cm^3; Log K_{ow} = -0.57; Log K_{oc} = 1.07
	*N*-Nitrosomorpholine (NMOR)	Yellow crystals; Soluble in organic solvents; Photosensitive; Molecular weight= 116.1; Water solubility = >100 mg/mL; Density = 1.11 g/cm^3; Log K_{ow} = -0.43

Table I continued

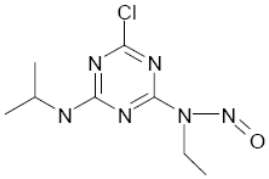
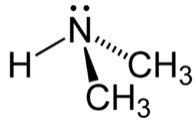
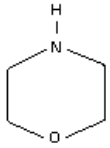
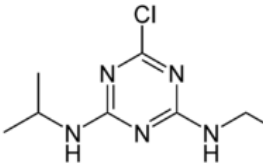
Structure and Name		Properties
	*N*-Nitrosoatrazine (NNAT)	Photosensitive; Molecular weight= 244.1; Water solubility= 0.29 mg/mL; Density= 1.42 g/cm^3

Table II. Physicochemical properties of parent compounds

Structure and Name		pKa	Properties
	Dimethylamine (DMA)	10.7	Colorless compressed liquefied gas with pungent odor ; Molecular weight= 450.1; Water solubility= 3540 mg/mL; Density= 0.67 g/cm^3; Log K_{ow} = -0.38
	Morpholine	8.7	Colorless oily volatile liquid; Water solubility- miscible; Molecular weight= 87.1; Density- 1.01 g/cm^3; Log K_{ow} = -0.86
	Atrazine	1.7	White solid; Molecular weight= 215.7; Water solubility= 0.033 mg/mL; Density= 1.187 g/cm^3; Log K_{ow} = 2.34

NDMA

NDMA is a known hepatotoxin (Lijinsky and Greenblatt 1972, Pegg 1980, Archer et al. 1994) and is classified as an IARC (International Agency for Research on Cancer) group 2A carcinogen (probably carcinogenic to humans) (IARC 1987, Lijinsky et al. 1972). NDMA may be present in air due to reactions between dimethylamine (DMA) and nitrogen oxides.

NDMA is widely used as an industrial solvent (Mirvish 1977). It can be synthesized by soil bacteria from various precursor substances, including nitrate, nitrite, and amine compounds. NDMA is also an inadvertent by-product of industrial processes, such as reaction of alkylamines DMA with nitrogen oxides, nitrous acid, or nitrite salts, or transnitrosation via nitro or nitroso compounds. NDMA may thus be present in discharges of rubber manufacturing, leather tanning, pesticide manufacturing, food processing, meat tinning and dye manufacturing industries (Mitch et al. 2003, Blicharz et al. 2005, Vocht et al. 2007). NDMA has also been identified in baby pacifiers, emissions from diesel vehicle exhaust and it can be released from industrial sources as a contaminant of products such as liquid rocket fuel (Sen et al. 1985, Mitch et al. 2003). NDMA has been detected in the air in chemical (0.05-0.5 $\mu g/m^3$) and rubber industries (0.07-0.14 $\mu g/m^3$) (Fajen et al. 1979).

Dietary sources of NDMA include beer, fish and fish products, dairy products, infant formula, cured meats, cereals and vegetables. In fact, NDMA accounts for almost 86% of the total nitrosamines in salted fish in China (Bulushi et al. 2009). The

contribution of NDMA from food is considered high even though concentrations in most food products are relatively low (Fristachi and Rice 2007, Shafer et al. 2010).

California's Department of Public Health (CDPH) has set 10 ng/L notification levels (advisory levels for chemicals in drinking water that lack maximum contaminant levels) for NDMA in drinking water. Most nitrosamine releases from industries are to sewage and subsequently water. NDMA concentrations as high as 0.11 mg/L have been detected in effluents from manufacturing industries (Mitch et al. 2003). DMA and nitrite may enter surface water streams from agricultural runoff. NDMA may also be formed during treatment of drinking water. Water treatment plants incorporating a chlorination process (e.g., sodium hypochlorite) produce NDMA from precursors (WHO, Fristachi and Rice 2007, Asami et al. 2009). High levels of NDMA have been detected in outdoor and indoor pools and hot tubs (Walse and Mitch 2008). Free chlorine (HOCl) may react with ammonia to form monochloramine (NH_2Cl) which in turn may react with DMA to form dimethylhydrazine ($(CH_3)_2NNH_2$) which then oxidizes to NDMA (Eq 5-7) (Mitch et al. 2003).

$$NH_3 + HOCl \rightarrow NH_2Cl\ (MC) + H_2O \quad (5)$$

$$NH_2Cl + (CH_3)_2NH \rightarrow (CH_3)_2NNH_2 + H^+ + Cl^- \quad (6)$$

$$(CH_3)_2NNH_2 + 2NH_2Cl + H_2O \rightarrow (CH_3)_2NNO + 2NH_3 + 2H^+ + 2Cl^- \quad (7)$$

NMOR

NMOR (nitrosated morpholine) is carcinogenic in many animal species (WHO, Tricker and Preussmann 1991) and it has been found to be mutagenic (Manson et al. 1978). NMOR is an IARC group 2B carcinogen (sufficient evidence of carcinogenesis in several experimental animal species) (WHO) as it is responsible for inducing liver, nasal-cavity, tracheal, oesophagus and stomach tumors in several animal models (Lofberg 1985).

Approximately 25000 metric tonnes of morpholine are produced industrially each year (WHO). It is a versatile chemical that is used as an intermediate for rubber polymerization accelerators, corrosion inhibitors, synthesis of optical brighteners, crop protection agents, dyes and drugs, polishes/waxes and food additives (WHO, Mirvish 1972, Sen et al 1987, Grosjean 1991, Vocht et al. 2007). Some countries still use morpholine in toiletry and cosmetic products, and in several direct and indirect food additive applications. Human exposure to morpholine arises from gaseous and aqueous emissions and directly from some of its uses. NMOR was produced in mice exposed to morpholine and NO_2 (Mirvish et al. 1981). NO_2 itself is a pollutant formed from combustion processes such as gas cooking, cigarette smoking, automobile exhaust, and flame drying of foods (Cooney and Ross 1987). Morpholine emissions mainly result from its manufacture and use in the chemical industry. Morpholine has been detected in a wide variety of foods and tobacco with the source being the coatings of wax on fruit or on packaging papers (WHO, Sen 1986). 90-4830 µg/kg of morpholine was detected in the waxed cardboard containers used to package snuff and snuff tobacco itself was found

to contain NMOR (10-690 µg/kg) (Brunnemann et al. 1982). NMOR levels of up to 140-670 µg/kg have been detected in waxes used to protect apples (Tricker et al. 1989).

Like NDMA, NMOR has been identified as an air pollutant in chemical (0.07 $\mu g/m^3$-5.1 $\mu g/m^3$) and aircraft tire industries (0.6 $\mu g/m^3$- 27 $\mu g/m^3$) (Fajen et al. 1979, Spiegelhalder and Preussmann 1983). NMOR has been detected in the rubber industry and in diesel engine crankcase emissions (Lofberg 1985). Disinfected wastewater effluents have also been shown to contain NMOR (Kulshrestha et al. 2010). NMOR has been identified in drinking water at maximum concentrations of about 3 ng/L (Padhye 2010).

NNAT

NNAT (nitrosated atrazine) can be formed from atrazine, a widely used broadleaf triazine herbicide that has been detected in groundwater (Spalding et al. 2003). Exposure to atrazine may occur on the application of the herbicide or consumption of contaminated food or water (Mirvish et al. 1991). Due to the widespread application of agrichemicals in the Midwest, some groundwaters are contaminated with nitrate and atrazine. Groundwater contaminated with atrazine often contains nitrate. A 1997 study (Gosselin et al. 1997) reported detection of atrazine in 70 Nebraska domestic wells. Wells contaminated by atrazine had a median nitrate-nitrogen concentration of 11.5 µg/L. High concentrations of atrazine (1500 µg/kg) have been detected in Wisconsin, where the wells also have high nitrate levels (Meisner et al. 1993). Elevated concentrations of nitrate and atrazine create the potential for NNAT formation after ingestion. Thus exposure to atrazine and nitrate may occur via consumption of contaminated water. Atrazine is a

secondary amine that can be readily nitrosated to form nitrosoatrazine under suitable conditions (Mirvish et al. 1991). Atrazine nitrosates almost 200 times faster than DMA at pH 2 (Wolfe et al. 1976) and NNAT is relatively stable at alkaline pH (Mirvish et al. 1991). NNAT is more photolabile than NMOR in water or CH_2Cl_2 which may be due to UV absorption by its triazine ring (Wolfe et al. 1976, Mirvish et al. 1991). NNAT is also mildly mutagenic in the Ames assay (Weisenburger et al. 1988, Gammon et al. 2005). Meisner (1993) found that exposure of lymphocytes to NNAT resulted in chromosome breakage.

Nitrosamine Sources and Exposure

Nitrosamines have been studied extensively in foods as they are found in certain foods that contain nitrite or are exposed to nitrogen oxides. These foods include dried salted fish, alcoholic beverages and cured meats (Mirvish 1977, Walker 1990), especially bacon in which concentrations of 10-100 µg/kg have been found (Lijinksy 1999). Human exposure to nitrosamines is estimated to be approximately 1.10 µmol/d. Major sources of exposure are the diet (0.79 µmol/d, 80-120 µg/d; 72% of the total exposure), occupational exposure (0.15-0.30 µmol/d; 25%), cigarette smoking (0.02 µmol/d, 3.4 µg /d; 2%), and miscellaneous sources, including pharmaceutical products (Brambilla et al. 1985, Dawson and Lawrence 1987), cosmetics, indoor and outdoor air (0.001 µmol/d, 0.1 µg/d; 1%) (Tricker 1997). Consumer products such as foods, beverages, pharmaceutical drugs and cosmetics that contain nitrosatable compounds and nitrite or have been exposed to nitrous oxides can be a source of nitrosamines (Rosenberg et al. 1980, Tenovuo 1986, Lijinksky 1999, Yurchenko and Molder 2006).

While nitrate levels are highest in vegetable products (189 mg of nitrate/serving), nitrite levels are highest in meat and bean products (1.84 mg/serving). Alcohol, meat and dairy products contain the highest concentrations of nitrosamines (0.531 µg/serving) (Griesenbeck et al. 2009) (serving indicates standard serving sizes in the United States as reported by the Centers for Disease Control).

While there is considerable research on nitrosamine exposure from food, concern has been expressed about nitrosamine formation, occurrence and exposure in the environment. A number of drugs are secondary or tertiary amines (e.g. chlorpheneramine, cefadroxil, diphenhydramine, ethambutol, furosemide, metoprolol, procainamide, propranolol, and ranitidine) and can be easily nitrosated to form NOC. Large doses of drugs are ingested by mouth and their chronic presence in the GI tract could be hazardous if they nitrosate to form carcinogenic NOCs. For example, oxytetracycline reacts endogenously with nitrite to form NDMA. Similarly, methapyrilene and chlorpheniramine can also react with nitrite to form NDMA (Mirvish 1995).

Occupational exposure to volatile nitrosamines occurs in rubber/latex and leather curing industries. NDMA and NMOR have been detected in the air of rubber industries. These are thought to arise from the reaction of exhaust NO_2 with amines arising from vulcanization accelerators (Mirvish 1995). Average daily intake of NDMA by individuals working in rubber industries is 0.8 $\mu g/m^3$ (Tricker et al. 1989). The estimated daily intake of NMOR by individuals working in rubber industries is 3.8 µg (Tricker 1989).

Of the three nitrosamines considered for this study, the presence of and exposure from NDMA has been examined most extensively. NDMA concentrations as high as 70 µg/L have been detected in German beer, although levels are usually much lower (5-10

µg/L). In the U.S., the estimated dietary exposure to NDMA from beer is almost 0.3-0.97 µg/d (Walker 1990). NDMA has also been detected in bacon (17 µg/kg), smoked pickled fish (32 µg/kg) and Japanese broiled squid (300 µg/kg) (Lijinksky 1999). NDMA has been identified in Korean dried seafood products and cooking has been shown to increase the NDMA content (from about 147 to 630.5 µg/kg) (Lee et al. 2003). NDMA concentrations of 0.003-0.4 mg/L have been found in groundwater near rocket engine testing facilities (Mitch et al. 2003). High concentrations of nitrosamines have been detected in latex gloves and studies have shown that these can migrate and be potentially toxic (Feng et al. 2009). NDMA is still found in some fish products (Mirvish 2008). A daily tolerance limit of 4 - 9.3 ng NDMA/kg/d or 280– 650 ng/d for a 70 kg person has been identified (Schafer et al. 2010).

Nitrosamine Carcinogenicity and Mutagenicity

In animal models, nitrosamines induce tumors of the liver, nose, kidneys, pancreas, esophagus and other organs. Several studies have shown that tumors are induced in mice after treatment with sodium nitrite together with various secondary amines (Mirvish 1972). More than 300 NOCs are carcinogenic in one or more animal species (Preussmann and Stewart 1984, Hasegawa et al. 1998). Exposure to nitrosamines has been associated with mortality from cancers of the oesophagus, oral cavity, and pharynx (Straif et al. 2000). NNAT was also mutagenic in the Ames assay (Weisenburger et al. 1988, Gammon et al. 2005).

Nitrosamines require metabolic activation to exert carcinogenic and mutagenic effects. Cytochrome P450 (P450) enzymes activate nitrosamines in the endoplasmic

reticulum to form α-hydroxynitrosamines, which spontaneously decompose to form monoalkylnitrosamines and alkyldiazohydroxides (Magee and Barnes 1967, Mirvish 1977, 1995). Formation of monoalkylnitrosamines followed by alkyldiazohydroxides is known as dealkylation. Alkyldiazohydroxides are capable of alkylating nucleophiles to form diazoalkanes, some of which can alkylate DNA bases especially at N-7 and O-6 of guanine and O-4 of thymine (Mirvish 1995). This induces mutations which are thought to initiate carcinogenesis. DNA damage due to alkylation of N-7 in guanine also generates reactive oxygen species such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Consequences include increased lipid peroxidation, protein adduct formation, and pro-inflammatory cytokine activation (Tong et al. 2010).

Teratogenic Potential of Nitrates, Nitrites and Nitrosamines

Maternal exposure to nitrosatable compounds may be related to birth defects (WHO, Takeuchi 1984, Carozza et al. 1995, Croen et al. 2001, Cowdin et al. 2003, Brender et al. 2004, Manassaram et al. 2007). Associations have been found between occupational exposure in agricultural work and pesticides and the risk of anencephaly (a neural tube defect that occurs when the cephalic end of the neural tube fails to close, resulting in the absence of a major portion of the brain, skull, and scalp) and other adverse pregnancy outcomes such as spontaneous abortion and preterm delivery (Greenlee et al. 2004, Lacasana et al. 2006).

