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Developmental Toxicity of Glyceryl Trinitrate in Quail Embryos

Ghalib K. Bardai,¹ Barbara F. Hales,^{1*} and Geoffrey I. Sunahara²

¹Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada, H3G 1Y6

²Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montreal, Quebec, Canada, H4P 2R2

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BACKGROUND: Although glyceryl trinitrate (GTN) is used extensively to treat angina and heart failure, little is known about its effects on the conceptus during organogenesis. The goal of these studies was to investigate the effects of GTN in a model organism, the quail (*Coturnix coturnix japonica*) embryo. **METHODS:** To identify the effects of GTN on quail embryo development, fertilized quail eggs (n = 10–12 eggs/group) were injected with GTN (0, 4.4, 44, or 440 μ M) at Hamburger–Hamilton (HH) stage 0, 9, or 19 and examined 7 days later. Next, HH 9 embryos were injected with GTN (0, 0.88, 4.4, 8.8, 44, 88, and 440 μ M, in 20 μ L per egg) and examined 24-hours, 48-hours, or 72-hours postinjection. Finally, the developing eye on one side was exposed to GTN (44 μ M) ex ovo and the tissue was probed for the presence of nitrated proteins. **RESULTS:** In ovo GTN exposure induced a dose-dependent increase in the number of malformed viable quail embryos with a maximal effect in HH 9 embryos. Microphthalmia, craniofacial, heart, and neural tube defects were elevated in GTN-exposed embryos. An increase in nitrated proteins was observed in the developing eye region of embryos exposed ex ovo to GTN. **CONCLUSIONS:** GTN treatment induced a variety of malformations in quail embryos. The presence of nitrated proteins suggests that organic nitrates, such as GTN, generate reactive nitrogen species. We hypothesize that GTN perturbations in the redox status of the embryo may underlie its developmental toxicity. *Birth Defects Research (Part A) 91:230–240, 2011.* © 2011 Crown in the right of Canada. Published by John Wiley & Sons, Inc.

Key words: organic nitrate; quail embryo; microphthalmia; reactive nitrogen species; redox status

INTRODUCTION

Organic nitrates (amyl nitrite, isosorbide mononitrate, isosorbide dinitrate, and glyceryl trinitrate) are used as anti-ischemic drugs; and they have been found to be effective therapeutic agents in osteoporosis (Jamal et al., 2006) and cancer pain management (Lauretti et al., 2002). These compounds are considered pro-drugs since they require bioactivation to nitric oxide (NO) and nitrite (NO₂⁻) to mediate their pharmacological effects; both NO and NO₂⁻ adversely affect embryo development in vitro (Barroso et al., 1998; Inoue et al., 2004). Given the widespread use of these compounds in cardiovascular diseases and their possible use in other therapeutic settings, it is important to determine the role of the parent compounds and metabolites in mediating any effects on the developing embryo.

The data from animal studies in which the effects of exposure to organic nitrates were determined are inconsistent in the literature. For example, treatment with isosorbide mononitrate in rats prolonged gestation and parturition and increased stillbirth and neonatal death

without an increase in embryotoxicity (Wyeth–Ayerst Laboratories, Philadelphia, PA), whereas isosorbide dinitrate in rabbits produced dose-related embryotoxicity (Wyeth–Ayerst Laboratories). Treatment with glyceryl trinitrate (GTN) did not produce adverse fetal effects in studies conducted with rats and rabbits. There is also some ambiguity with respect to the effects of organic nitrates in humans. The Collaborative Perinatal Project recorded 4 malformed children from a group of 15 patients exposed to GTN and amyl nitrite during the first trimester and 8 other patients exposed to vasodilators (as cited in Briggs et al., 2008). Due to the lack of data, GTN has a “non determined” rating of risk in TERIS, an automated teratology resource, and carries a Food and Drug Administration

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*Correspondence to: Barbara F. Hales, Department of Pharmacology & Therapeutics, McGill University, 3655 Promenade Sir-William-Osler Montreal, Quebec, H3G 1Y6. E-mail: Barbara.hales@mcgill.ca

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warning level of "C", signifying that a risk cannot be ruled out (Friedman et al., 1990). Thus, a detailed teratological evaluation of organic nitrates is needed.

GTN is a structurally simple compound containing three nitrates linked by an ester bond to a glycerol backbone. The metabolic breakdown products of GTN are the di (1,2–1,3 glyceryl) and mono (1,2 glyceryl) nitrates of the parent compound, as well as NO and NO₂ (Hashimoto and Kobayashi, 2003). GTN induces the formation of reactive oxygen species such as superoxide (Münzel et al., 1995), activates second messenger pathways (Bryan et al., 2005), induces post-translational protein modifications (Perlman et al., 2009), and alters pyrimidine nucleotide ratios (Garcia et al., 2010).

The quail embryo is an excellent model system for developmental biology, embryology, and teratology studies since it is accessible and easily manipulated during embryonic stages. This embryo undergoes rapid organogenesis; the availability of a comprehensive description of quail embryo development (Padgett and Ivey, 1959; Padgett and Ivey, 1960; Huss et al., 2008) permits the use of experimental exposures that target specific developmental stages (Drake et al., 2006). In this in ovo model, maternal metabolism is absent allowing the ascertainment of the direct effects of drugs on the embryo. Furthermore, this embryo can be cultured ex ovo, allowing an examination of the effects of exposing specific areas of embryos at one stage of development. Thus, the quail embryo model provides a convenient model system in which to ascertain how a teratogen may interfere with organogenesis in the absence of the mother.

A series of in ovo and ex ovo dose response experiments were done to determine the effects of GTN on quail embryo development. Furthermore, the impact of GTN on protein nitration was investigated.

MATERIALS AND METHODS

Chemicals and reagents.

A stock solution of GTN (99.9% purity; CAS 118-96-7) in corn oil was supplied by General Dynamics Ordnance and Tactical Systems – Canada (Valleyfield, Quebec, Canada) at a concentration of 0.2 g/mL. Authentic standard solutions of 1,2 and 1,3 glyceryl dinitrate solutions in acetonitrile (1 g/L) were purchased from Cerilliant Corporation (Round Rock, TX). Sodium nitrite (1000 mg/L) standards were obtained from Alltech (Deerfield, IL). Acetonitrile and acetone (high performance liquid chromatography [HPLC] grade) were obtained from EM Science (Darmstadt, Germany). All other reagents were from Sigma Chemical (St. Louis, MO).

Glyceryl trinitrate determinations.

GTN determinations were done using a previously described method (Groom et al., 2002) modified for GTN determinations. Briefly, GTN determinations were done using a Waters (Milford, MA) HPLC-UV chromatographic system composed of a Model 600 pump, a Model 717 Plus injector, a Model 2996 Photodiode-Array Detector, and a temperature control module. A Supelcosil LC-CN column (250 × 4.6 mm, 5 μm particles; Supelco, Bellefonte, PA) was used for separation with a column heater set at 35°C. The isocratic mobile phase consisted of methanol/water (30/70, v/v) delivered at 1.5 mL/mi-

nute for 8 minutes. A linear gradient was then run from 30 to 65% methanol, v/v over 12 minutes; after this, the solvent ratio was returned to initial isocratic conditions over 5 minutes. These initial conditions were then held for another 5 minutes. The sample volume injected was 50 μL with a total run time of 25 minutes. The detector was set to scan from 200 to 325 nm with extraction of chromatograms at 205 nm. A calibration curve using known concentrations of GTN was run before analysis. The limit of quantification was 0.05 mg/L. Relative SD for the instrument precision was <1.3% for concentrations equal or higher than 0.5 mg/L, and 7.5% for a concentration of 0.05 mg/L.

Preparation of test solutions.

Dilutions of the stock GTN solution (0.2 g/mL) were made in corn oil to achieve nominal concentrations of 0, 0.88, 4.4, 8.8, 44, 88, and 440 μM (0, 0.1, 0.5, 1, 5, 10, and 50 μg/μL) of GTN. Concentrations of dissolved GTN in the corn oil vehicle were analyzed for the presence of GTN and its metabolites using HPLC, as described above. Actual respective concentrations of GTN (μg/μL) were found to be 0.09 ± 0.03 (SE; n = 3), 0.64 ± 0.05 (n = 3), 1.3 ± 0.1 (n = 3), 5.5 ± 0.48 (n = 3), 9.8 ± 0.8 (n = 3), 54.8 ± 2.5 (n = 3), and 102 ± 1.2 (n = 3). HPLC analysis revealed the absence of known GTN metabolites in the test solutions on the day of injection.

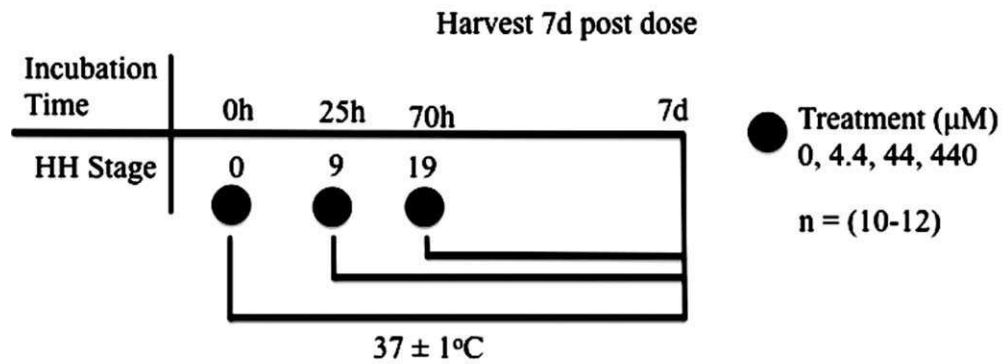
Handling and preparation of Japanese quail embryos.

All animal studies were carried out in accordance with the established protocols of the National Research Council of Canada, Biotechnology Research Institute for the use of animals. Fertilized Japanese quail (*Coturnix coturnix japonica*) eggs (n = 120 for study 1, and n = 1050 for study 2) were obtained from a local breeder (Couvoir Simetin, Mirabel, Quebec, Canada) on the day of laying. Eggs that showed signs of physical stress (cracks or dents) were discarded; the remaining eggs were incubated at 22°C for 2 hours, horizontally on their long axis to allow the germ cells to be positioned close to the topmost point on the yolk and to avoid trauma from the introduction of the needle. During this time, embryo development ceases. After 2 hours of incubation, the eggs were placed in an environment-controlled incubator (Octagon 40 forced draft incubator; Brinsea, Titusville, FL) set at 37 ± 1°C and 60 ± 5% relative humidity. All embryos were staged according to the criteria of Hamburger and Hamilton (HH; Hamburger and Hamilton, 1992).

Glyceryl trinitrate injection procedure.

GTN in corn oil was injected directly into the center of the egg yolk using an established protocol that does not cause developmental anomalies in control embryos (Drake et al., 2006). Before injection, the blunt end of the egg was wiped with sterile gauze moistened with 70% ethanol; a small hole (1 mm) was made using a Dremel (Racine, WI) tool without penetrating the membrane. Eggshell residue remaining after the drilling was brushed away. Vehicle (corn oil) or test substance was slowly injected into the center of the yolk using a Hamilton glass syringe with a 0.8 × 40 21GA 11/2 needle. After a single injection of GTN, the hole was sealed with paraffin

Study 1



Study 2

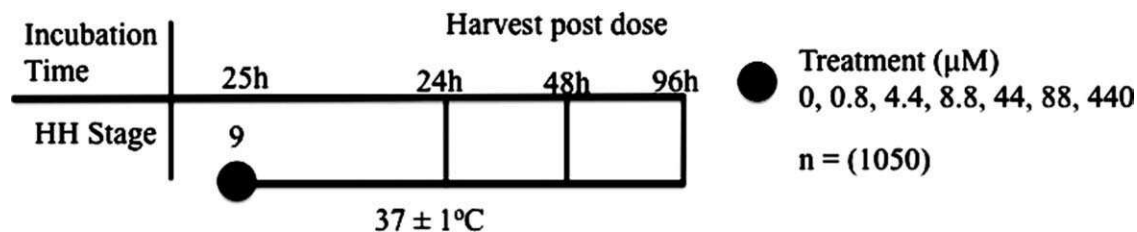


Figure 1. Treatment protocol for glyceryl trinitrate (GTN) exposure studies: study 1 and study 2. For study 1, three groups of eggs ($n = 10\text{--}12/\text{group}$) were incubated to the desired stage of development (Hamburger and Hamilton [HH] stages 0, 9, and 19), GTN was then administered to the eggs and embryos were assessed 7 days later. For study 2, GTN was administered at HH stage 9 and embryos were assessed 24 hours, 48 hours, and 72 hours post-dose.

wax and the eggs were placed into a forced draft incubator, as described above.

Experimental design.

The temporal and concentration dependency of the developmental toxicity of GTN were investigated in separate studies. The objective for study 1 was to identify the effects of GTN during critical periods of development (Fig. 1 Top). Eggs were randomly divided into control (corn oil) and GTN treated groups and allowed to incubate to HH stage 0, 9, or 19. In our laboratory, quail embryos reached HH 9 and HH 19 in an incubation time of 25 hours and 70 hours, respectively. Since quail eggs are highly mottled and colored, making windowing unreliable, a subset ($n = 5$) of eggs was removed to check that embryos had developed to the desired stage (Padgett and Ivey, 1960; Bellairs and Osmond, 2005). Each HH group was then injected with a constant volume of $10 \mu\text{L}$ GTN solution ($n = 10$ eggs/group) to give final concentrations, based upon the volume of the egg (10 mL), of 4.4, 44, and $440 \mu\text{M}$ per egg and examined after incubation for 7 days. The objective for study 2 (Fig. 1 Bottom) was to increase the range of treatment concentrations, based upon our observations in study 1. HH 9 embryos were injected with a constant volume of $20 \mu\text{L}$ GTN solution to give a final concentration of 0, 0.88, 4.4, 8.8, 44,

88, or $440 \mu\text{M}$ per egg; embryos were examined 24-hours, 48-hours, or 72-hours postinjection.

Analysis of embryos treated in ovo.