NDMA decreased the hatching of fertilized eggs in carp (Bieniarz et al. 1996). Ten mg/kg of methylnitrosourea induced limb and other defects in murine models (Koyama et al. 1970, Iannaccone et al. 1982). Exposure to *N*-methyl-*N*-nitrosourea also increased

gross malformations in mice (Bossert and Iannaccone, 1985) and *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine induced mid line defects in mice (Inouye and Murakami 1978). NDMA is lethal to or inhibits the growth of chick embryos (Maduagwu and Bassir 1979). A methyl derivative of NDMA, *N*-Methyl-*N*-(α-acetoxy)methyl-nitrosamine is a strong teratogen in a mouse limb bud culture system (Stahlmann et al. 1983). Takeuchi (1984) showed that exposure to *N*-nitroso compounds was associated with neural tube defects and cleft palate in mice. Certain nitrosatable drugs have been associated with an increased risk of craniosynostosis (premature fusion of the sutures of the skull). It has been suggested that ischaemia (restriction in blood supply) and reperfusion injury (tissue damage caused after a period of ischemia) leading to an increase in the rate of formation of NO may be the cause of dysmorphogenesis (Gardner et al. 1998).

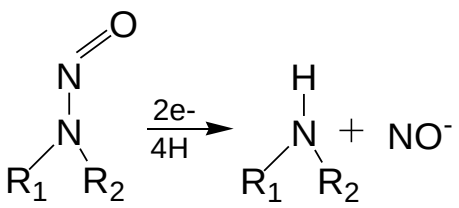
A 2004 epidemiological study reported an association between intake of dietary nitrite and neural tube defects in humans (Brender et al. 2004). Croen showed that maternal exposure to nitrate-contaminated drinking water was associated with risk of neural tube defects (Croen et al. 2001). Exposure to nitrate in drinking water at levels greater than 45 mg/L (the maximum contaminant level) and in groundwater at concentrations below the maximum contaminant level has been associated with an increased risk for anencephaly (Croen 2001). Many other studies have indicated links between drinking water containing nitrate and neural tube defects (NTDs) (Ward et al. 2005). The risk of NTDs from high levels of nitrates in food/water increases if mothers are exposed to nitrosatable drugs (Brender et al. 2004, Manassaram 2007).

Elevated concentrations of dietary nitrosamines have been significantly associated with gastroschisis (failure of the abdominal wall to close) (Torfs et al. 1998).

In fact, several studies have shown an increase in the rates of gastroschisis and other abdominal wall defects in the past two decades (Laughon et al. 2003, Alvarez et al. 2007, Collins et al. 2007, Vu et al. 2008). Annola et al. (2009) demonstrated that the human fetus can be exposed to NDMA from maternal blood circulation.

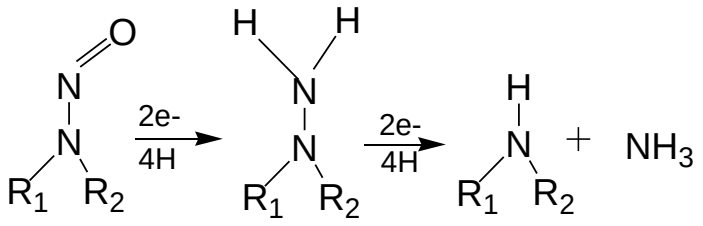
Nitrosamine Dealkylation and Denitrosation

Research is limited regarding the denitrosation of nitrosamines and fate in environmental media and in the mammalian digestive tract. Nitrosamines can be reduced across the N-N bond or the N-O bond. Reduction across the N-N bond releases the parent amine and nitrous acid. The nitrous acid released can decompose to nitrogen dioxide, nitric oxide and water. It may also decompose to nitric acid, nitrous oxide and water (Eq. 8). Near the site of NO production, NO reacts with dissolved oxygen to form N_2O_3 and N_2O_4 which react with water at neutral pH to form nitrite and nitrate, and with amines to form nitrosamines (Mirvish 1995). Reduction across the N-O bond yields a hydrazine which can be further reduced to the parent amine and ammonia (Eq. 9). Hydrazine formation may be a pathway for bioactivation as it is a highly reactive base and reducing agent.



N-nitrosoamine

(8)



N, N-Dialkyl Hydrazine

(9)

Metabolism of nitrosamines includes dealkylation and denitrosation. Denitrosation in the endoplasmic reticulum may be a pathway of detoxification (Lee 1996, Williams 2004). There are two denitrosation mechanisms mediated by P450 isoenzymes. One electron reduction produces NO and the secondary amine which may be dealkylated. One electron abstraction liberates NO via an oxidative mechanism involving the formation of an aminium cation radical. An alkylidenaminoalkane is formed due to the loss of a proton which in turn hydrolyses to the primary amine and the corresponding aldehyde (Haussmann and Werringloer 1987, Appel et al. 1991). Studies with rat liver microsomes have demonstrated that denitrosation of NDMA accounts for the formation of methylamine and the production of NO via an oxidative mechanism, which is a precursor of nitrite (Keefer et al. 1987, Haussmann 1987). Although denitrosation is a possible mechanism of detoxification of the nitrosamine, toxic effects due to NO and its conversion to nitrite and nitrate (due to intermediate formation of the NO_2 radical) are possible (Appel et al. 1991).

Denitrosation can increase NO and can lead to cell injury (Lee 1996, Williams 2004). The liberated NO also may result in formation of nitrosamines via nitrosation. Hiramoto (2002) demonstrated that on contact with reactive oxygen species (ROS), *N*-nitrosamines decomposed, with release of NO. Nitrosamine metabolism may result in the formation of ROS, including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and

hydroxyl radicals ($OH^{\bullet}$). These ROS may cause oxidative stress and possible induction of hepatocellular necrosis, carcinogenesis, tumor formation and other cell damage (Farombi et al. 2009).

Nitric oxide, Peroxynitrite and Nitrotyrosine

Nitric oxide (NO) is an important biological messenger that mediates critical physiological processes, including gene regulation, immune regulation, apoptosis, neurotransmission and vascular smooth muscle cell relaxation (Stamler et al. 1992, Tiboni and Clementini 2004). NO synthesis via oxidation of L-arginine involves unusual chemistry that has uncovered novel aspects of eukaryotic enzymology. L-arginine synthesizes NO by the enzyme nitric oxide synthase (NOS) (Stamler et al. 1992, Tiboni and Clementini 2004). Of the three isoforms of NOS, the constitutive isoforms nNOS (neuronal NOS) and eNOS (endothelial NOS) produce small amounts of NO while iNOS (inducible NOS) produces much larger amounts of NO (Dijkstra et al. 1998). The biological activity of NO is due to its direct actions on the enzyme guanylyl cyclase. The activation of guanylyl cyclase by low concentrations of NO is the major pathway of NO signaling that is involved in the regulation of many physiological functions such as neurotransmission and vascular smooth muscle relaxation (Moncada et al. 1991).

Studies show that a change in NO can alter NO^{-} mediated intracellular signaling which can adversely affect embryonic and fetal development (Fantel and Person 2002, Trapp et al. 2006). Excessive or inadequate NO can lead to reproductive and developmental failure (Tiboni and Clementini 2004). Inhibition of NO may lead to limb

defects (Tiboni and Clementini 2004). Abnormally high levels of NO are involved in the hypotension associated with endotoxic shock (Kilbourn and Belloni 1990) and inflammatory response-induced tissue injury (Mulligan et al. 1991). A recent study by Alexander et al. (2007) demonstrated that changes in NO levels resulted in morphological defects of the heart, neural tube and eyes in chick embryos. While NO itself can be cytotoxic, it may also exert adverse effects via production of other oxidizing agents (Dijkstra et al. 1998).

Peroxynitrite ($ONOO^-$) is produced by the reaction of NO with superoxide radical ($O_2^{\bullet}$) which occurs at a high rate (Kaur 1994). Peroxynitrite is a potent oxidizing agent that has been implicated in various pathological conditions. The adverse effects of $ONOO^-$ include tissue damage by lipid peroxidation and DNA strand breaks leading to apoptosis and oxidation of protein sulfydryl groups (Dijkstra et al. 1998). The half-life of $ONOO^-$ *in vivo* is quite short which is why it is usually measured in terms of the formation of the comparatively more stable nitrotyrosine (Takizawa et al. 1999). Peroxynitrite can decompose to products that nitrate aromatic amino acids, which can be markers of NO^- dependent oxidative damage *in vivo* (Kaur 1994). One of these markers is 3-nitrotyrosine. Measuring 3-nitrotyrosine may be a useful way to provide evidence of NO^- mediated pathology as it is indicative of a more intense oxidative stress (Kaur 1994, Pacher et al. 2007).

Nitrotyrosine is produced when $ONOO^-$ reacts with tyrosine (i.e. it induces nitration of tyrosine) or to proteins containing tyrosine residues (Halliwell et al. 1997, Gal et al. 1997). Tyrosine nitration involves addition of a $–NO_2$ group near the –OH group on the tyrosine aromatic ring. For tyrosine nitration to occur, a hydrogen atom is

abstracted from tyrosine to form a tyrosyl radical. The tyrosyl radical then rapidly reacts with nitrogen dioxide to form 3-nitrotyrosine (Pacher et al. 2007). This nitration of tyrosine residues can subsequently result in the loss of protein structure, function and activity, which may compromise cell signal transduction, alter cytoskeletal organization and bring about a change in the catalytic activity of enzymes. This is why tyrosine nitration is considered a vital aspect of peroxynitrite-mediated cytotoxicity (Pacher et al. 2007).

Nitrotyrosine levels are elevated in many pathological conditions where inflammation is observed. Nitrotyrosine is also elevated in the plasma protein of people with chronic renal failure, atheroschelrotic plaque, and ischemia-reperfusion injury. High nitrotyrosine levels are also observed in people with diseases that have a high oxidative stress burden such as diabetes (Takizawa et al. 1999**,** Nakazawa et al. 2000**,** Mohiuddin et al. 2006**)**. Elevated nitrotyrosine concentrations have been found in malformed murine embryos (Trapp et al. 2006).

Use of chicken egg and embryo model systems to assess bioavailability and potential teratogenicity

Health risks do not always correlate with the total amount of toxicant in an environmental matrix or a biological system. While all of the nitrosamine that is present may be considered *bioaccessible*, assessment of potential impacts requires a determination of *bioavailability*. Bioavailability is the rate and extent to which an active agent is absorbed and becomes available at the site of action. The bioaccessible fraction

is considered to represent the maximum amount of contaminant that is available for intestinal absorption. Bioaccessible fractions of nitrosamines may be absorbed and transferred into the blood (or lymph) stream (Oomen et al. 2002) and bioavailability depends, in part, on the route of exposure (Caussy 2003, Harmsen 2007).

Partitioning between liquid and solid phases, membrane penetration, entry in the organs of the host, and subsequent biochemical effects determine nitrosamine toxicity. The partitioning behavior of nitrosamines can be studied using model systems. Aside from serving as a model of embryological development, chicken eggs can be used to study the transfer of nitrosamines among biological compartments.

In chicken eggs, the yolk is centered in the albumin or egg white and is surrounded by the vitelline membrane (Fig.1). The egg white (pH 7.9-8.0) makes up 60% of the total egg weight and is comprised of the proteins ovomucin, globulins, conalbumin, ovalbumin, lysozyme, ovotransferrin and ovomucoid (Palmer 1944, Mine 2007). Water is the major constituent of egg white (88%) while proteins account for 11%. Polar amino acids alternate along the peptide chain in egg white. Egg yolk contains nonpolar triacylglycerols and polar phospholipids. In the yolk (pH 5.9-6.0), polyedric droplets are surrounded by a membrane in which high density lipoprotein granules and low density lipoprotein micelles are held in the aqueous phase. Egg yolk lipids include cholesterol, triglycerides, cerebrosides and phospholipids (Palmer 1944, Mine 2007). The vitelline membrane is composed of glycoprotein, carbohydrate and lipids (Ternes 2001). Thus the egg serves as an ideal model to study the transfer of the selected nitrosamines between the hydrophilic and lipophilic phases.

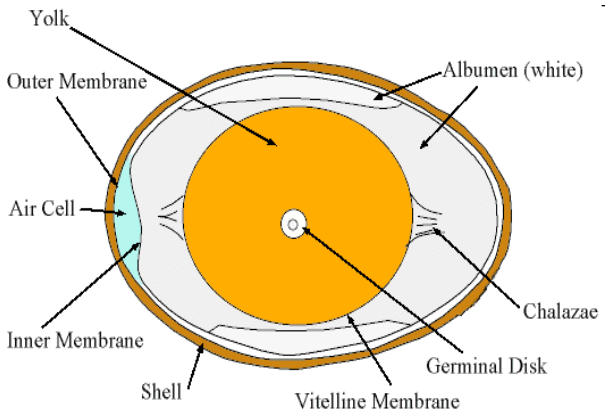


Fig.1. Structure and components of the chicken egg.

The embryotoxic potential of a compound depends on factors such as dose, critical window of exposure and sensitivity of the developing morphogenetic system at the time of administration. Also important is the metabolic activity of the morphogenetic system that transforms a substance to active or inactive metabolites.

The chicken egg and developing embryo are useful models for the study of teratogenicity. Chicken embryos are widely used to study development and developmental abnormalities (Rosenquist et al. 2001, 2007, 2010, Lie et al. 2010). The chick embryo *in ovo* represents a morphogenetic system that includes epigenetic tissue interactions and it possesses a drug metabolizing capacity. The chick embryo provides an acceptable measure of embryotoxic potency (Jelinek et al. 1985). It is an inexpensive and rapid *in vitro* model system. Hamburger and Hamilton staged the chicken embryo in 1951, describing the various features of the chicken that can be observed at specific times (mean of a range) after fertilization. The chicken embryo reaches the blastoderm stage by the time the egg is laid. Three h after fertilization the newly formed single cell divides and division continues until there are many cells grouped in a small, whitish spot visible

on the upper surface of the egg yolk. Within the first 24 h, the alimentary tract appears and the brain crease, nervous system and head fold begin to form. The heart starts to beat within 48 h (Hamburger and Hamilton 1951). The external form of chicken embryos at various stages of development can be studied beginning with the second or third day of incubation by carefully breaking open or windowing an egg each day (Matthew et al. 2007). Defects such as neural tube abnormalities can also be detected by observing the developing embryo within 36 h of incubation (Madeleine et al. 2005). Thus the chick embryo model can provide a useful tool to screen for toxicity and developmental abnormalities.

MATERIALS AND METHODS

Chemical Reagents and Eggs

All chemicals used in the experiments were analytical grade. Atrazine (98%) was obtained from Chem Service (West Chester, PA). NDMA and NMOR were purchased from Sigma-Aldrich (St. Louis, MO). NNAT was synthesized using the method of Mirvish et al. (1991). Fertilized chicken eggs were obtained from Charles River Laboratories International, Inc (Wilmington, MA).