After incubation, the eggs were removed from the incubator. Embryonic survival was determined after carefully removing the shell from a 1 to 2 cm area directly over the embryo and observing the presence of a beating heart. Live embryos were kept at 4°C for 2 hours; this was sufficient to arrest all physiologic function. Body lengths were measured after embryo harvest. Craniofacial development was compared in treated and control embryos harvested 48 hours post-treatment in the following manner. We first assessed the presence of a meso-metencephalic fold; this fold delineates the hindbrain from the mesencephalon (Fig. 2A). The mesencephalon was then evaluated to ensure that it was a completely rounded and closed. We next evaluated both the diencephalon and telencephalon (Fig. 2A) to ensure that these were present as discrete structures divided by a well-defined fold. The eye and its size and position relative to the telencephalon and the diencephalon were examined. Cardiac development was also assessed in comparison to control embryos. The position of the developing heart inside the body cavity and proper looping, as well as the conus arteriosus, ventricle, and atrium, were examined. For neural tube defects, embryos were examined from

the dorsal view. Indications of failure of the neural tube to close were considered as neural tube defects. After assessment of embryos as described above, photomicrographs of the craniofacial region of the embryos were taken using a stereomicroscope. In these photomicrographs, both the eye and mesencephalon were present as well-defined discrete structures that allowed for tracing using the program ImageJ. Briefly, the entire craniofacial area was traced in the following manner: a perimeter was traced, using ImageJ, starting at the meso-metencephalic fold, over the mesencephalon, diencephalon, around the telencephalon, and ending at the first pharyngeal arch. A direct line was then traced from the first pharyngeal arch to join the starting point (meso-metencephalic fold).

Histologic analysis.

Embryos were cut away from the vitelline and allantoic circulatory system, rinsed with isotonic saline, weighed, and a gross evaluation was done. Embryos were then fixed overnight in 4% paraformaldehyde. Embryos destined for ossified bone and cartilaginous double staining, including viable embryos from all dose groups and those embryos displaying microphthalmia, were fixed according to the method described by Nakane and Tsudzuki (1999) for 7-day-old embryos. Briefly, embryos were simultaneously fixed and stained for 2 days at 37°C in freshly prepared 95% ethanol containing 20 mL acetic acid, and 15 mg Alcian blue 8GX. After this, embryos were dehydrated in a fresh solution of 95% ethanol. Embryos were then transferred to 0.002% alizarin red S/0.2% KOH solution for 1 day and cleared in increasing concentrations (25, 50, and 75%) of glycerin/H₂O solutions for 7 days and then stored in 100% glycerin.

Embryos for cryosectioning were processed as described below. After overnight incubation in 4% paraformaldehyde, specimens were rinsed in phosphate buffered saline (PBS), placed in a 25% sucrose-PBS solution overnight, and then transferred to a 35% sucrose-PBS solution. After this, half of the sucrose-PBS solution was removed and replaced with optimum cutting temperature (OCT) compound. Embryos remained in the OCT solution for 2 hours before being transferred into 100% OCT for 1 hour at 40°C. Embryo heads were then dissected and immersed into OCT compound (Shandon Scientific, Pittsburgh, PA) and frozen at -80°C until use. Pre-frozen samples equilibrated to -15°C in a Leica 1900 cryostat were placed into the cryostat chamber. The cryostat and knife temperatures were set to -18°C and -25°C, respectively. Tissue sections (8 µm thick) were cut and placed onto slides for storage at -80°C until required.

Paraffin processing of quail embryos was carried out as previously described (Simard et al., 2006). Briefly, embryos were collected and fixed in a 3.7% formaldehyde/60% ethanol/30% water solution for 1 hour, washed twice in 70% ethanol for 15 minutes, and stored at -20°C. Embryos were dehydrated through a series of ethanol washes and washed twice for 30 minutes in xylene before being embedded in paraffin. Embryos were then sectioned at 10 µm and sections were placed on Fisherbrand Superfrost Plus glass slides.

Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay.

Apoptosis was assessed by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) assay using ApopTag Peroxidase In Situ Apoptosis Detection kit (S7100, Chemicon International, Temecula, CA), according to the manufacturer's instructions. After TUNEL staining, sections were counterstained with methyl green and mounted under glass coverslips with Permount (Fisher Scientific, Montreal, Quebec, Canada).

Preparation of ex ovo embryo cultures.

After incubation at 37.7 ± 0.5°C and 55 to 60% relative humidity, eggs were processed according to Chapman et al. (2001) at HH 9 when primary optic vesicle development occurs. Briefly, the yolk was placed into a glass Petri dish and the albumen over the blastoderm was removed. A filter paper (2 cm × 2 cm) with a small hole (5 mm diameter) punched in the middle was placed over the embryo. After cutting through the vitelline membranes, the filter paper with attached, blastoderm was placed ventral side up into a Petri dish with an agar-albumen substrate. Any remaining yolk adhering to the filter paper was gently washed away. Using a stereomicroscope, a filter paper soaked in 88 µM GTN in corn oil was placed next to the left eye, while another filter paper soaked in the corn oil vehicle was placed next to the opposite eye. The embryos were placed in a humidified, heated chamber for 18 hours. Embryos were then removed, photographed, and processed as required.

Western blot analysis.

Protein concentration was determined with bicinchoninic acid protein assay kit from Pierce Chemical Company (Rockford, IL) using bovine serum albumin as the standard. Samples were loaded on 10% gels using a Bio-Rad Mini Protean II electrophoresis system and run at room temperature for 2 hours at 110 volts. Separated proteins were electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane overnight at 4°C; membranes were developed with One-Step Western Advanced Kit for mouse primary antibody (GenScript Corporation, Piscataway, NJ). Primary anti-nitrotyrosine mouse monoclonal antibody (clone 1A6, Millipore Canada, Etobicoke, Ontario, Canada) and anti-beta actin mouse monoclonal antibody, as an internal standard (V10178, GenScript Corporation), were used at 200-fold and 10,000-fold dilutions, respectively.

Statistical analysis.

Measurements of embryo were done from photomicrographs using ImageJ, version 1.43 software (National Institutes of Health, Bethesda, MD). The data were expressed as mean ± SD and were evaluated by chi-square for linear trends, one-way or two-way analysis of variance (ANOVA) or Spearman on ranks, where appropriate. The a priori level of significance was $p \leq 0.05$. Differences between exposure groups and their respective controls were considered significant when $p \leq 0.05$ using the Dunnett's post hoc test. Statistical analysis was done

Table 1
Effects of In Ovo GTN Exposure after 7 Days Incubation: Study 1

Stage (HH)	GTN (μM)	Eggs ^a	Fertile eggs ^b	Mortality (%)	Crown rump (mm) ^c	Weight (g) ^c	Microphthalmia (%)
0	0	10	7	0	20.4 \pm 2.7	0.81 \pm 0.13	0
	4.4	10	9	22	19.4 \pm 1.4	0.76 \pm 0.17	0
	44	10	9	44	16.5 \pm 2.5*	0.48 \pm 0.33***	0
	440	10	10	70	19.5 \pm 1.4	0.84 \pm 0.01	0
9	0	11	10	0	20.6 \pm 1.8	0.94 \pm 0.07	0
	4.4	11	10	10	18.2 \pm 2.4	0.76 \pm 0.15*	20
	44	11	10	20	19.8 \pm 2.2	0.79 \pm 0.15*	40
	440	11	9	56	18.3 \pm 3.5	0.68 \pm 0.16**	0
19	0	11	10	0	20.8 \pm 2.1	0.93 \pm 0.08	0
	4.4	11	10	10	19.4 \pm 2.0	0.81 \pm 0.09	0
	44	11	9	11	19.5 \pm 1.7	0.78 \pm 0.10	22
	440	11	11	18	19.2 \pm 1.8	0.76 \pm 0.06*	9

^aNumber of eggs treated.

^bNumber of fertile eggs.

^cMean \pm SD.

*Significantly different from control (** $p \leq 0.0001$; ** $p \leq 0.001$; * $p \leq 0.05$) when examined using Dunnett's post hoc test. GTN, glyceryl trinitrate; HH, Hamburger-Hamilton.

using GraphPad Prism version 5.0 for Macintosh, GraphPad Software (San Diego, CA).

RESULTS

Developmental Toxicity Induced by Glyceryl Trinitrate Treatment (Study 1)

The effects of GTN treatment (0, 4.4, 44, or 440 μM) on HH 0, 9, or 19 stage quail embryos were assessed according to the exposure protocol shown in Figure 1 (study 1). GTN treatment produced a dose-dependent increase in embryo mortality (Table 1). GTN was highly embryotoxic to HH 0 embryos while HH 9 and HH 19 embryos were more resistant. HH 19 embryos were least susceptible to GTN insult, as seen by the increased number of surviving embryos across dose groups when compared to the HH 0 and HH 9 groups. Embryo growth, as assessed by crown rump lengths (Table 1), was not significantly affected except at 44 μM ($p \leq 0.05$) in the HH 0 group. An ANOVA two-way 4 (dose) \times 3 (groups) factorial design between groups was conducted to analyze the effects of GTN treatment on embryo weights. Developmental stage did not have a significant impact ($F^{2,83} = 2.99$; $p = 0.05$). However, the interaction effect (dose \times stage) and GTN treatment both had significant effects ($F^{6,83} = 2.85$; $p = 0.01$) and ($F^{3,83} = 8.91$; $p \leq 0.001$), respectively. Post hoc comparisons of embryo weights within dose groups was performed using the Dunnett's multiple comparison test. The HH 0 group showed a significant difference ($p \leq 0.001$) between control and the 44 μM GTN concentration. However, post hoc analysis of the highest concentration tested in this dose group was not possible due to the low number of survivors ($n = 3$). In the HH 9 group, significant differences were observed between controls, the 4.4 μM ($p \leq 0.05$) and 440 μM concentration tested ($p \leq 0.001$). Multiple comparison testing showed significant differences ($p \leq 0.05$) between control and the highest concentration tested in the HH 19 treatment group. An increased incidence of microphthalmia was observed in the 44 μM treatment groups in both HH 9 (40% incidence) and HH 19 (22% incidence) treated-embryos. The absence of microphthalmia in the HH 9 embryos exposed to the highest dose of GTN (440 μM)

may be a consequence of embryo mortality. Skeletal anomalies were not observed in combined Alcian blue-Alizarin red stained embryos (data not shown). Taken together, these data indicate that GTN treatment was embryotoxic to early stage embryos (HH 0), retarded embryo growth in a dose-dependent manner independent of the stage and induced microphthalmia.

Histologic Analysis

A histologic analysis was performed on the embryos presenting with microphthalmia (Fig. 3A). Embryos presenting with microphthalmia had upper beaks that were slightly bent toward the microphthalmic eye (Fig. 3A). A cross sectional analysis through the sagittal plane (Fig. 3B) clearly showed that the affected eye (designated as ae) was decreased in volume and had a small round lens compared to the oval lens of the unaffected eye (ue). In a higher magnification view of the ae region (Fig. 3C), the neural retinal (nr) cell layer was smaller and detached from the retinal-pigmented epithelium (rpe) compared to the ue region (Fig. 3D); the rpe of the ae was thin and highly disorganized.

Because the underlying matrix is critical to normal organ growth and development, we investigated the matrix of connective tissue surrounding both the ua and ae. The deposition of glycosaminoglycans was investigated using the Alcian blue staining technique while cells undergoing apoptosis were detected with the TUNEL technique (Fig. 4). A uniform distribution of glycosaminoglycans in the ue (Fig. 4A) and an absence of apoptosis (Fig. 4B) were observed in the ue. In contrast, there was an abnormal, enlarged amount of glycosaminoglycan staining in the ae (Fig. 4C); furthermore, TUNEL staining revealed increased apoptosis at the periphery of this glycosaminoglycan staining (Fig. 4D; *arrow*). These data demonstrate that the underlying matrix was affected in the microphthalmic eye, coinciding with an increase in the presence of apoptotic cells in comparison to the ue.

Detailed Dose-response Study (Study 2)

A detailed dose-response study was conducted at HH 9 to characterize embryos treated with GTN. Fertile quail eggs ($n = 1050$) were injected with GTN (0, 0.88, 4.4, 8.8,

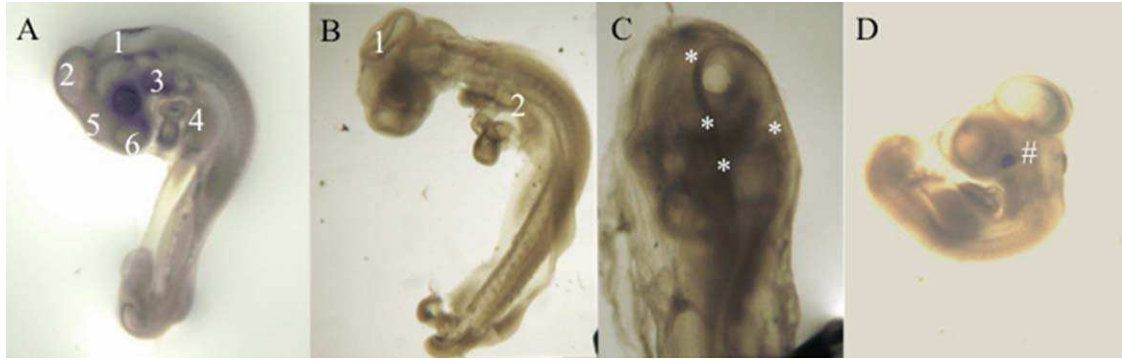


Figure 2. Effects of glyceryl trinitrate (GTN) treatment on quail embryo development. Embryos were observed under a stereomicroscope. **(A)** Control embryo 48-hours post-dose; (1) hindbrain, (2) mesencephalon, (3) eye, (4) heart, (5) diencephalon, and (6) telencephalon. **(B)** GTN-exposed embryo (44 μM) 48-hours post-dose showing failure of the cranial neural tube (1) to close, absence of mesencephalon, and an underdeveloped hind and forebrain; the eye is still oval and craniofacial distance is reduced when compared to the control embryo. The heart (2) is protruding from the body cavity. **(C)** GTN-exposed embryo 48-hours post dose (440 μM), seen from the dorsal view, showing incomplete closure of neural tube (*). **(D)** GTN-exposed embryo 72-hour post-dose (44 μM) with microphthalmia (#).

44, 88, or 440 μM ; $n = 50$ eggs/concentration) at HH 9 of development; after treatment with GTN, the eggs were returned to the incubator and examined for 24 hours ($n = 350$), 48 hours ($n = 350$), and 72 hours ($n = 350$) post dose (pd) for body length, craniofacial development (mesencephalon, metencephalon, prosencephalon, telencephalon, optic vesicle, and eye), heart (atrium, ventricle), and neural tube defects compared to timed-matched controls. Figure 2 shows examples of the types of defects observed. A normal 48 hour pd individual is shown in Figure 2A with a well developed hindbrain, mesencephalon, eye, heart, and forebrain. Figure 2B shows a GTN (44 μM) exposed embryo 48-hours pd in which there is a failure of cranial neural tube closure, absence of mesencephalon, and an underdeveloped hind and forebrain when compared to control (Fig. 2A). The heart and the

developing vessels are protruding from the body cavity. Figure 2C depicts failure of complete neural tube closure after GTN (440 μM) exposure in an embryo 48-hour pd, seen from the dorsal view. Figure 2D shows a GTN (44 μM) exposed embryo with microphthalmia 72-hour post-treatment.