Nitrosamine Stability and Transfer among Biological Compartments

Unfertilized chicken eggs were used to characterize the stability and transfer of the selected nitrosamines. To determine the stability of NDMA, NMOR and NNAT, the egg white, membrane and yolk were separated and weighed. One mL of deionized, distilled (DD) water containing 1 mg nitrosamine/mL was added to individual fractions of the egg. After incubation on a rotary shaker at room temperature (ca. 25 °C) for different time intervals (4, 6, 10, 16 and 24 h), the yolk, egg white and membrane fractions were separated and weighed. Using methodology similar to the EPA SW846 method for nitroaromatics and nitramines, nitrosamines were extracted from each egg fraction. In this procedure, acetonitrile was added to each fraction and the samples were placed on the shaker for 16 h. The supernatant was centrifuged, filtered and analyzed by HPLC (Shimadzu, Kyoto, Japan), using a 250 × 4.6 mm Keystone NA column (Thermo Hypersil-Keystone, Bellefonte, PA).

To characterize the transfer of the selected nitrosamines between the biological compartments, NDMA, NMOR and NNAT were added to the air cell, via a window, and

the treated eggs were placed on a rotary shaker at room temperature for varying time intervals (1, 4, 6, 10, 16, 24, 48, 72 and 120 h). After incubation and subsequent separation of the fractions, the same method of extraction, detection and quantification was used to determine the amount of nitrosamine in each fraction of the egg.

To determine nitrosamine transfer within the egg, its distribution between just two fractions was studied. To determine the distribution between the egg white and membrane, the yolk was removed and the nitrosamine was added to the egg white-membrane matrix. For the distribution between membrane and yolk, the egg white was removed after which the nitrosamine was added. To determine the distribution between egg white and yolk, the nitrosamine was added to the egg. Treated eggs were placed on a rotary shaker at room temperature for varying time intervals (1, 4, 10 and 24 h), after which the amount in each fraction was determined. Extraction and analysis of the nitrosamine were carried out using the method described above. All values were corrected for recovery.

Teratogenic Potential

Experiments were performed using a chick embryo model to determine dose-response and evaluate the teratogenic potential of atrazine, NDMA, NMOR and NNAT. Pathogen-free fertilized chicken eggs were incubated in a forced air incubator at 38 °C and 65–75% relative humidity for 30 h (HH stage 9 – 10; Hamburger and Hamilton 1951). The eggs were treated in the air cell at HH stage 9 -10, when there are 7 to 10 somites (Hamburger and Hamilton 1951), by delivering 50 µL of DD water containing various concentrations of each of the selected nitrosamines using a micropipette.

Negative controls included fertilized eggs injected with DD water. The eggs were returned to the incubator after dosing. The embryos were harvested at days 3 (72 h, HH stage 20) and 5 (120 h, HH stage 27) and examined microscopically for soft tissue and skeletal abnormalities, including: neural tube defects (failure of the neural tube to close), craniofacial hypoplasia (tissue deficiency or agenesis, failure of an organ to develop during embryo development), microphthalmia (abnormally small eye/s), anophthalmia (absence of one or both eyes), ectopic heart (displacement of the heart outside the thoracic cavity), gastroschisis (intestines and other organs develop outside the fetal abdomenfailure of the abdominal wall to close), and caudal regression (lack of or the degenerative regression of the caudal aspect of the embryo leading to absence or lack of caudal stuctures).

Embryos were compared to the negative controls to identify developmental anomalies and assess mortality and abnormality rates. The amounts of nitrosamine in the 50 µL injections and concentrations of the three nitrosamines used to treat fertilized chicken eggs are reported in Table III.

Table III. Dose and concentration of selected nitrosamines

Compound	Dose (µg in 50 µL DD water)	Concentration (µg/g egg matrix (excluding egg shell))
NDMA	12.5	0.2
	25.0	0.5
	50.0	0.9
NMOR	25.0	0.5
	250.0	4.7
	1250.0	23.6
	2500.0	47.2
	5000.0	94.3
	7500.0	141.5
NNAT	0.06	0.001
	0.01	0.002
	0.2	0.004
	0.5	0.009
	0.9	0.017

Table III continued

Compound	Dose (µg in 50 µL DD water)	Concentration (µg/g egg matrix (excluding egg shell))
NNAT	1.8	0.03
	3.6	0.07
	5.5	0.10
	7.3	0.14
	14.5	0.27
Atrazine	16.5	0.31

Nitrotyrosine Determination

Measuring the stable 3-nitrotyrosine may be a useful way to provide evidence of NO-mediated pathology as it is indicative of intense oxidative stress. Nitrotyrosine was measured in tissue sonicates of embryos treated with DD water (negative controls), embryos treated with NNAT that appeared normal and embryos treated with NNAT that were malformed using the *Oxis*Research™ Bioxytech® Nitrotyrosine Enzyme Immunoassay (EIA) for Nitrotyrosine (Portland, OR). The EIA is a "sandwich" ELISA. The antigen that is captured by a solid phase monoclonal antibody is detected with a biotin labeled goat polyclonal anti-nitrotyrosine. A streptavidin peroxidase conjugate then

binds to the biotinylated antibody. A tetramethylbenzidine (TMB) substrate is added and the yellow product is measured at 450 nm. Nitrotyrosine concentrations (expressed in terms of nmol/µg Protein) were measured in tissue sonicates of five-day-old embryos treated with different doses of NNAT (0.06, 0.23, 0.46, 0.91 and 3.63 µg/50 µL) and deionized, distilled water (controls). Protein concentrations (expressed as µg/µL) were determined in control and treated embryos using the Bradford assay (Bradford 1976, Kruger 2002).

HPLC Analyses

The nitrosamines (NDMA, NMOR and NNAT) and atrazine were identified and quantified by HPLC. HPLC analyses were carried out by injecting 10 µL of sample onto a 250 × 4.6 mm Keystone NA column (Thermo Hypersil-Keystone, Bellefonte, PA). HPLC mobile phases and operating conditions for the nitrosamine analyses are given in Table IV.

TABLE IV. HPLC conditions for nitrosamine analyses

Compound	Wavelength (nm)	Flow rate (mL/min)	Mobile phase
NDMA	220	1.0	70:30 Methanol:Water
NMOR	248	1.0	50:50 Water:Acetonitrile
NNAT	246	1.0	50:50 Water:Acetonitrile
Atrazine	235	1.0	50:50 Water:Acetonitrile

Statistical Analyses

Chi square tests (SAS, Cary, NC) were used to examine the relationship between NDMA, NMOR and NNAT treatment and the subsequent effect observed (i.e., embryo death and malformation). Differences in nitrotyrosine concentrations between and within groups (i.e., treated malformed, treated normal and controls) were determined by analysis of variance (ANOVA) in SAS (SAS Institute Inc., Cary, NC). Risks of mortality and malformations from exposure to the three nitrosamines (versus the non-exposed group i.e. controls) were determined by Relative Risk in SAS (SAS Institute Inc.). Relative risk is the risk of developing a particular condition (in this case malformations and mortality) for one group compared to another group: Relative risk = $P_{exposed} / P_{non\text{-}exposed}$. Analysis of relative risk is used frequently in the statistical analysis of binary outcomes where the outcome of interest has relatively low probability. For all analyses, statistical significance was accepted at a p value = 0.05.

RESULTS AND DISCUSSION

Stability and Transfer of Selected Nitrosamines

To study the stability and transfer of nitrosamines, experiments were carried out using unfertilized chicken eggs. Results indicate that NDMA, NMOR and NNAT partition into the yolk where they are fairly stable and it is hypothesized that this may impact NO concentrations during embryo development in fertilized eggs. Standard deviations (n=3) were typically small for egg white and yolk measurements but large for membrane measurements due to the difficulty in separating this fraction.

Stability of NDMA

To determine the stability of NDMA in the various fractions of the egg, a known amount was added to each fraction and the amount remaining was determined over time.

The amounts of NDMA in the egg white and yolk were relatively constant over time (Fig. 2), indicating that NDMA is quite stable in the egg white and yolk.

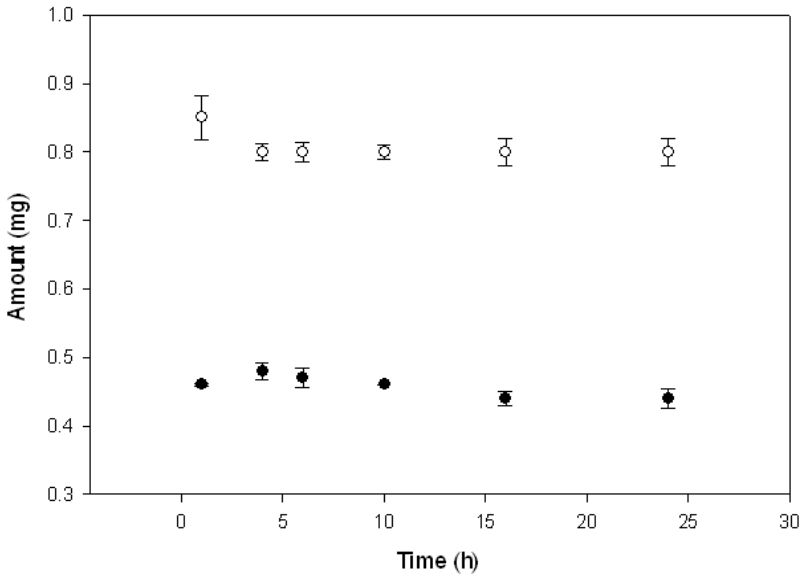


Fig. 2. NDMA stability in egg white (albumin) (●) and yolk (○) in a 24-h experiment. Error bars indicate mean standard deviations; where absent bars fall within symbols.

NDMA Transfer among Egg Fractions

NDMA amount and concentration within the egg white decreased during 120 h of incubation (Fig. 3). The NDMA content increased in the yolk, while it decreased in the membrane from 1 to 24 h and then increased to 72 h after which a decrease was observed (Fig. 3). The decrease in NDMA in the egg white and concomitant increase in the yolk with time suggests that NDMA partitions from the egg white into the yolk.

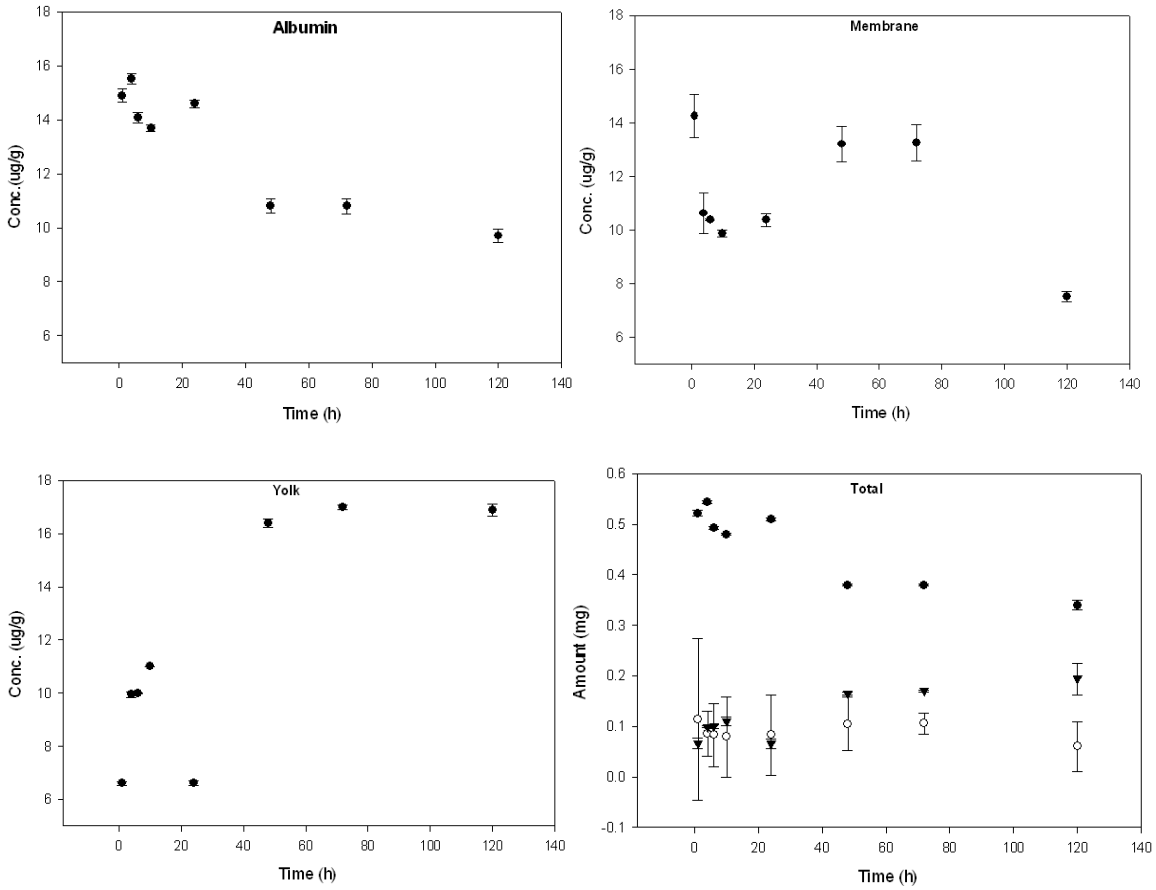


Fig. 3. NDMA concentrations (µg/g) in the albumin (egg white), membrane and yolk, and total amount of NDMA (mg) in the albumin (egg white) (●), membrane (○) and yolk (▼) during 120 h of incubation. Error bars indicate mean standard deviations; where absent bars fall within symbols.

The total amount of NDMA in the egg decreased during 120 h of incubation (Table V). This suggests that NDMA gradually decomposes. The NDMA may be denitrosating to the parent compound (DMA) and/or is being transformed to other products (not determined).

TABLE V. Total NDMA in the egg during a 120-h experiment (1 mg added)

Time (h)	NDMA in Egg (mg)	NDMA Lost or Unaccountable (mg)	(%)
1	0.70 (0.009)[a]	0.30	30
4	0.73 (0.004)	0.27	27
6	0.68 (0.003)	0.32	32
10	0.67 (0.010)	0.33	33
24	0.66 (0.007)	0.34	34
48	0.65 (0.009)	0.35	35
72	0.65 (0.008)	0.34	34
120	0.60 (0.013)	0.40	40

[a] Values in parenthesis indicate standard deviations (n = 3)

Transfer between Fractions

On analyzing the distribution of NDMA between two layers, NDMA decreased in the egg white with time while the amount in the vitelline membrane increased (Table VI). This shows transfer of NDMA from the egg white to the membrane. In the membrane-yolk matrix, the amount of NDMA in the membrane decreased with time while that in the yolk remained constant after an initial increase (Table VII), suggesting transfer from the membrane to the yolk. The distribution of NDMA between the egg white and yolk also indicated transfer from the egg white into the yolk over a period of time (Table VIII).