All viable embryos (normal and malformed; Table 2) in the 24-hour and 72-hour pd groups were subjected to a one-way between patients ANOVA to compare the effects of GTN treatment on embryo growth as assessed by body length. There was a significant effect of treatment on embryo length at the $p \leq 0.05$ level for the seven concentrations tested in both the 24-hour and 72-hour pd groups ($F^{6,241} = 17$; $p = 0.0002$) and ($F[6230] = 4$; $p = 0.0003$), respectively (Table 2). Post hoc comparisons using Dunnett's multiple comparison test for the 24-hour

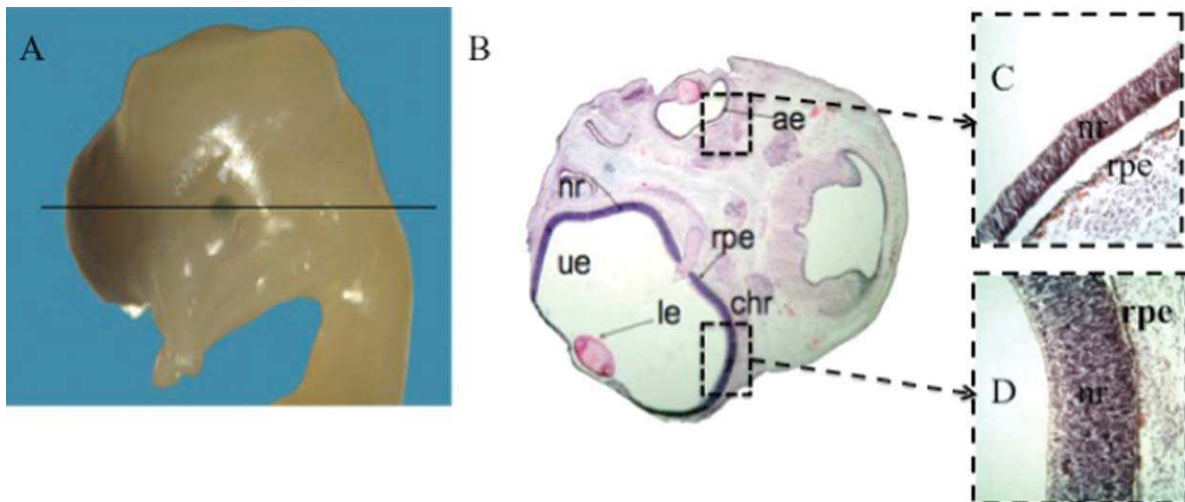


Figure 3. Effects of glyceryl trinitrate (GTN) on eye development. **(A)** A stereomicroscope representation of a 7-day-old embryo treated at Hamburger and Hamilton (HH) 9 with 440 μM GTN presenting with unilateral microphthalmia. Line represents where a sagittal cross section was taken; **(B)** is a hematoxylin and eosin stained cross section of **(A)**. affected eye (ae); unaffected eye (ue); lens (le); neural retina (nr); retinal pigment epithelium (rpe); choroid (chr). **(C and D)** Are higher magnification ($\times 40$) views of the ae and ue. The ae shows a thin and highly disorganized nr detached from the rpe.

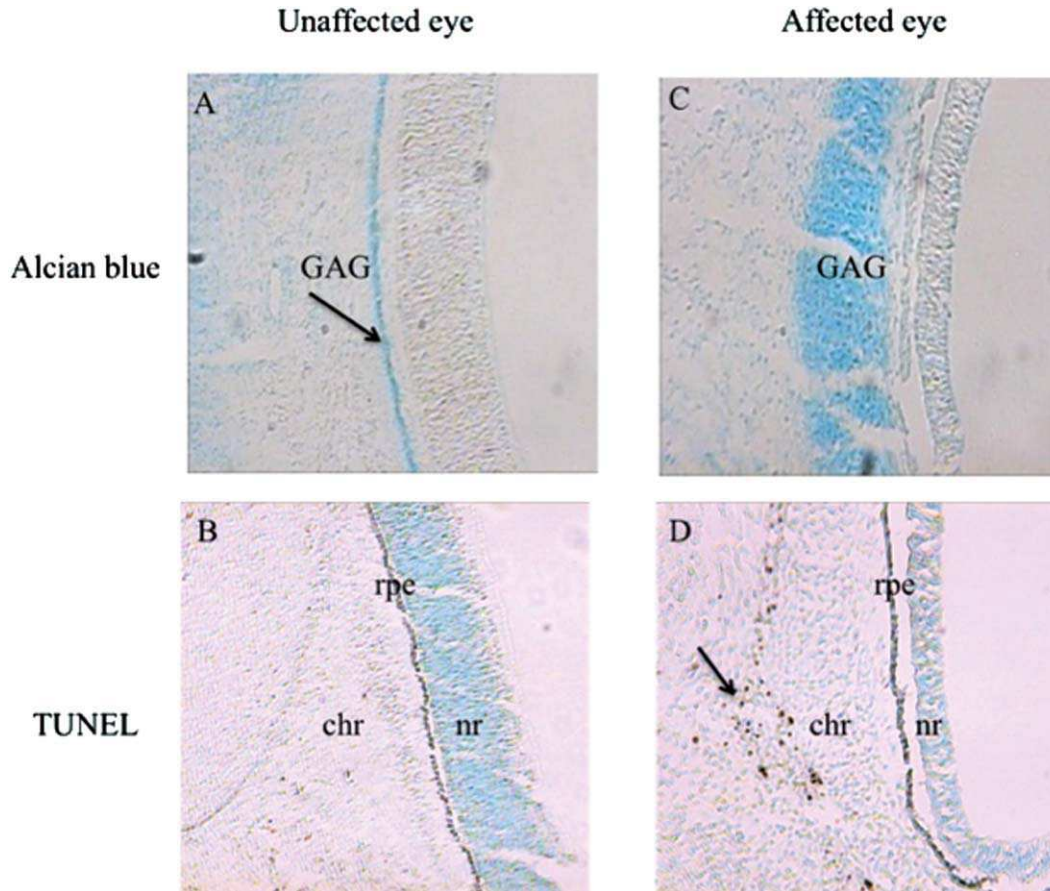


Figure 4. Alcian blue and transferase biotin-dUTP nick end labeling (TUNEL) staining in the underlying supporting matrix of both the unaffected and affected eye ($\times 40$). Alcian blue staining for glycosaminoglycans (GAG) in the unaffected eye (A) clearly shows an even distribution of staining when compared to the affected eye (C), which shows increased, abnormal glycosaminoglycan distribution. TUNEL staining shows increased apoptosis in the choroid (chr) of the affected (D) compared to the unaffected eye (B) at the periphery of the abnormal GAG distribution of the affected (D, arrow) eye. RPE, retinal pigment epithelium; neural retina (nr).

pd group showed significant differences ($p \leq 0.001$) across all dose groups when compared to control at $p \leq 0.05$; however, in the 72-hour pd group, significant differences were seen between control and the 4.4 ($p \leq 0.05$), 44 ($p \leq 0.01$), and 440 μM ($p \leq 0.05$) GTN concentrations. Survival of embryos across all dose groups was not significantly affected, with only the highest dose (440 μM) showing an increase in embryo mortality at 72-hour post-treatment. Taken together, these results suggest that the exposure of early embryos to GTN decreased embryo growth; embryos displayed substantial recovery from the effects of treatment by 72-hour post-treatment.