TABLE VI. NDMA in the membrane and egg white of an egg white-membrane matrix during a 24-h experiment (1 mg added)

	Egg white		Membrane		Total	
Time	Amount	Relative percent	Amount	Relative percent	Amount	Loss
(h)	(mg)	(%)	(mg)	(%)	(mg)	(%)
1	0.33 (0.004)[a]	60	0.22 (0.008)	40	0.55	45
4	0.30 (0.021)	52	0.28 (0.005)	48	0.58	42
10	0.16 (0.001)	33	0.32 (0.005)	67	0.48	52
24	0.11 (0.024)	24	0.35 (0.003)	76	0.46	54

[a] Values in parenthesis indicate standard deviations (n = 3)

Table VII. NDMA in the membrane and yolk of a membrane-yolk matrix during a 24-h experiment

	Membrane		Yolk		Total	
Time	Amount	Relative Percent	Amount	Relative Percent	Amount	Loss
(h)	(mg)	(%)	(mg)	(%)	(mg)	(%)
1	0.88 (0.008)[a]	69	0.39 (0.009)	31	1.27	23
4	0.60 (0.006)	60	0.40 (0.023)	40	1.00	0
6	0.44 (0.017)	47	0.49 (0.013)	53	0.93	7
10	0.39 (0.019)	42	0.54 (0.006)	58	0.93	7
16	0.34 (0.011)	43	0.45 (0.010)	57	0.79	21
24	0.24 (0.014)	38	0.40 (0.011)	63	0.64	36

[a] Values in parenthesis indicate standard deviations (n = 3)

Table VIII. NDMA in the egg white, membrane and yolk during a 24-h experiment

	Egg white		Membrane		Yolk		Total	
Time	Amount	Relative Percent	Amount	Relative Percent	Amount	Relative Percent	Amount	Loss
(h)	(mg)	(%)	(mg)	(%)	(mg)	(%)	(mg)	(%)
1	0.42 (0.009)[a]	63	0.16 (0.019)	23	0.10 (0.005)	14	0.68	32
4	0.33 (0.013)	31	0.27 (0.001)	26	0.45 (0.012)	43	1.05	20
6	0.43 (0.015)	43	0.31 (0.014)	31	0.26 (0.011)	26	1.00	0
10	0.34 (0.014)	66	0.11 (0.002)	22	0.07 (0.106)	13	0.52	48
16	0.21 (0.019)	15	0.27 (0.107)	20	0.89 (0.015)	65	1.37	13
24	0.32 (0.104)	36	0.07 (0.036)	8	0.51 (0.039)[a]	57	0.90	10

[a] Values in parenthesis indicate standard deviations (n = 3)

Stability of NMOR

To determine NMOR stability, a known amount of NMOR was added to individual fractions of the egg. The amount of NMOR in the egg white and yolk were constant for the duration of the experiment (Fig. 4), suggesting stability in both fractions.

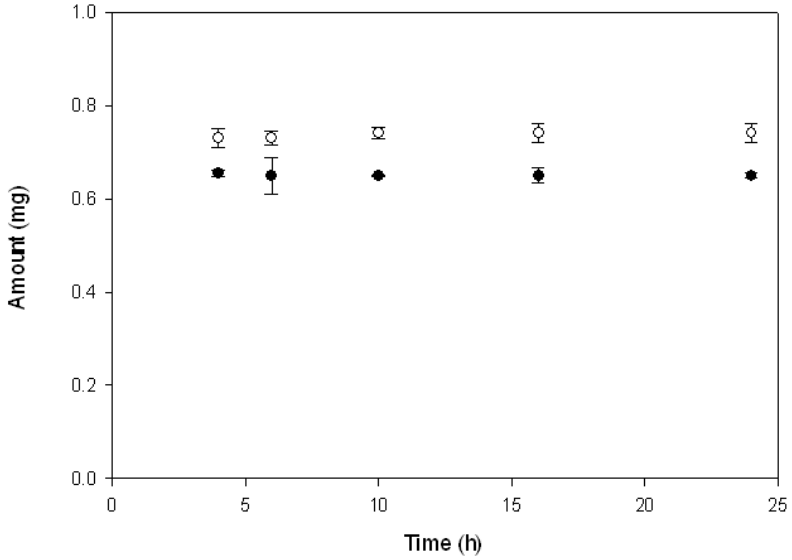


Fig. 4. NMOR in albumin (egg white) (●) and yolk (○) in a 24-h experiment. Error bars indicate mean standard deviations; where absent bars fall within symbols.

NMOR Transfer among Egg Fractions

The amount and concentration of NMOR in the egg white decreased with time. Membrane-associated NMOR increased up to 24 h after which it decreased. The amount and concentration of NMOR within the yolk steadily increased with time up to 120 h (Fig. 5).

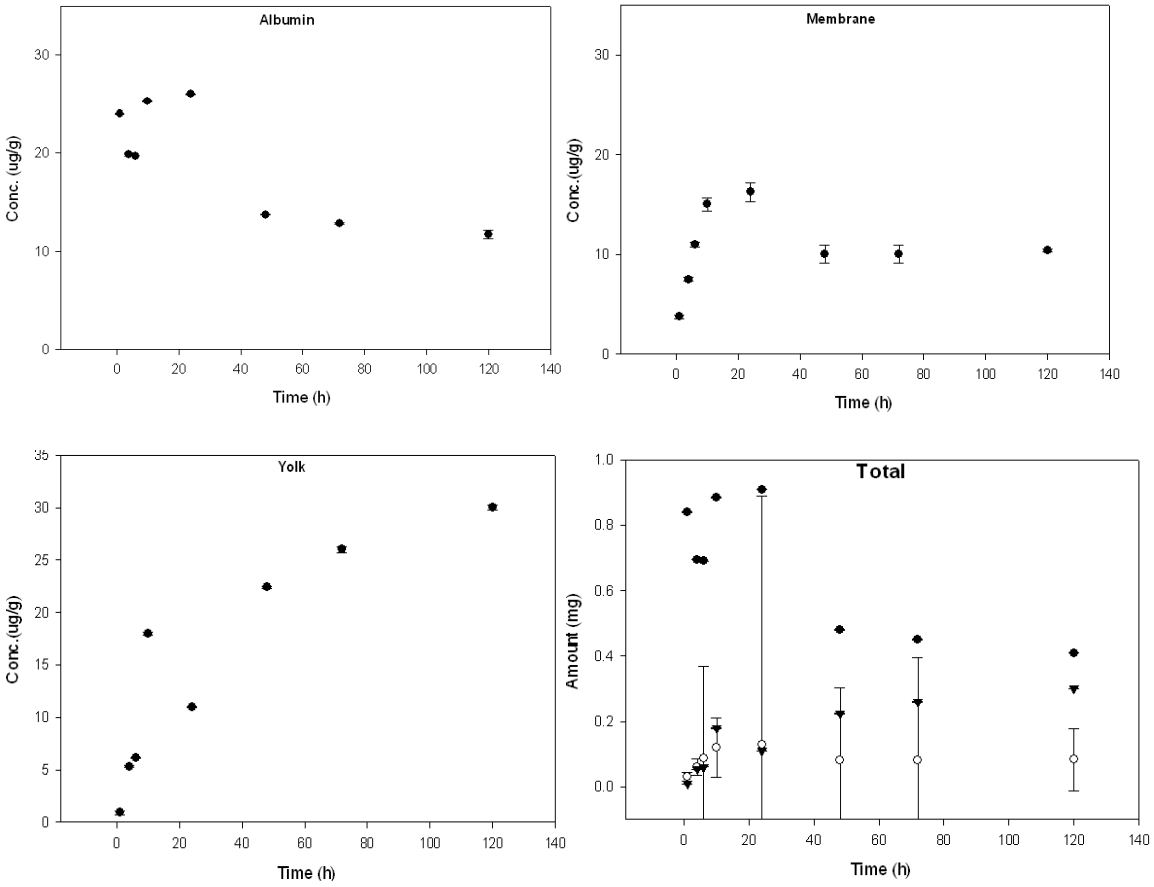


Fig. 5: NMOR concentrations (µg/g) in the albumin (egg white), membrane and yolk, and total NMOR (mg) in the albumin (egg white) (●), membrane (○) and yolk (▼) during 120 h of incubation. Error bars indicate mean standard deviations; where absent bars fall within symbols.

The total amount of NMOR in the egg remained constant for the first 24 h then decreased through the remainder of the 120-h experiment (Table IX).

TABLE IX. Total NMOR in the egg during a 120-h experiment (1 mg added)

Time (h)	Amount of NMOR in egg (mg)	NDMA Lost or Unaccountable (mg)	(%)
1	0.88 (0.004)[a]	0.12	12
4	0.81 (0.013)	0.19	19
6	0.84 (0.054)	0.16	16
10	1.18 (0.014)	0.06	5
24	1.15 (0.012)	0.10	8
48	0.78 (0.019)	0.22	22
72	0.79 (0.121)	0.21	21
120	0.79 (0.118)	0.21	21

[a] Values in parenthesis indicate standard deviations (n = 3)

Transfer between Fractions

On analyzing the distribution of NMOR between the egg white and membrane (Table X), NMOR transferred from the egg white to the membrane. NMOR differed from NDMA as less NMOR transferred from the egg white to the membrane. NMOR transferred from the membrane to the yolk during the 24-h incubation period. The results suggest dynamic movement of NMOR between the membrane and yolk (Table XI). Transfer to the yolk also was observed in the egg white-yolk matrix (Table XII).

TABLE X. NMOR transfer from the egg white to the membrane during a 24-h experiment (1 mg added)

	Egg white		Membrane		Total	
Time (h)	Amount (mg)	Relative Percent (%)	Amount (mg)	Relative Percent (%)	Amount (mg)	Loss (%)
1	0.49 (0.009)[a]	68	0.23 (0.029)	32	0.72	28
4	0.58 (0.053)	75	0.19 (0.009)	25	0.77	23
6	0.54 (0.009)	64	0.30 (0.001)	36	0.84	16
10	0.61 (0.015)	75	0.20 (0.007)	25	0.81	19
16	0.53 (0.035)	71	0.22 (0.071)	29	0.75	25
24	0.52 (0.139)	71	0.22 (0.001)	30	0.74	26

[a] Values in parenthesis indicate standard deviations (n = 3)

TABLE XI. NMOR transfer from the membrane to the yolk during a 24-h experiment (1 mg added)

	Membrane		Yolk		Total	
Time (h)	Amount (mg)	Relative Percent (%)	Amount (mg)	Relative Percent (%)	Amount (mg)	Loss (%)
1	0.46 (0.017)[a]	53	0.41 (0.037)	47	0.87	13
4	0.41 (0.006)	49	0.43 (0.091)	51	0.84	16
6	0.29 (0.055)	26	0.82 (0.040)	74	1.11	14
10	0.40 (0.012)	50	0.40 (0.011)	50	0.8	20
16	0.56 (0.135)	65	0.30 (0.021)	35	0.86	14
24	0.37 (0.002)	42	0.52 (0.012)	58	0.89	11

[a] Values in parenthesis indicate standard deviations (n = 3)

Table XII. NMOR transfer from the egg white to the yolk during a 24-h experiment (1 mg added)

	Egg white		Membrane		Yolk		Total	
Time	Amount	Relative Percent	Amount	Relative Percent	Amount	Relative Percent	Amount	Loss
(h)	(mg)	(%)	(mg)	(%)	(mg)	(%)	(mg)	(%)
1	0.49 (0.001)[a]	64	0.13 (0.012)	17	0.15 (0.002)	20	0.77	23
4	0.56 (0.013)	78	0.07 (0.015)	10	0.09 (0.009)	13	0.72	28
6	0.53 (0.012)	62	0.12 (0.151)	14	0.21 (0.006)	24	0.86	14
10	0.58 (0.009)	74	0.08 (0.004)	10	0.12 (0.010)	15	0.78	22
24	0.57 (0.003)	76	0.10 (0.041)	13	0.08 (0.070)	11	0.75	25

[a] Values in parenthesis indicate standard deviations (n = 3)

Stability of NNAT

To determine the stability of NNAT in the various fractions of the egg, a known amount was added to each fraction and the amount remaining was determined with time. The amounts of NNAT in the egg white and yolk decreased slightly during the first 10 h, but then remained relatively constant for at least 24 h (Fig. 6).

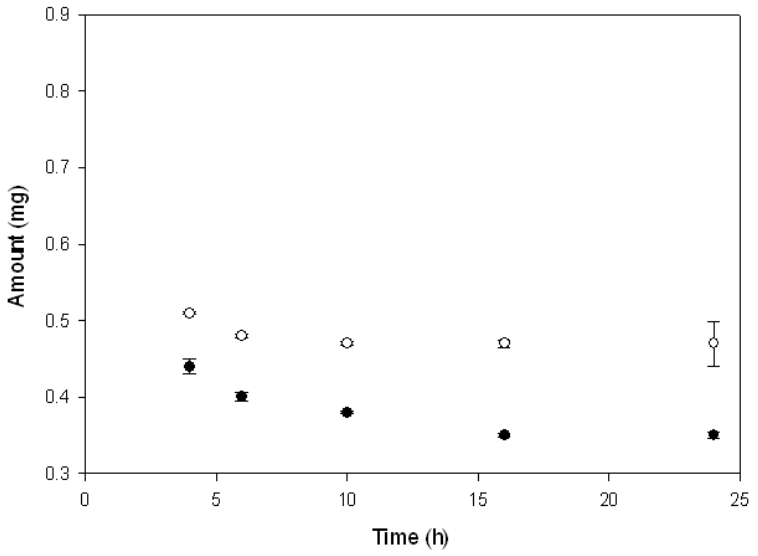


Fig. 6. NNAT in albumin (egg white) (●) and yolk (○) in a 24-h experiment. Error bars indicate mean standard deviations; where absent bars fall within symbols.

NNAT Transfer among Egg Fractions

In the egg white and membrane, the amount of NNAT reached a maximum at 4 h and then progressively decreased (Fig. 7). The amount of NNAT decreased in the yolk after 1 h. After 24 h no NNAT was detected in any fraction. The lack of change in atrazine concentrations (atrazine was an impurity in NNAT) indicated that NNAT was not decomposing to atrazine.

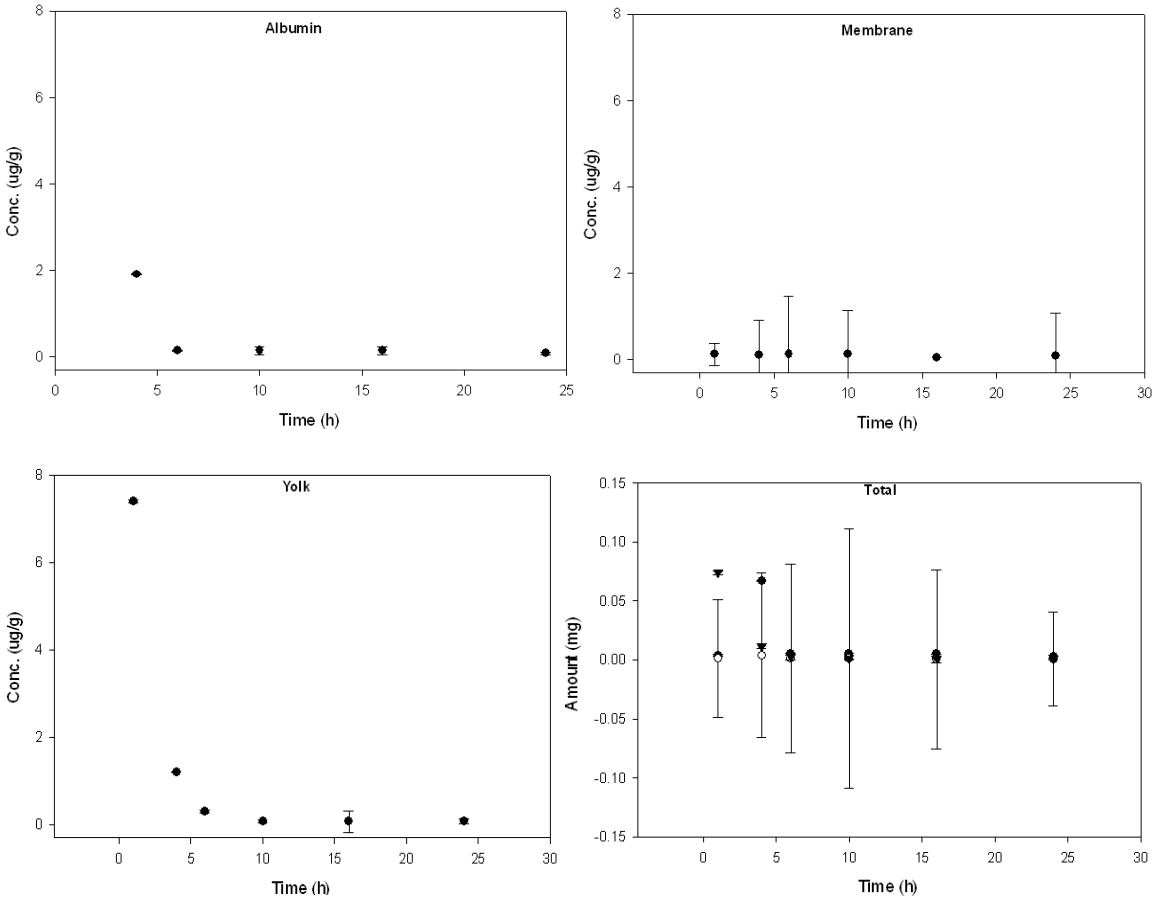


Fig. 7. NNAT concentrations (µg/g) in the albumin (egg white), membrane and yolk, and total NNAT (mg) in the albumin (egg white) (●), membrane (○) and yolk (▼) during 120 h of incubation. Error bars indicate mean standard deviations; where absent bars fall within symbols.

The total amount of NNAT in the egg decreased during the 24 h experiment and none was detected after 24 h (Table XIII).

Table XIII. Total NNAT in the egg during a 24-h experiment (1 mg added)

Time (h)	Amount of NNAT in egg (mg)	Amount of NNAT lost/ unaccounted for (mg)	(%)
1	0.08 (0.007)[a]	0.92	92
4	0.08 (0.009)	0.92	92
6	0.01 (0.003)	0.99	99
10	0.01 (0.001)	0.99	99
16	0.01 (0.001)	0.99	99
24	0.0 0 (0.001)	1.00	100

[a] Values in parenthesis indicate standard deviations (n = 3)

Transfer between Fractions

On analyzing the distribution of NNAT between two layers, the amount of NNAT in the egg white decreased while the amount in the membrane increased within the first 4 h in the egg white-membrane matrix (Table IV). Within 4 h, the amounts of NNAT in the egg white and the membrane decreased and neither could be detected after 24 h. This shows that although NNAT transferred from the egg white into the membrane within the first few hours, NNAT degraded on longer incubation. In the membrane-yolk matrix, the amount of NNAT in the membrane fraction decreased with time while that in the yolk remained relatively constant after an initial increase (Table XV), suggesting transfer from the membrane to the yolk. The distribution of NNAT between the egg white and yolk also indicated increased transfer from the egg white into the yolk with time (Table XVI).

TABLE XIV. NNAT transfer from the egg white to the membrane in a 24-h experiment (1 mg added)

	Egg white		Membrane		Total	
Time (h)	Amount (mg)	Relative Percent (%)	Amount (mg)	Relative Percent (%)	Amount (mg)	Loss (%)
1	0.31 (0.003)[a]	84	0.06 (0.010)	16	0.37	63
4	0.23 (0.075)	70	0.1 (0.011)	30	0.33	67
10	0.02 (0.030)	50	0.02 (0.050)	50	0.04	96
16	0.01 (0.001)	-	Not detected	-	-	99
24	Not detected	-	Not detected	-	-	100

[a] Values in parenthesis indicate standard deviations (n = 3)

Table XV. NNAT transfer from the membrane to the yolk during a 24-h experiment (1 mg added)

	Membrane		Yolk		Total	
Time (h)	Amount (mg)	Relative Percent (%)	Amount (mg)	Relative Percent (%)	Amount (mg)	Loss (%)
1	0.26 (0.002)[a]	87	0.04 (0.004)	13	0.30	70
4	0.13 (0.007)	48	0.14 (0.006)	52	0.27	73
10	0.07 (0.070)	39	0.11 (0.051)	61	0.18	82
16	0.13 (0.018)	42	0.18 (0.005)	58	0.31	69
24	0.05 (0.011)	39	0.08 (0.008)	61	0.13	87

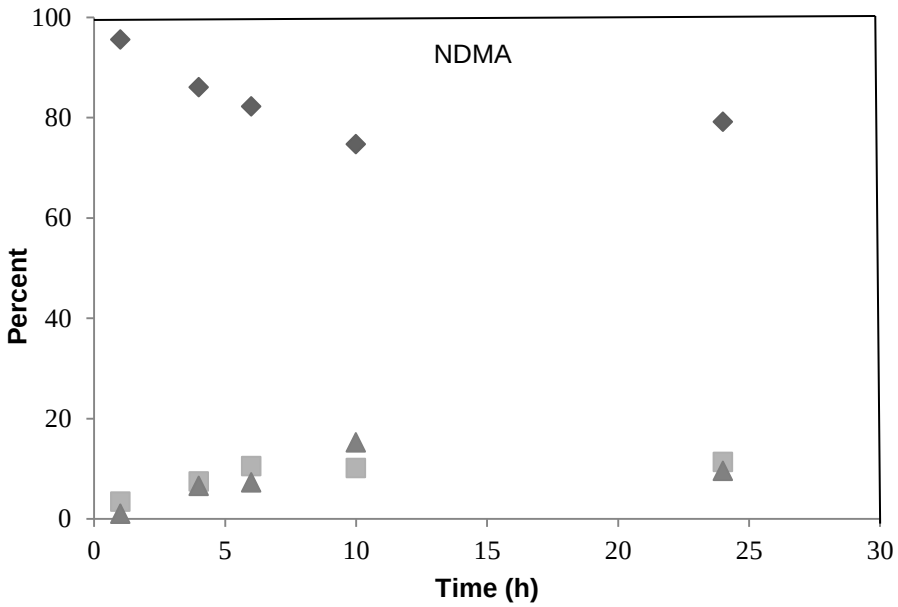
[a] Values in parenthesis indicate standard deviations (n = 3)

Table XVI. NNAT transfer from the egg white to the yolk during a 24-h experiment (1 mg added)

	Egg white		Membrane		Yolk		Total	
Time	Amount	Relative Percent	Amount	Relative Percent	Amount	Relative Percent	Amount	Loss
(h)	(mg)	(%)	(mg)	(%)	(mg)	(%)	(mg)	(%)
1	0.06 (0.003)[a]	60	0.01 (0.001)	10	0.03 (0.010)	30	0.1	90
4	0.03 (0.001)	50	0.01 (0.070)	17	0.02 (0.027)	33	0.06	94
10	0.02 (0.012)	40	0.01 (0.053)	20	0.02 (0.004)	40	0.05	95
16	0.02 (0.070)	40	0.01 (0.006)	20	0.02 (0.021)	40	0.05	95
24	Not detected	-	0.01 (0.016)	33	0.02 (0.001)	67	0.03	97

[a] Values in parenthesis indicate standard deviations (n = 3)

The distribution of the nitrosamines in unfertilized chicken eggs indicates that while most of the NDMA and NMOR remained in the egg white, NNAT rapidly moved into the yolk. The total amount of NNAT decreased with time (Fig. 8).


NDMA
Percent
Time (h)
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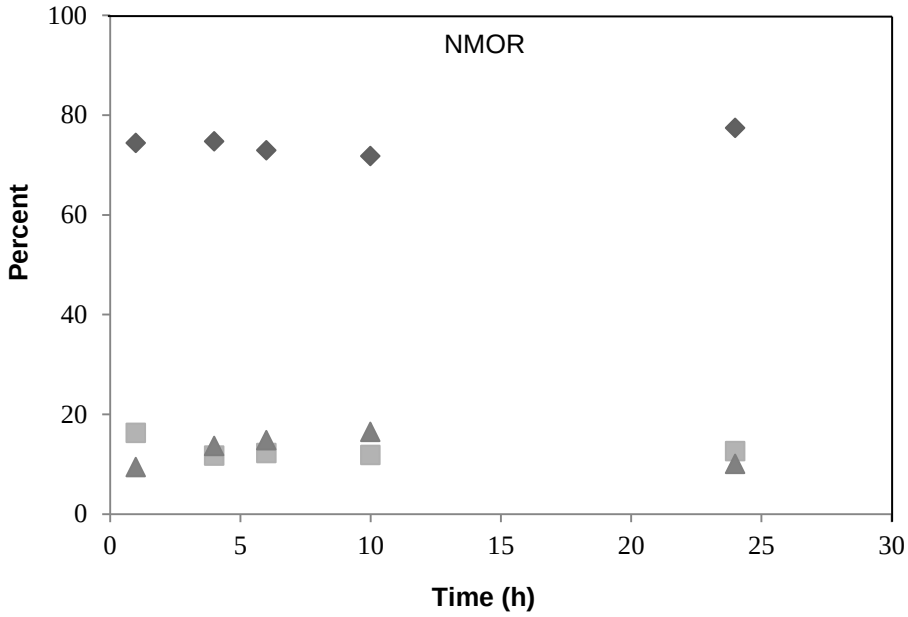
20
40
60
80
100
0
5
10
15
20
25
30


NMOR
Percent
Time (h)
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40
60
80
100
0
5
10
15
20
25
30

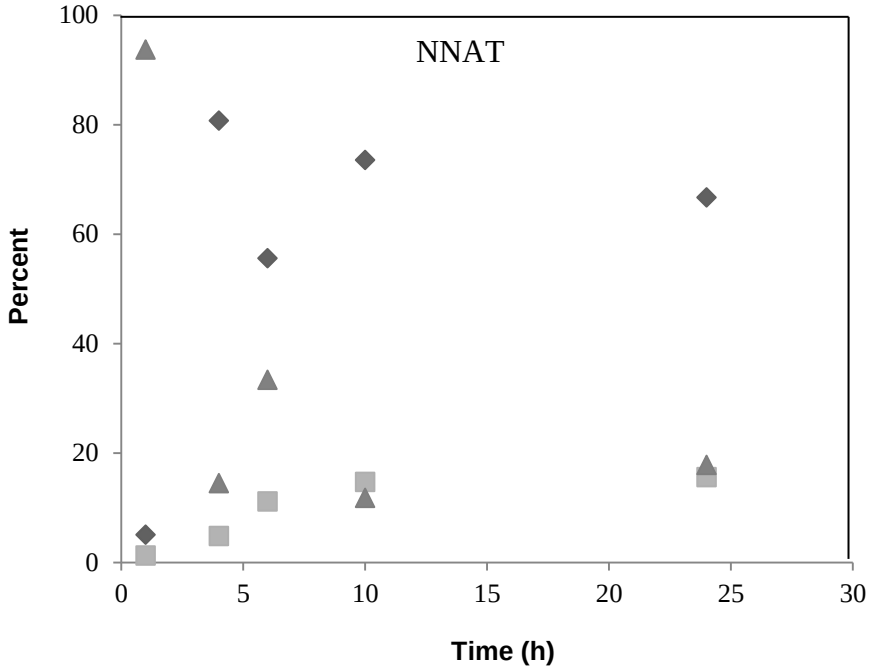


Fig. 8. Distribution of NDMA, NMOR and NNAT in the albumin (egg white) (♦), membrane (■) and yolk (▲) during a 24-h incubation.

A comparison among the three compounds shows that NDMA and NMOR are similar in their transfer behavior in that both have a greater affinity for the yolk fraction over the egg white fraction (Table XVII). Although the concentration of NNAT is very low compared to NDMA and NMOR, it readily moves into the yolk. The larger Yolk/Egg white distribution coefficient for NNAT indicates that it has a greater affinity for yolk (larger bioconcentration potential) than NDMA or NMOR.

Table XVII. Maximum nitrosamine concentrations in the egg fractions

Highest concentration	NDMA	NMOR	NNAT
Egg white (µmol/g)	0.24 (0.02)[a]	0.4 (0.02)	0.03 (0.02)
Membrane (µmol/g)	0.22 (0.15)	0.24 (0.02)	0.002 (0.02)
Yolk (µmol/g)	0.26 (0.005)	0.46 (0.02)	0.11(0.02)
Yolk/Egg white (Distribution coefficient)	1.08	1.15	3.67

[a] Values in parenthesis indicate standard deviations (n = 3)

The biological effects of nitrosamines depend in part on their stability and transfer behavior. The studies with unfertilized chicken eggs indicate that NDMA, NMOR and NNAT partition from the egg white into the yolk because temporal decreases in their amounts in the egg white and membrane were accompanied by concomitant increases in the yolk. All three compounds were also fairly stable in the yolk fraction. NNAT has a higher affinity than NDMA and NMOR for the more lipophilic yolk fraction, consistent with its octanol-water partition coefficient (K_{ow}). This suggests that it may have a greater potential to bioconcentrate than NDMA and NMOR.

A loss in the total amount of the nitrosamines with time suggests that they may be decomposing or denitrosating. Haussmann and Werringloer (1987) and Appel et al. (1991) demonstrated two possible pathways by which nitrosamines denitrosate in biological media: one electron reduction that produces NO and the secondary amine or one electron abstraction that liberates NO via an oxidative mechanism involving the

formation of a primary amine and the corresponding aldehyde. Because the parent compounds of the nitrosamines were not detected in this study, decomposition or denitrosation in the yolk likely occurred via an oxidative mechanism (i.e. one electron abstraction). This mechanism also liberates NO, which may subsequently affect development of chicken embryos.

Teratogenic Potential of Selected Nitrosamines

From the studies with unfertilized eggs, it was determined that all three nitrosamines partition into the yolk where they are all fairly stable. Decreases in the total amounts of nitrosamines may be due to denitrosation or decomposition. It was hypothesized that the presence of the nitrosamine may alter NO levels and affect development. Thus, studies were conducted with fertilized chicken eggs to determine the impact of the three nitrosamines on the development of chicken embryos. The embryos were harvested at days 3 and 5 and examined microscopically for any soft tissue or skeletal abnormalities.

All three nitrosamines adversely affected development of the chicken embryos to varying degrees. Malformations were observed in embryos exposed to much lower concentrations of NNAT than NDMA or NMOR.