Viable embryos with craniofacial (mesencephalon, metencephalon, prosencephalon, telencephalon, optic vesicle, and eye), heart (atrium, ventricle), and neural tube defects were observed in all GTN-treatment groups. The dose-response relationship for malformed embryos with craniofacial, heart, and neural tube defects is illustrated in Figure 5A (48-hour pd) and Figure 5B (72-hour pd). The $\text{EC}_{50\text{s}}$ (concentrations at which GTN induced malformations in 50% of the exposed embryos) in the 48-hour and 72-hour pd treatment groups were 10 and 16 μM , respectively (Fig. 5A and B).

A Spearman correlation matrix showed a significant correlation between craniofacial defects and heart and

neural tube defects in the 48-hour pd ($r = 0.99$, $p = 0.0004$; $r = 0.97$, $p = 0.0028$) embryos with $\text{EC}_{50\text{s}}$ of 12 and 29, respectively. A significant correlation was also observed between craniofacial and heart defects in the 72-hour pd ($r = 0.87$, $p = 0.012$) group with $\text{EC}_{50\text{s}}$ of 7.3 and 50, respectively (Fig. 5B). In contrast, there was not a significant correlation between craniofacial defects and microphthalmia ($r = 0.65$, $p = 0.39$) ($\text{EC}_{50} > 100$). Thus, the GTN treatment-induced dose-dependent increase in craniofacial defects was strongly correlated with both cardiac and neural tube defects in the 48-hour and 72-hour pd groups, suggesting a common mechanism. Microphthalmia was not correlated with these defects, suggesting a different mechanism of toxicity.

Since craniofacial defects were one of the predominant malformations that we observed, we measured craniofacial size by tracing the perimeter of the craniofacial structure using ImageJ in embryos 24-hours, 48-hours, and 72-hours pd. A trend toward a decrease in craniofacial size was significant for the seven GTN concentrations tested at the $p \leq 0.05$ level ($F[6241] = 13.7$, $p = 0.02$), ($F[6261] = 6.1$, $p = 0.002$) and ($F[6255] = 11.1$, $p = 0.002$; Table 2). Post hoc comparisons using Dunnett's multiple comparison test was done for all the pd groups at $p \leq 0.05$. While the 24-hour pd group showed a significant difference

Table 2
Effects of In Ovo GTN Treatment on Body Length, Cranio-Facial, and Mesencephalon Circumference and Eye Diameter: Study 2

Post-dose (h)	GTN (μ M)	(n) ^a	Body length (mm) ^b	Craniofacial circumference (mm)	Mesencephalon (mm)	Eye circumference (mm)
24	0	37	6.6 \pm 0.72	1.3 \pm 0.26	n/d	n/d
	0.88	32	5.7 \pm 0.76***	1.1 \pm 0.19**	n/d	n/d
	4.4	44	5.5 \pm 0.58***	1.0 \pm 0.18***	n/d	n/d
	8.8	29	5.5 \pm 0.59***	0.97 \pm 0.19***	n/d	n/d
	44	38	5.8 \pm 0.65***	1.0 \pm 0.21***	n/d	n/d
	88	36	5.3 \pm 0.70***	0.91 \pm 0.16***	n/d	n/d
	440	32	5.1 \pm 1.1***	0.96 \pm 0.27***	n/d	n/d
48	0	35	n/d	3.6 \pm 0.63	1.0 \pm 0.28	0.39 \pm 0.13
	0.88	43	n/d	4.1 \pm 1.0	1.1 \pm 0.30	0.52 \pm 0.17***
	4.4	36	n/d	3.9 \pm 1.2	1.0 \pm 0.32	0.45 \pm 0.21
	8.8	40	n/d	3.4 \pm 0.68	0.93 \pm 0.21	0.35 \pm 0.09
	44	39	n/d	3.6 \pm 1.0	0.97 \pm 0.26	0.40 \pm 0.14
	88	36	n/d	3.6 \pm 0.91	0.90 \pm 0.22	0.38 \pm 0.12
	440	39	n/d	2.9 \pm 0.91*	0.80 \pm 0.25**	0.32 \pm 0.14
72	0	35	15.9 \pm 0.84	12.0 \pm 1.8	3.7 \pm 0.54	2.4 \pm 0.71
	0.88	46	15.6 \pm 0.76	12.9 \pm 1.8	3.9 \pm 0.57	2.4 \pm 0.56
	4.4	46	14.9 \pm 1.6*	13.5 \pm 2.2	3.9 \pm 0.74	2.7 \pm 0.73
	8.8	35	15.6 \pm 1.0	13.9 \pm 2.6*	4.1 \pm 0.90	2.6 \pm 0.68
	44	48	14.8 \pm 1.0**	10.9 \pm 2.4	3.3 \pm 0.79	1.6 \pm 0.85***
	88	43	15.2 \pm 1.2	11.9 \pm 2.7	3.5 \pm 1.0	2.1 \pm 0.82
	440	24	14.6 \pm 1.8*	9.8 \pm 2.1*	3.0 \pm 0.58**	1.3 \pm 0.50***

^aNumber of viable individuals.

^bMean \pm SD.

*Significantly different from control (*** p \leq 0.0001; ** p \leq 0.001; * p \leq 0.05) when examined using Dunnett's post hoc test. GTN, glyceryl trinitrate; HH, Hamburger-Hamilton.

across all dose groups when compared to control, in the 48-hour pd group and the 72-hour pd group craniofacial size was significantly reduced compared to control in the highest dose groups tested.

To determine the relative contributions of discrete structures that contribute to craniofacial size, the mesencephalon, and the eye, we undertook an examination of the sizes of these structures in both the 48-hour and 72-hour pd GTN treatment groups (Table 2) by tracing these discrete structures within the craniofacial area as described in MATERIALS AND METHODS. Measurements were done only in embryos that had apparently normal eyes. Examination of embryos in the 48-hours pd group showed significant effects of GTN exposure on both the mesencephalon and eye across treatment groups (p = 0.0064 and p = 0.001, respectively). The increase in eye circumference in the 0.88 μ M GTN treatment group may reflect increased growth or be a consequence of edema. Dunnett's post hoc comparisons of the size of the mesencephalon showed a significant difference between control and the highest dose group (p \leq 0.01) in the 48-hour pd group. Measurements of mesencephalon and eye circumference performed on the 72-hour pd treatment group showed significant treatment-related effects (p = 0.009 and p = 0.03, respectively). Dunnett's multiple comparison test showed a difference between the control group and the highest concentration tested (p \leq 0.01) for mesencephalon measurements. Differences were seen between control and the 44 (p \leq 0.001) and 440 (p \leq 0.001) GTN-treatment groups for eye diameter measurements. Taken together, these results suggest that GTN had an early effect on embryo growth and craniofacial size (24-hour pd) and that this effect persisted in the embryos examined 72-hours pd. Since the mesencephalon

and the eye contribute to the overall size of the craniofacial area, the decrease in craniofacial size may be attributed to decreases in the size of these structures.