Impact of NDMA on developing embryos

Fertilized chicken eggs were treated with 12.5, 25.0, or 50.0 µg NDMA in 50 µL of DD water. Mortality and deformities were observed only in embryos treated with 25 µg and higher doses of NDMA (Fig 9).

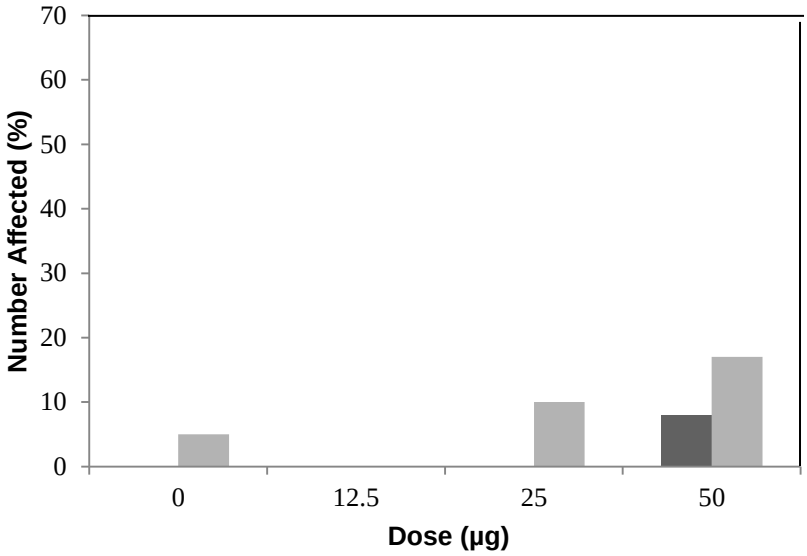


Fig. 9. Impact of NDMA dose (in 50 µL DD water) on chicken embryo mortality ( ) and malformations ( )

The most common defects observed on exposure to NDMA included gastroschisis (33%), heart defects, neural tube defects (22% each), microphthalmia (11%) and caudal regression (11%).

Analysis of the frequency of mortality or abnormalities resulting from NDMA exposure using chi square test (SAS, Cary, NC) indicated that NDMA does not significantly affect development in chicken embryos ($p > 0.05$). There was also no difference within treatments; i.e., between dosage groups of NDMA ($p > 0.05$) and thus there was no dose – response relationship. No significant association was observed between exposure to NDMA and embryo mortality observed in the embryos ($p > 0.05$). Analysis of the relative risk of mortality or abnormalities from exposure to NDMA is very low (RR < 1.0, 95% CI).

Impact of NMOR on developing embryos

Fertilized chicken eggs were treated with 25, 250, 1250, 2500, 5000, and 7500 µg NMOR in 50µL DD water. Mortality and abnormalities were observed in embryos treated with high doses of NMOR (≥2500 µg) (Fig 10).

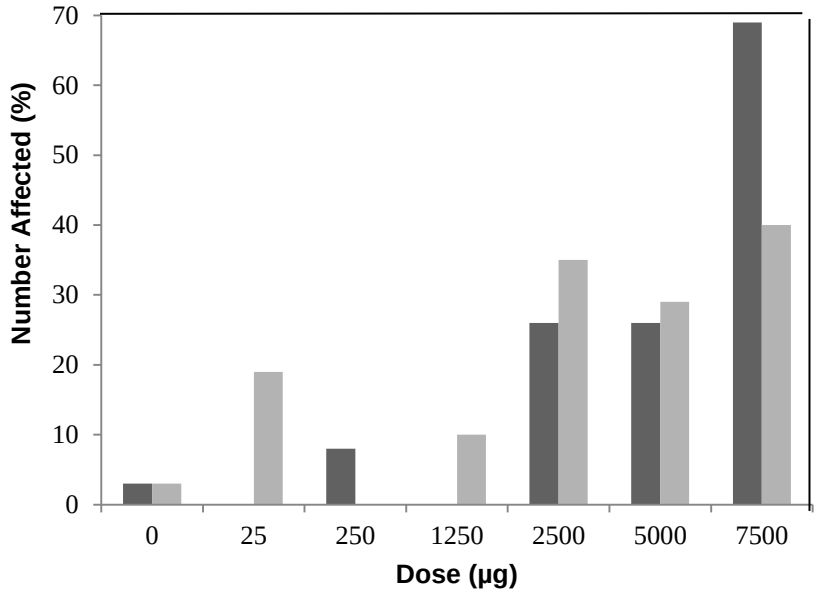


Fig. 10. Impact of NMOR dose (in 50 µL DD water) on chicken embryo mortality ( ) and malformations ( )

Gastroschisis was the most frequently occurring malformation (30%), followed by heart defects (~ 26%). Other defects observed include neural tube defects, microphthalmia and caudal regression (11, 11, and 22%, respectively).

A chi square test was used to assess relationships between exposure to varying doses of NMOR and the effects observed (mortality and malformations). On comparing overall exposure to NMOR and subsequent effects (mortality and deformity), NMOR had

a significant adverse effect on the developing embryos ($p < 0.05$). There also was a difference between doses of NMOR ($p < 0.05$) with respect to the effect they produce.

NMOR exposure (combining all doses) was associated with embryo death ($p < 0.05$). There also was a significant dose – response relationship; larger doses of NMOR induced a high level of mortality in exposed chicken embryos. An analysis of relative risk indicated a greater risk of mortality in embryos treated with NMOR than in the controls (RR < 1.0, 95% CI).

NMOR exposure (combining all doses) was associated with malformations ($p < 0.05$). However, there were no significant differences among the NMOR doses. Relative risk analysis indicated that the chance of the embryos being malformed is greater for embryos treated with NMOR than for controls (95% CI). A comparison (by chi square test) of each NMOR dose with the control shows that there is a significant association between exposure and malformations and mortality at doses ≥2500 µg.

Impact of NNAT on developing embryos

Fertilized chicken eggs were treated with 0.06, 0.01, 0.23, 0.46, 0.91, 1.82, 3.63, 5.50, 7.25, and 14.50 µg NNAT in 50µL DD water. Adverse effects (mortality and malformations) were observed in embryos exposed to doses as low as 0.06 µg NNAT (Fig 11). NNAT exposure resulted in malformations at lower doses than NDMA or NMOR, therefore of the three nitrosamines, NNAT was the most potent embryotoxic compound.

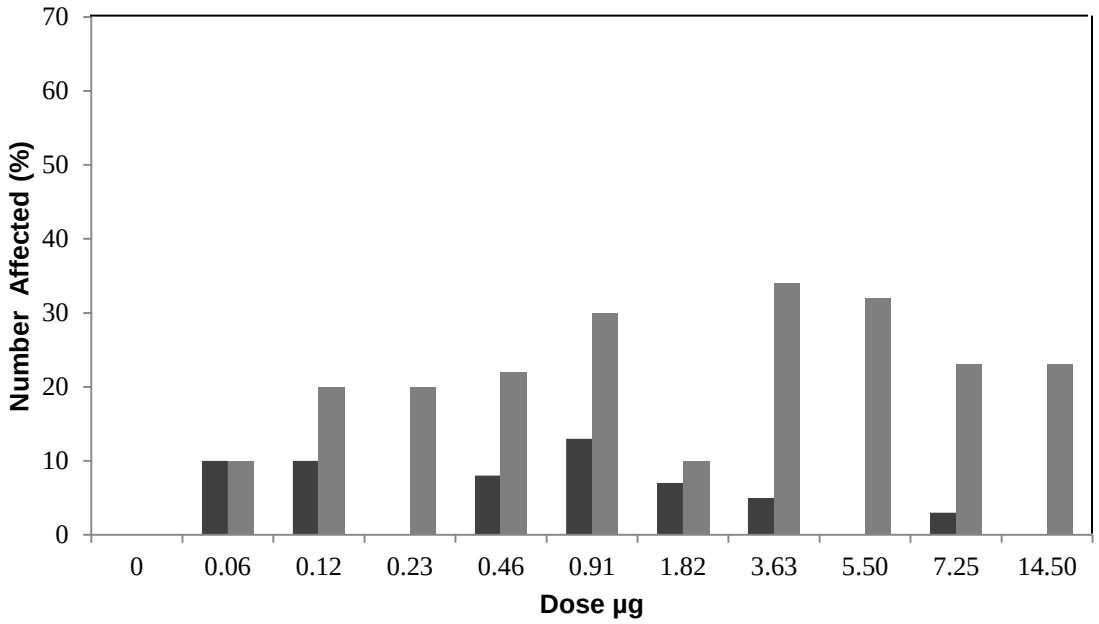
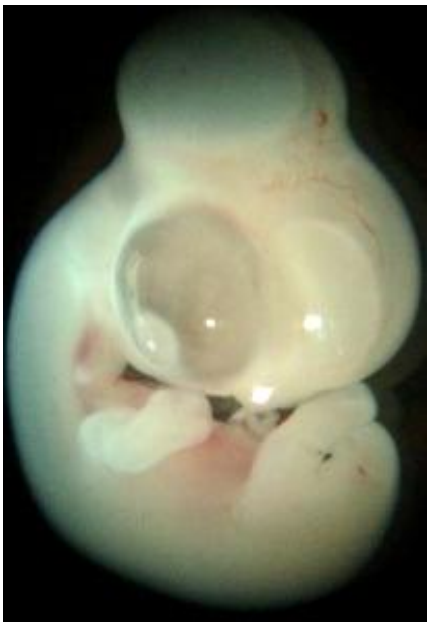
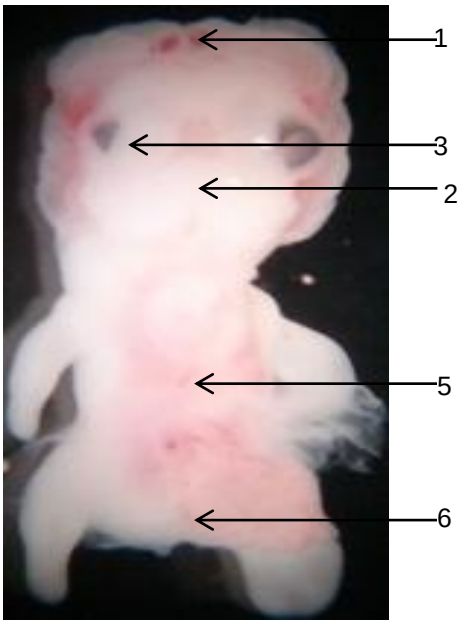


Fig. 11. Impact of NNAT dose (in 50 µL DD water) on mortality ( ) and malformations ( )

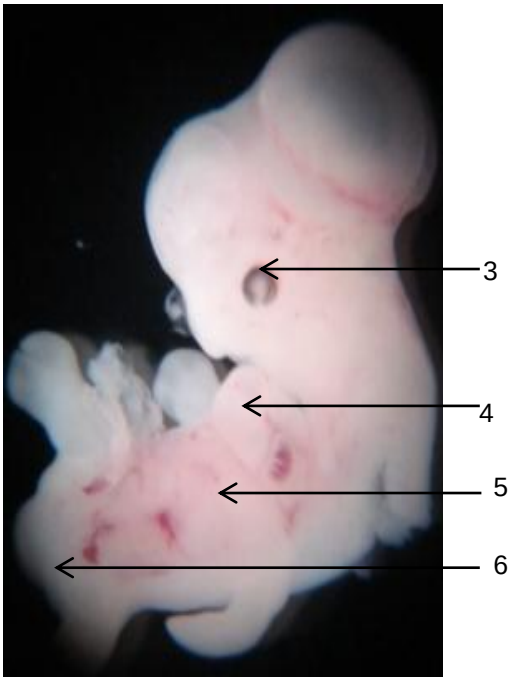
Heart defects (ectopic and abnormally looped heart) and gastroschisis (each 24%) were the most frequently observed defects observed following exposure to NNAT. Other defects include caudal regression (19%), craniofacial hypoplasia, microphthalmia (each 11%) and neural tube defects (8%). Some of the embryos (4%) showed anophthalmia, which was not observed in embryos exposed to either NDMA and NMOR. Some of the defects are shown in Fig 12.



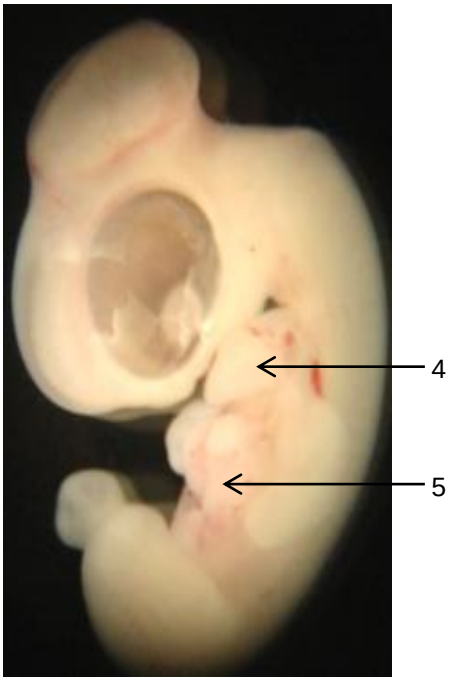
Normal 5 day old embryo



NNAT 3.63 µg/50 µL



NNAT 1.82 µg/50 µL



NNAT 0.91 µg/50 µL

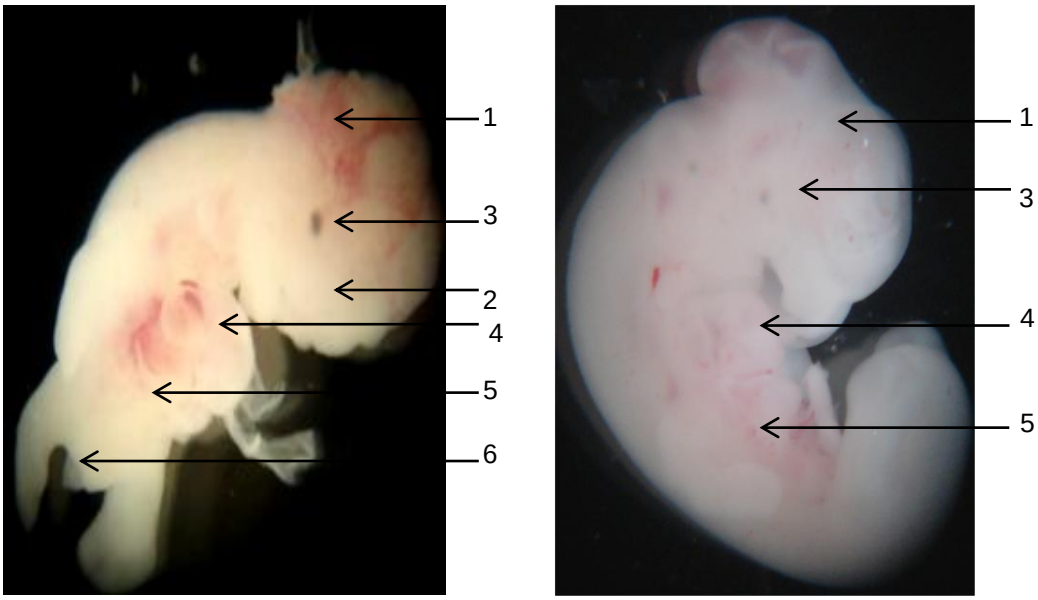


Fig. 12. Malformations in 5-day-old chicken embryos following exposure to NNAT. 1= neural tube defect, 2=craniofacial hypoplasia, 3=microphthalmia, 4=ectopic heart, 5= gastroschisis, 6=caudal regression

A chi square test was used to evaluate the impact of NNAT on mortality and malformations in chicken embryos. Exposure to NNAT adversely affected (mortality and malformation) chicken embryos ($p < 0.05$), although there is no clear dose-response relationship between various doses of NNAT and either lethality or observed abnormalities.