Effects of Glyceryl Trinitrate Treatment of Ex Ovo Embryo Cultures

The ex ovo culture method was used to analyze the effects of GTN exposure on the development of the optic vesicle in more detail. Using this method, localized GTN exposure to one side of the developing eye field is possible while the other eye field serves as a control. Figure 6A shows the developing optic vesicle. On the non-treated side, two very distinct dark banding patterns (*dashed lines*) are observed when compared to the treated side. These darker banding patterns on the treated side are a developing lens and the regressing presumptive neural retina and retinal pigmented epithelium, as seen in a cross section of this area (Fig. 6B). The GTN treated side shows the presence of an optic vesicle devoid of a lens, neural retina, and retinal pigmented epithelium. Taken together, these data demonstrate that localized GTN exposure to embryos ex ovo arrested the development of the eye, specifically by inhibiting the development of the neural retinal and retinal pigment epithelium.

GTN has been shown to post translationally modify proteins by nitrating amino acids (Perlman et al., 2009). To determine if GTN treatment results in the nitration of embryonic proteins, we determined the levels of nitrated proteins in embryo homogenates from vehicle and GTN-exposed embryos using Western blot analysis with a monoclonal antibody to nitrotyrosine (anti-NT). While nitrated embryonic proteins were detected in control embryos, a dose-dependent increase in the amounts of

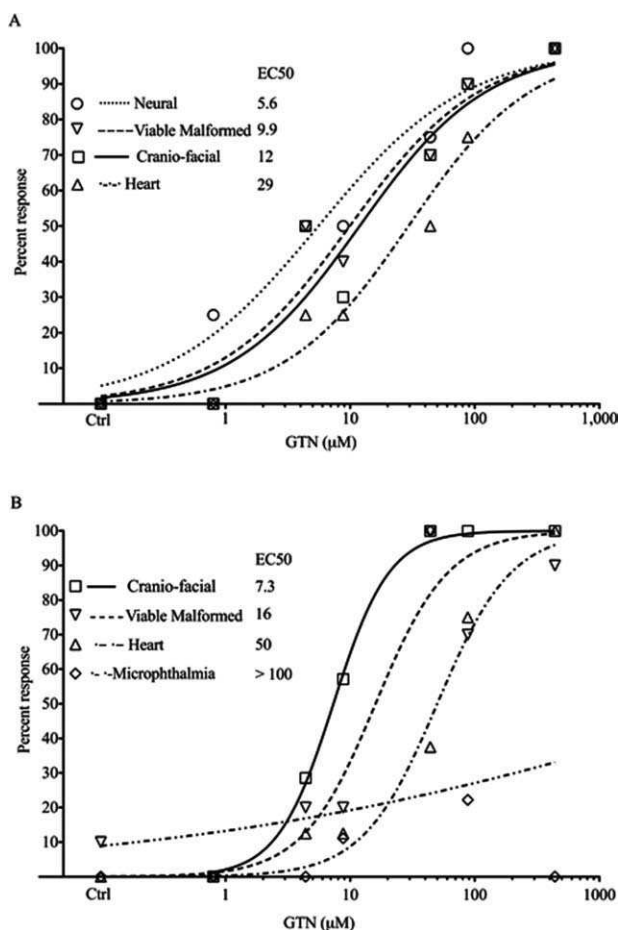


Figure 5. Dose-response analysis of the malformations observed in glyceryl trinitrate (GTN)-exposed embryos from study 2 at 48-hours (A) and 72-hours (B) post-dose. Symbols indicate viable malformed embryos (-▽-), craniofacial (-□-), heart (-△-), neural tube (-○-), and microphthalmic (-◇-) defects. Data have been normalized on the y-axis from 0 to 100%.

these nitrated proteins was observed in GTN-exposed embryos (Fig. 7) with predominant bands appearing in the 216 and 16 kDa regions.

DISCUSSION

The paucity of data on the toxicological effects of organic nitrates prompted us to carry out studies with a well-characterized organic nitrate, GTN, and the quail embryo, a biologic model representative of mammalian development. Our studies demonstrate that GTN is highly embryotoxic to early stage quail embryos; the embryo lethality of GTN was greater in HH 0 embryos than in HH 9 and HH 19 stage embryos (Table 1). Significant decreases in embryo weight were observed in GTN exposed embryos at all three HH stages. In addition, GTN exposure resulted in numerous malformations, including craniofacial, heart, and neural tube defects, as well as microphthalmia. Localized ex ovo treatment of GTN also led to ocular malformations.

GTN exposure may result in the generation of pro-oxidative reactive nitrogen species (Fig. 7) or reactive oxy-

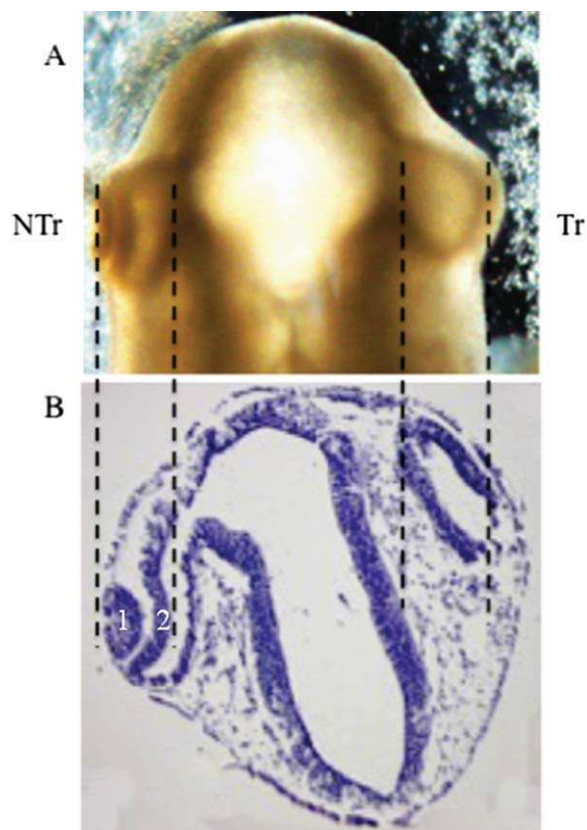


Figure 6. Effects of ex ovo glyceryl trinitrate (GTN) treatment of the quail embryo. (A) One side of a Hamburger and Hamilton (HH) 9 embryo was treated with 44 μM GTN (Tr) the other with corn oil (NTr) and examined 18 hours later. (B) Hematoxylin and eosin stained cross sectional analysis of the same embryo shows the NTr side with a developing lens (1) an invaginating optic vesicle (2). The Tr side eye field shows the failure of the lens to develop and the absence of an invaginating optic vesicle (×40).