The relationship between mortality and NNAT exposure was evaluated using a chi square test and relative risk analysis. There was an association between NNAT exposure (all doses) and embryo mortality ($p < 0.05$) but there was no difference among the doses.

Relative risk analysis showed a greater chance of mortality in chicken embryos exposed to NNAT (the chance of control embryos remaining alive was 1.1 times greater than those exposed to NNAT (95% CI)).

Analysis of the impact of NNAT (all doses) on malformations showed a significant association ($p < 0.05$) between exposure to NNAT and subsequent deformity. However, there was no significant association between NNAT dose and defect. Assessment of relative risk indicates that the chance of an embryo developing normally is 1.3 times greater for controls than for those treated with NNAT (95% CI); i.e. the risk of malformations from exposure to NNAT is greater than if there is no exposure. A comparison (chi square test) of each NNAT dose with the control showed a significant association between exposure and defect at 0.46, 0.91, 3.63, 5.50, and 7.25 µg in 50 µL DD water.

There is conflicting evidence about the adverse effects of atrazine. Atrazine may be an endocrine disrupter in some animal models (mainly amphibian) (Gammon et al. 2005). Atrazine has been shown to affect development in rats and rabbits (delayed skeletal ossification) and the reproductive NOEL is 25 mg/kg body weight/d (Gammon et al. 2005). The present studies with chicken embryos showed that 16.5 µg atrazine was neither teratogenic nor lethal to chicken embryos.

Teratogenic potential and lethality varied among the three nitrosamines tested. NDMA, a known hepatotoxin and carcinogen, was neither lethal nor teratogenic in chicken embryos at the doses administered. Previous work with chicken embryos showed that NDMA is lethal or inhibits growth (Maduagwu and Bassir 1979). Inoculating White Leghorn chicken eggs with 2.25 mg/mL NDMA (similar to the methodology of the

present studies) on the tenth day of development resulted in 100% mortality. However, these effects were not observed in the present study. NMOR was teratogenic and lethal at relatively high doses (≥2500 µg). NNAT proved to be the most potent teratogen (lowest dose at which malformations were observed was 0.455 µg). There was no clear dose-response relationship with NNAT because it was teratogenic at 0.91 µg but not at the higher dose of 1.82 µg. It also induced defects at doses of 3.63 µg to 7.25 µg but not at the largest dose of 14.50 µg.

Despite the lack of a clear dose-response relationship between NNAT and the observed teratogenic effects, this study shows that exposure to NNAT or the endogenous formation of NNAT could be potentially harmful. Research is limited regarding exposure to atrazine and NNAT and the potential for *in vivo* formation of NNAT from exposure to atrazine and nitrate. Atrazine, due to its weak basicity, nitrosates rapidly (200 times faster than dimethylamine at pH 2 (Mirvish et al. 1991) and thus exposure to atrazine poses a risk for endogenous formation of NNAT. High concentrations of atrazine (1500 µg/kg) have been detected in well water in Wisconsin, where some wells also have high nitrate levels (Meisner et al. 1993). This creates the potential for NNAT formation after ingestion. As observed in experiments with unfertilized eggs, NNAT has a high affinity for the lipophilic yolk fraction and rapidly moves into the yolk in a short period of time. NNAT showed a greater bioconcentration potential than NDMA and NMOR, which may be contributing to the occurrence of the biological effects observed in the experiments with fertilized eggs.

Several factors may explain the variability in observed effects of the nitrosamines on the chicken embryos, despite measures taken to control treatment conditions. Embryo

maturity at the time of treatment/exposure will vary due to small differences in temperature. There is variability in embryo robustness and genetic susceptibility to a particular compound or to certain developmental defects. Variability may result from the combined effects of multiple factors. These factors cannot be excluded with certainty as contributors to the variability in observed defects. Despite these drawbacks, the chicken embryo is a useful model for preliminary screening of compounds for teratogenicity. For a clearer, in-depth understanding of potential teratogenic effects of these and other environmentally relevant nitrosamines, further study with murine models are warranted as are epidemiological studies to evaluate associations between nitrosamine exposure and birth defects.

Impact of NNAT on nitrotyrosine concentrations in chicken embryos

Teratology studies with chicken embryos showed a significant association between exposure to NNAT and developmental abnormalities. Because transfer studies show NNAT bioconcentration in yolk and suggest loss with time may be due to denitrosation or nitrosamine decomposition, it was hypothesized that the defects observed in chicken embryos may be due to alterations in NO concentrations. As previously mentioned, measurement of the stable 3-nitrotyrosine is a useful way to provide evidence of NO-dependent damage and may reflect one pathway by which NNAT exerts teratogenic effects. Nitrotyrosine was measured in tissue sonicates from five-day-old chicken embryos treated with DD water (controls), embryos exposed to NNAT that appeared normal, and embryos exposed to NNAT that were malformed.

Malformed five-day-old embryos had higher concentrations of nitrotyrosine than normal-appearing embryos of the same treatment group (Fig. 13). Paired t-tests were used to compare nitrotyrosine values in normal and malformed embryos for each treatment. Malformed embryos treated with 0.06, 0.46, 0.91 and 3.63 µg NNAT had significantly higher concentrations of nitrotyrosine than treated embryos that appeared normal.

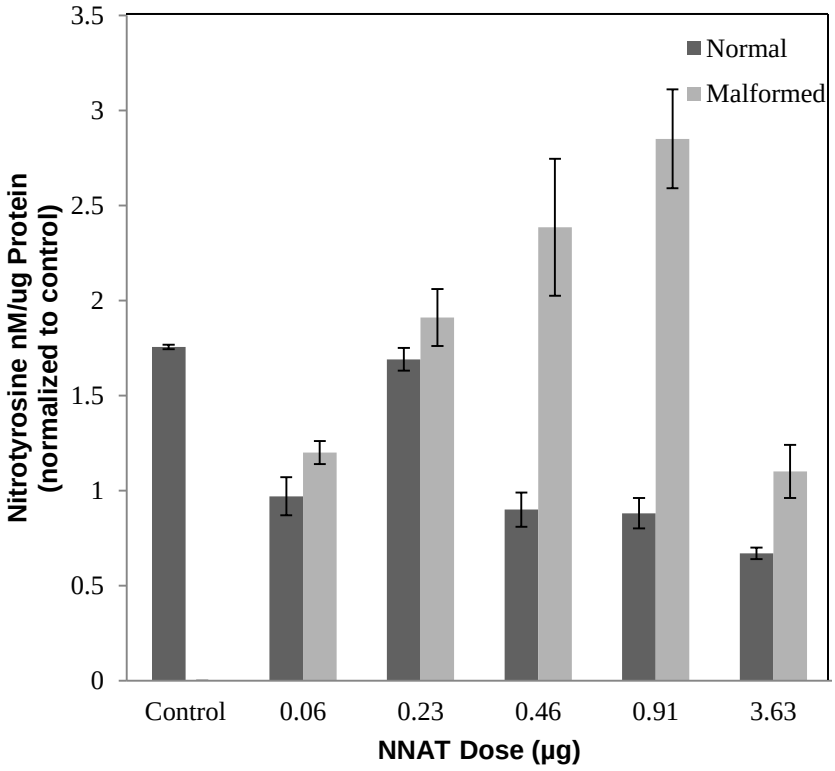


Fig. 13. Nitrotyrosine concentrations in malformed 5-day-old embryos exposed to NNAT

One-way analysis of variance (ANOVA) showed that nitrotyrosine concentrations in normal-appearing embryos treated with 0.06, 0.46 and 3.63 µg NNAT were significantly smaller than those in controls ($p < 0.05$). Nitrotyrosine concentrations were greater ($p < 0.05$) in malformed embryos treated with 0.46 and 0.91 µg NNAT than in the controls. What comprises "normal" nitrotyrosine concentrations in developing chicken

embryos is uncertain. In our study we observed high levels of nitrotyrosine in the controls which could indicate stress induced by rapid mitotic divisions and subsequent cellular differentiation during embryogenesis and organogenesis. At higher doses of NNAT exposure, where nitrotyrosine concentrations were observed to be lower than controls, other mechanisms of denitrosation/dealkylation may be influencing NO and subsequently nitrotyrosine levels.

One-way ANOVA analysis also was used to compare nitrotyrosine concentrations in embryos (normal and malformed combined) receiving different doses of NNAT. Nitrotyrosine was greater ($p < 0.05$) in embryos treated with 0.91 µg NNAT than in embryos treated with 0.06 µg NNAT. Embryos treated with 0.23, 0.46 and 0.91 µg NNAT had significantly larger concentrations of nitrotyrosine than those treated with the largest dose of NNAT (3.63 µg). The large 3.63 µg dose may have induced a different deleterious response, resulting in overall lower nitrostyrosine concentrations.

The ANOVA showed that nitrotyrosine levels were significantly larger in treated malformed embryos than in treated, normal-appearing embryos. These experiments show that exposure to NNAT can affect nitrotyrosine concentrations in five-day-old chicken embryos. NO-mediated stress may reflect one pathway by which NNAT exerts teratogenic effects.

CONCLUSIONS AND RECOMMENDATIONS

The main objectives of this study were to use unfertilized chicken eggs and developing chicken embryos to evaluate the stability, biological transfer and potential teratogenicity of three environmentally relevant nitrosamines: NDMA, NMOR and NNAT. Experiments using unfertilized chicken eggs showed that all three nitrosamines transfer from the egg white into the yolk where they are relatively stable. NNAT has a greater potential to bioconcentrate than NDMA and NMOR due to its higher affinity for the yolk fraction of the egg. A decrease in the total amounts of the three nitrosamines in the egg yolk suggests that they may be denitrosating (or otherwise decomposing) and releasing NO. Alterations in NO levels can affect intracellular signaling and adversely impact embryonic and fetal development. Studies with fertilized chicken eggs showed the three nitrosamines were detrimental to the development of chicken embryos to varying degrees. Observed abnormalities include neural tube defects, craniofacial hypoplasia, microphthalmia, anophthalmia, heart defects, gastroschisis, and caudal regression. Malformations were observed in embryos exposed to much lower concentrations of NNAT than NDMA or NMOR proving that NNAT was the most potent teratogen.

Exposure to NNAT was associated with developmental abnormalities in chicken embryos. However, atrazine, the parent compound of NNAT, did not affect development. It was hypothesized that defects observed in chicken embryos may be due to alterations in NO concentrations resulting from exposure to NNAT. Subsequent test showed significantly higher levels of nitrotrotyrosine, a stable marker of NO and subsequent nitrosative stress, in NNAT-treated, five-day-old embryos with malformations than in treated, normal-appearing embryos.

Considering the increasing prevalence of nitrates in ground and drinking water and the widespread use of atrazine, data are inadequate about potential exposure to NNAT. Epidemiological studies concerning potential exposure to NNAT and other nitrosamines are needed. Laboratory studies also need to be conducted to quantify the exposure and kinetics of a given dose of the nitrosamine absorbed by chicken/rodent embryos, which can then be used to estimate exposure levels in humans. Although much is known about the carcinogenic effects of nitrosamines, there is limited research regarding their teratogenic impacts and the mechanisms by which they induce abnormal development. More epidemiological studies are needed to assess the association between exposure to these and other nitrosamines and adverse fetal outcomes.

NNAT may be teratogenic and nitrotyrosine, a marker of NO-dependent oxidative stress, may reflect one pathway through which nitrosamines could exert their teratogenic effects. Further studies are needed to understand this and other possible mechanisms. The activation of guanylyl cyclase by low concentrations of NO is the major pathway of NO signaling that is involved in the regulation of many physiological functions. An increase or decrease in the NO levels due to nitrosamine exposure could disrupt the normal activation of guanylyl cyclase. Levels of NO and guanylyl cyclase could be determined after nitrosamine exposure to evaluate this possible mechanism.

Apoptosis is crucial during development (sculpting digits and extremities and governing the connection between central nervous system, distal structures and cardiac development). Therefore another focus for future investigations would be to extend areas of apoptosis leading to malformations. Depending on which organ is malformed, embryos (chicken or rodent) can be evaluated for disruption of apoptosis in the

primordial tissues that develop into the affected tissue. Cellular injury resulting from reactive nitrogen species is another parameter that can be studied.

The unfertilized chicken egg model provides a useful model to study the biological stability and transfer of chemical compounds. While the chicken embryo is a faster and relatively inexpensive model to screen compounds for potential teratogenicity, further studies with murine models are required to reach firm conclusions regarding the teratogenic behavior of nitrosamines.

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APPENDIX A: STABILITY AND TRANSFER OF SELECTED NITROSAMINES

Average weight of egg fractions

Egg Fraction	Average weight (g)
Egg white	30.0
Membrane	8.0
Yolk	12.0
Whole egg (without shell)	50.0

Percent recovery of nitrosamines from each fraction of egg

Egg Fraction	NDMA (%)	NMOR (%)	NNAT (%)
Egg White	100	100	49
Membrane	80	88	63
Yolk	60	99	37

APPENDIX B: TERATOGENIC POTENTIAL OF SELECTED NITROSAMINES

NDMA

Number of eggs treated with NDMA

Dose (µg)	No. treated	No. unfertilized	No. dead	No. malformed
Controls	25	0	1	1
12.5	10	2	0	0
25.0	10	1	0	1
50.0	20	1	2	5

SAS output

Case-Control Study of Malformation

The FREQ Procedure

Table of Treatment by Outcome

Treatment Outcome

Frequency	Dead	Malforme	Normal	Total
0.25	0	0	8	8
0.5	0	1	9	10
1.00	2	5	17	24
Control	1	1	22	24
Total	3	7	56	66

Statistics for Table of Treatment by Outcome

Statistic	DF	Value	Prob
Chi-Square	6	6.6753	**0.3519**

Chi square test shows that no statistically significant relationship exists between treatment with NDMA and subsequent adverse effects (i.e. mortality and deformity) in chicken embryos.