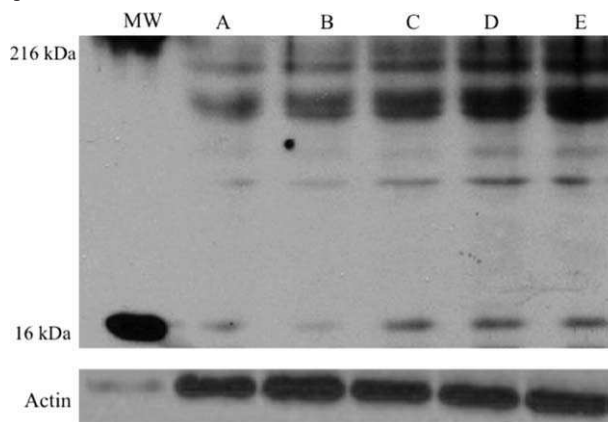


Figure 7. Effects of glyceryl trinitrate (GTN) treatment on protein nitration. Hamburger and Hamilton (HH) 9 embryos were treated with GTN and examined 18 hours later for the presence of nitrated proteins using Western blot analysis. Lanes MW (nitrated standards), (A) untreated embryo, (B) vehicle treated, (C) 4.4 μM GTN, (D) 44 μM GTN, and (E) (440 μM GTN). An increase in the presence of nitrated proteins is seen by the increase in band intensities at 216 and 16 kDa.

gen species (Münzel et al., 1995; Haqqani et al., 2002). Such free radicals would perturb redox homeostasis or the equilibrium between pro-oxidative and anti-oxidative processes. Anti-oxidative defenses consist of small molecules (glutathione [GSH], ascorbic acid, and tocopherols) and the enzymatic activities of antioxidant enzymes (GSH peroxidases, GSH reductase), which are generally reduced in embryos compared to the adult (Wilson et al., 1992; Parman et al., 1999; Winn and Wells, 1999). Disturbances in redox homeostasis may also affect cellular energy metabolism (Eriksson and Borg, 1991; Akazawa, 2005; Wentzel and Eriksson, 2005) and redox sensitive transcription factors.

One of the predominant malformations observed in GTN-exposed quail embryos is microphthalmia. At HH 9 in avian development, the optic vesicles (OVs), symmetrical bilateral evaginations from the diencephalon, expand through the mesenchyme to contact the surface ectoderm at which point the ectoderm thickens into a lens placode, which then begins to invaginate (Bellairs and Osmond, 2005). This invagination generates two distinct cellular layers, an internal layer, the neural retina and an external layer, the retinal pigment epithelium, while the ventral portion of the OV forms the optic nerve (Chow and Lang, 2001; Adler and Canto-Soler, 2007). Our histologic data of the sagittal cross sections shows the affected eye, with a small round lens and an undeveloped optic nerve (Fig. 3B). However, at a higher magnification, it is apparent that the neural retina of the affected eye is thin, disorganized, and detached from the retinal-pigmented epithelium (Fig. 3C and D). The effects of GTN exposure on early stages of eye development, when the OV is in contact with the surface ectoderm, may reflect an effect on the specification of the neural retina and retinal pigmented epithelium by inductive signals originating in the surface ectoderm via FGF-8 (Vogel-Höpker et al., 2000) and in the extra-ocular mesenchyme through an activin-like signal (Fuhrmann et al., 2000).

Using whole embryo cultures, Fantel et al. (1986) and Greenaway et al. (1986) demonstrated that the nitro-based compound niridazole (1-(5'-nitro-2-thiazolyl)-2-imidazolidinone) induced microphthalmia. The conclusions drawn in these studies were related to the reactive species of the parent structure (nitro anion radical, nitroso, and hydronitroxide radical) generated under relatively hypoxic conditions, rather than to the covalent binding of nitro compounds or their denitrated metabolites to proteins (Fantel et al., 1988; Fantel et al., 1989). However, new data show that the nitrite anion is a biologically active molecule (Bryan et al., 2005; Garcia et al., 2010) under both hypoxic and normoxic conditions (Cao et al., 2009), suggesting an alternate hypothesis that implicates the nitrite anion. Niridazole-induced microphthalmia was positively correlated with the formation of the denitrated metabolite of niridazole, 1-thiocarbamoyl-2-imidazolidinone; 4'-methylniridazole (MNDZ), a structural analog of niridazole without a nitro group, did not induce this specific malformation. These data suggest that release of the nitro moiety was critical in inducing microphthalmia. While GTN and niridazole are different structures, and, therefore, have differing chemistries, nitrite is a byproduct of GTN metabolism and should not be ruled out. Therefore, the extent to which the embryo metabolizes GTN to release nitrite may be a decisive factor in the developmental toxicity of this chemical.

Since the extracellular matrix serves as a source of morphogenetic factor and facilitates complex tissue interactions, movements, and shape changes during early stages of lens and optic vesicle morphogenesis (Peterson et al., 1995), we examined the regional distribution of this component as assessed by Alcian blue staining. Regional differences in staining patterns (Fig. 4A and C) were found that correlated with increased apoptosis (Fig. 4B and D), suggesting that glycoprotein deposition was perturbed. A possible explanation for this may be that signaling between the cellular layers of the developing eye (neural retina and retinal pigmented epithelium) was perturbed since both these cellular structures were affected in the microphthalmic eye.

GTN exposure induced a dose-dependent increase in the number of malformed embryos (Fig. 5A and B). Interestingly, there was a significant positive correlation between craniofacial and heart defects in both the 48-hour and the 72-hour pd groups ($r = 0.99$, $p = 0.0004$ and $r = 0.87$, $p = 0.012$, respectively; Fig. 5A and B). This positive correlation suggests that GTN affects a pathway that is common to craniofacial and heart development. The neural crest cells may represent such a "shared" pathway. Neural crest cells give rise to many structures in the craniofacial region and a subpopulation of neural crest cells, the cardiac neural crest cells migrate from discrete areas in the hindbrain into the developing aortic arch arteries (Kirby et al., 1983; Hutson and Kirby, 2003). Most of the heart defects we observed included aortic arch anomalies (Fig. 2B). It is interesting to speculate on how GTN may affect neural crest cells. Retinoic acid, generated from retinol by retinaldehyde dehydrogenases, constitutes the most important signaling pathway in neural crest cell migration (van Gelder et al., 2010). GTN can irreversibly inactivate mitochondrial aldehyde dehydrogenase (Beretta et al., 2008), an enzyme that shares 83% sequence similarity with retinaldehyde dehydrogenase (Yoshida et al., 1998). Therefore, it is possible that GTN inhibits retinaldehyde dehydrogenase, resulting in decreased retinoic acid, which has been shown to result in neural crest related malformations (Wilson et al., 1953).

The observation that the induction of microphthalmia by GTN was not correlated to craniofacial or heart defects ($r = 0.65$, $p = 0.39$) suggests a different mechanism of action. This is surprising since abnormal neural crest cell differentiation and distribution have been implicated in ocular anomalies, including microphthalmia (Warburg and Friedrich, 1987; Warburg, 1992). However, since the eye is derived from three different tissues, the anterior neuroectoderm, the neuroepithelium, and the periocular mesenchyme, with the neuroepithelium giving rise to both the neural retina and the pigment retinal epithelium while the periocular mesenchyme originates from both the head mesoderm and the cranial neural crest (Creuzet et al., 2005; Gage et al., 2005), it is possible that GTN or its metabolites specifically affect the neuroepithelium, resulting in abnormal development of the retinal pigment epithelium or neural retina, as observed in our first study (Fig. 3C and D).

GTN exposure localized to the ocular region arrested eye development (Fig. 6A and B), and caused an increase in nitrated proteins (Fig. 7). GTN may be metabolized to release nitrite, which then reacts with tyrosine containing amino acids, increasing nitrated proteins. Interestingly, recent *in vitro* studies have shown that physiologic levels of GTN or nitrite cause an increase in extracellular ATP

levels (Cao et al., 2009; Garcia et al., 2010); purine mediated signaling by ATP has been suggested to trigger, once converted to ADP by nucleases, both the expression of eye field transcription factors and eye development in *Xenopus* (Massé et al., 2007). Further studies are needed to elucidate the role of protein nitration in the embryotoxicity of organic nitrates such as GTN.

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