Case-Control Study of Malformation

The FREQ Procedure

Statistics for Table of trt by Outcome

Estimates of the Relative Risk (Row1/Row2)

Type of Study	Value	95% Confidence Limits	
Case-Control (Odds Ratio)	0.2576	0.0290	2.2874
Cohort (Col1 Risk)	0.2899	0.0372	2.2604
Cohort (Col2 Risk)	**1.1253**	**0.9622**	**1.3162 (>1)**

Analysis of relative risk indicates that the risk of mortality or malformations in chicken embryos from exposure to NDMA is very low (95% CI)

NMOR

Number of eggs treated with NMOR

Dose (µg)	No. treated	No. unfertilized	No. dead	No. malformed
Controls	35	3	1	1
25.0	15	2	0	3
250.0	15	2	1	0
1250.0	10	1	0	1
2500.0	30	4	10	8
5000.0	15	0	5	4
7500.0	15	1	11	2

SAS output

Case-Control Study of Malformation
The FREQ Procedure

Table of Treatment by Outcome

Treatment Outcome

The FREQ Procedure

Table of Treatment by Outcome

Treatment Outcome

Frequency	Dead	Malforme	Normal	Total
0.5	0	3	13	16
100.0	5	4	10	19
150.0	11	2	3	16
25.0	0	1	9	10
5.0	1	0	12	13
50.0	10	8	16	34
Control	1	1	31	33
Total	28	19	94	141

Statistics for Table of Treatment by Outcome

Statistic	DF	Value	Prob
Chi-Square	12	54.5979	**<.0001**

Chi square test shows that NMOR has a statistically significant impact on development of chicken embryos (malformations and death).

Case-Control Study of Malformation

The FREQ Procedure

Table of Treatment by Outcome

Treatment Outcome

Frequency	Malforme	Normal	Total
0.5	3	13	16
100.0	4	10	14
150.0	2	3	5
25.0	1	9	10
5.0	0	12	12
50.0	8	16	24
Control	1	31	32
Total	19	94	113

Statistics for Table of Treatment by Outcome

Statistic	DF	Value	Prob
Chi-Square	6	15.0753	**0.0197**

Chi square test shows that there is a statistically significant relationship between exposure to NMOR and subsequent malformations in chicken embryos.

Case-Control Study of Malformation
The FREQ Procedure

Statistics for Table of trt by Outcome

Estimates of the Relative Risk (Row1/Row2)

Type of Study	Value	95% Confidence Limits	
Case-Control (Odds Ratio)	0.1129	0.0144	0.8850
Cohort (Col1 Risk)	0.1406	0.0196	1.0100
Cohort (Col2 Risk)	**1.2455**	**1.0915**	**1.4213**

Analysis of relative risk indicates that risk of malformations in chicken embryos from exposure to NMOR is significantly high.

Case-Control Study of Malformation

The FREQ Procedure

Table of Treatment by Outcome

Treatment Outcome

Frequency	Dead	Normal	Total
0.5	0	13	13
100.0	5	10	15
150.0	11	3	14
25.0	0	9	9
5.0	1	12	13
50.0	10	16	26
Control	1	31	32
Total	28	94	122

Statistics for Table of Treatment by Outcome

Statistic	DF	Value	Prob
Chi-Square	6	44.3219	**<.0001**

Results from chi square test show that there is a statistically significant relationship between exposure to NMOR and subsequent mortality in chicken embryos.

Case-Control Study of Malformation
The FREQ Procedure

Statistics for Table of trt by Outcome

Estimates of the Relative Risk (Row1/Row2)

Type of Study	Value	95% Confidence Limits	
Case-Control (Odds Ratio)	0.0753	0.0098	0.5799
Cohort (Col1 Risk)	0.1042	0.0148	0.7356
Cohort (Col2 Risk)	**1.3839**	**1.1925**	**1.6061**

Analysis of relative risk indicates that risk of death in chicken embryos from exposure to NMOR is significantly high.

NNAT

Number of eggs treated with NNAT

Dose (µg)	No. treated	No. unfertilized	No. dead	No. malformed
Controls	75	2	0	0
0.06	30	4	3	3
0.12	25	2	3	6
0.23	25	0	0	6
0.46	40	2	4	11
0.91	25	2	4	9
1.82	30	3	2	3
3.63	40	0	3	16
5.50	15	0	0	6
7.25	25	2	1	7
14.50	25	2	0	7

SAS output

Case-Control Study of Malformation

The FREQ Procedure

Table of Treatment by Outcome

Treatment Outcome

Frequency	Dead	Malforme	Normal	Total
0.00115	3	3	23	29
0.0023	3	6	20	29
0.00455	0	6	24	30
0.0091	4	11	34	49
0.0181	4	9	17	30
0.0363	2	3	25	30
0.0725	3	16	37	56
0.11	0	6	13	19
0.145	1	7	22	30
0.29	0	7	23	30
Control	0	0	75	75
Total	20	74	313	407

Case-Control Study of Malformation

The FREQ Procedure

Statistics for Table of Treatment by Outcome

Statistic	DF	Value	Prob
Chi-Square	20	50.2383	**0.0002**

Chi square test shows that NNAT has a statistically significant impact on development of chicken embryos (malformations and death).

Case-Control Study of Malformation

The FREQ Procedure

Table of Treatment by Outcome

Treatment Outcome

Frequency	Malforme	Normal	Total
0.00115	3	23	26
0.0023	6	20	26
0.00455	6	24	30
0.0091	11	34	45
0.0181	9	17	26
0.0363	3	25	28
0.0725	16	37	53
0.11	6	13	19
0.145	7	22	29
0.29	7	23	30
Control	0	75	75
Total	74	313	387

Case-Control Study of Malformation

The FREQ Procedure

Statistics for Table of Treatment by Outcome

Statistic	DF	Value	Prob
Chi-Square	10	32.0366	**0.0004**

Chi square test shows that there is a statistically significant relationship between exposure to NNAT and subsequent malformations in chicken embryos.

Case-Control Study of Malformation

The FREQ Procedure

Statistics for Table of trt by Outcome

Estimates of the Relative Risk (Row1/Row2)

Type of Study	Value	95% Confidence Limits	
Cohort (Col2 Risk)	**1.3109**	**1.2323**	**1.3946**

Analysis of relative risk indicates that risk of malformations in chicken embryos from exposure to NNAT is significantly high.

Case-Control Study of Malformation

The FREQ Procedure

Table of Treatment by Outcome

Treatment Outcome

Frequency	Dead	Normal	Total
0.00115	3	23	26
0.0023	3	20	23
0.00455	0	24	24
0.0091	4	34	38
0.0181	4	17	21
0.0363	2	25	27
0.0725	3	37	40
0.11	0	13	13
0.145	1	22	23
0.29	0	23	23
Control	0	75	75
Total	20	313	333

Case-Control Study of Malformation

The FREQ Procedure

Statistics for Table of Treatment by Outcome

Statistic	DF	Value	Prob
Chi-Square	10	20.1202	**0.0281**

Chi square test shows that there is a statistically significant relationship between exposure to NNAT and subsequent mortality in chicken embryos.

Case-Control Study of Malformation

The FREQ Procedure

Statistics for Table of trt by Outcome

Estimates of the Relative Risk (Row1/Row2)

Type of Study	Value	95% Confidence Limits	
Cohort (Col2 Risk)	**1.0840**	**1.0464**	**1.1231**

Analysis of relative risk indicates that risk of death in chicken embryos from exposure to NNAT is significantly high.

APPENDIX C: IMPACT OF NNAT ON NITROTYROSINE CONCENTRATIONS IN CHICKEN EMBRYOS

Nitrotyrosine concentrations in controls and embryos treated with different doses of NNAT

No.	Treatment	Nitrotyrosine nmol/µg Protein
1	Control 1	824.0
2	Control 2	787.6
3	Control 3	14292.4
4	Control 4	699.5
5	Control 5	474.9
6	Control 6	450.8
7	Control 7	438.9
8	Control 8	318.7
9	NNAT 0.06 µg Normal (1)	281.0
10	NNAT 0.06 µg Normal (2)	293.2
11	NNAT 0.06 µg Normal (3)	256.9
12	NNAT 0.06 µg Normal (4)	402.7
13	NNAT 0.06 µg Malformed (1)	407.6
14	NNAT 0.06 µg Malformed (2)	384.7
15	NNAT 0.06 µg Malformed (3)	327.8

16	NNAT 0.06 µg Malformed (4)	410.0
17	NNAT 0.23 µg Normal (1)	501.2
18	NNAT 0.23 µg Normal (2)	959.9
19	NNAT 0.23 µg Normal (3)	568.7
20	NNAT 0.23 µg Normal (4)	548.6
21	NNAT 0.23 µg Malformed (1)	579.5
22	NNAT 0.23 µg Malformed (2)	501.2
23	NNAT 0.23 µg Malformed (3)	617.5
24	NNAT 0.23 µg Malformed (4)	737.8
25	NNAT 0.46 µg Normal (1)	311.8
26	NNAT 0.46 µg Normal (2)	293.1
27	NNAT 0.46 µg Normal (3)	182.1
28	NNAT 0.46 µg Normal (4)	195.7
29	NNAT 0.46 µg Normal (5)	358.2
30	NNAT 0.46 µg Normal (6)	388.7
31	NNAT 0.46 µg Normal (7)	287.3
32	NNAT 0.46 µg Normal (8)	280.1
33	NNAT 0.46 µg Malformed (1)	676.0
34	NNAT 0.46 µg Malformed (2)	1697.0
35	NNAT 0.46 µg Malformed (3)	1099.0
36	NNAT 0.46 µg Malformed (4)	1105.1
37	NNAT 0.46 µg Malformed (5)	380.8
38	NNAT 0.46 µg Malformed (6)	326.5

39	NNAT 0.46 µg Malformed (7)	372.8
40	NNAT 0.46 µg Malformed (8)	426.4
41	NNAT 0.91 µg Normal (1)	338.9
42	NNAT 0.91 µg Normal (2)	257.9
43	NNAT 0.91 µg Normal (3)	241.4
44	NNAT 0.91 µg Normal (4)	886.7
45	NNAT 0.91 µg Malformed (1)	1146.4
46	NNAT 0.91 µg Malformed (2)	864.7
47	NNAT 0.91 µg Malformed (3)	758.4
48	NNAT 0.91 µg Malformed (4)	861.3
49	NNAT 3.63 µg Normal (1)	223.6
50	NNAT 3.63 µg Normal (2)	227.7
51	NNAT 3.63 µg Normal (3)	550.7
52	NNAT 3.63 µg Normal (4)	193.2
53	NNAT 3.63 µg Malformed (1)	372.9
54	NNAT 3.63 µg Malformed (2)	306.9
55	NNAT 3.63 µg Malformed (3)	257.0
56	NNAT 3.63 µg Malformed (4)	466.7

SAS output

Paired t-test to compare nitrotyrosine concentrations in NNAT treated malformed and NNAT treated normal embryos

Dose: 0.06 µg:

The TTEST Procedure

Statistics

Difference	N	Std Err
Normal - Malformed	4	0.0755

T-Tests

Difference	DF	t Value	Pr > |t|
Normal - Malformed	3	-3.14	**0.0515- Difference is statistically significant**

Dose: 0.23 µg:

The TTEST Procedure

Statistics

Difference	N	Std Err
Normal - Malformed	3	0.1

T-Tests

Difference	DF	t Value	Pr > |t|
Normal - Malformed	2	-3.32	**0.08- Difference is not statistically significant**

Dose: 0.46 µg:

The TTEST Procedure

Statistics

Difference	N	Std Err
Normal - Malformed	8	0.59

T-Tests

Difference	DF	t Value	Pr > |t|
Normal - Malformed	7	-2.54	**0.0388- Difference is statistically significant**

Dose: 0.91 µg:

The TTEST Procedure

Statistics

Difference	N	Std Err
Normal - Malformed	3	0.27

T-Tests

Difference	DF	t Value	Pr > |t|
Normal - Malformed	2	-7.49	**0.0173- Difference is statistically significant**

Dose: 3.63 µg:

The TTEST Procedure

Statistics

Difference	N	Std Err
Normal - Malformed	4	0.11

T-Tests

Difference	DF	t Value	Pr > |t|
Normal - Malformed	3	-5.49	**0.0119- Difference is statistically significant**

One way analysis of variance (ANOVA) to compare nitrotyrosine concentrations in NNAT treated and control embryos

The SAS System

The Mixed Procedure

Number of Observations Read	55
Number of Observations Used	55
Number of Observations Not Used	0

Differences of Least Squares Means

Effect	Treatment	Treatment	Adj P
Treatment	NNAT 0.06 Normal	Control	**0.4877**
Treatment	NNAT 0.06 Malformed	Control	**0.8318**
Treatment	NNAT 0.23 Malformed	Control	**1.0000**
Treatment	NNAT 0.23 Normal	Control	**1.0000**
Treatment	NNAT 0.46 Malformed	Control	**0.6430**
Treatment	NNAT 0.46 Normal	Control	**0.1917**
Treatment	NNAT 0.91 Malformed	Control	**0.2082**
Treatment	NNAT 0.91 Normal	Control	**0.9655**
Treatment	NNAT 3.63 Malformed	Control	**0.6921**

Treatment	NNAT 3.63 Normal		Control	**0.2468**

The adjusted P values indicate that nitrotyrosine concentrations were not statistically significantly different between NNAT treated (malformed and normal) five day old embryos and the controls.

<u>One way analysis of variance (ANOVA) to compare nitrotyrosine concentrations in embryos treated with various doses of NNAT</u>

The Mixed Procedure

Differences of Least Squares Means

Effect	Trt	Trt	Pr > |t
Trt	NNAT 0.06	NNAT 0.23	**0.0829**
Trt	NNAT 0.06	NNAT 0.46	**0.1034**
Trt	NNAT 0.06	NNAT 0.91	**0.0125**
Trt	NNAT 0.06	NNAT 3.63	**0.6283**
Trt	NNAT 0.23	NNAT 0.46	**0.6562**
Trt	NNAT 0.23	NNAT 0.91	**0.4628**
Trt	NNAT 0.23	NNAT 3.63	**0.0351**
Trt	NNAT 0.46	NNAT 0.91	**0.1807**
Trt	NNAT 0.46	NNAT 3.63	**0.0390**
Trt	NNAT 0.91	NNAT 3.63	**0.0047**

The p-values indicate that there was a significant difference in the nitrotyrosine concentrations of embryos treated with 0.06 µg NNAT and those treated with NNAT dose 0.91 µg. Similarly, there was a significant difference in the nitrotyrosine concentrations of embryos treated with 0.23 µg NNAT and those treated with the highest NNAT dose of 3.63 µg. There was also a significant difference in the nitrotyrosine concentrations of embryos treated with 0.46 µg and 0.91 µg NNAT and those treated with 3.63 µg NNAT.

One way analysis of variance (ANOVA) to compare nitrotyrosine concentrations in controls with embryos exposed to NNAT that were normal

The SAS System

The Mixed Procedure

Differences of Least Squares Means

Effect	Trt	Trt	Adj P
Trt	NNAT 0.06	Control	**0.0586**
Trt	NNAT 0.23	Control	**0.9989**
Trt	NNAT 0.46	Control	**0.0089**
Trt	NNAT 0.91	Control	**0.5314**
Trt	NNAT 3.63	Control	**0.0145**

The p-values indicate that the nitrotyrosine concentrations in normal – appearing embryos treated with 0.46µg and 3.63 µg NNAT were significantly different from nitrotyrosine in control embryos.