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Glyceryl trinitrate metabolism in the quail embryo by the glutathione S-transferases leads to a perturbation in redox status and embryotoxicity

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9 Figures, 3 Tables and 1 Supplemental Figure

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Abstract

Exposure of stage 9 quail (*Coturnix coturnix japonica*) embryos to glyceryl trinitrate (GTN) induces malformations that were associated in previous studies with an increase in protein nitration. Increased nitration suggests metabolism of GTN by the embryo. The goals of this study were to characterize the enzymes and co-factors required for GTN metabolism by quail embryos, and to determine the effects of *in ovo* treatment with N-acetyl cysteine (NAC), a precursor of glutathione (GSH), on GTN embryotoxicity. GTN treatment of quail embryo resulted in an increase in nitrite, a

decrease in total GSH, and an increase in the ratio of NADP⁺/NADPH, indicating that redox balance may be compromised in exposed embryos. Glutathione S-transferases (GSTs; EC 2.5.1.18) purified from the whole embryo (K_m 0.84 mM; V_{max} 36 μ M/min) and the embryonic eye (K_m 0.20 mM; V_{max} 30 μ M/min) had GTN-metabolizing activity (1436 and 34 nmol/min/mg, respectively); the addition of ethacrynic acid, an inhibitor of GST activity, decreased GTN metabolism. Peptide sequencing of the GST isozymes indicated that alpha- or mu-type GSTs in the embryo and embryonic eye had GTN metabolizing activity. NAC co-treatment partially protected against the effects of GTN exposure. Thus, GTN denitration by quail embryo GSTs may represent a key initial step in the developmental toxicity of GTN.

Key Words: Glutathione, Ethacrynic acid; Nitrated proteins; N-acetylcysteine, Redox homeostasis, Embryotoxicity.

Abbreviations: Glyceryl trinitrate, GTN; N-acetyl cysteine, NAC; Glutathione, GSH; Glutathione S-transferase, GST; Nitric oxide, NO; Nitrite, NO₂⁻; Hamilton Hamburger stage, HH;

1. Introduction

Glyceryl trinitrate (GTN) is used to treat a variety of cardiovascular diseases, including coronary artery disease, congestive heart failure, and acute myocardial infarction. In addition, organic nitrates may be beneficial in several other diseases and conditions, such as osteoporosis (Wimalawansa et al., 1996) and cancer pain management (Lauretti et al., 2002). GTN is a pro-drug, thought to exert its action through the release of nitric oxide (NO) or nitrite (NO₂⁻). While both NO and NO₂⁻ have pharmacologically beneficial effects, their chemical and biological reactivity may also lead to detrimental consequences to the organism. Nitric oxide is a free radical; its reaction with reactive oxygen species leads to the formation of peroxynitrite which interacts with lipids, DNA, and proteins via direct oxidative reactions or via indirect, radical-mediated mechanisms, leading to cytotoxicity (Radi, 2004). Although NO₂⁻ was thought to be a biologically inert molecule, it has been shown to act under physiological conditions as a signaling molecule and to post-translationally modify proteins independent of NO (Bryan et al., 2005; Veselik et al., 2008).

Despite the extensive use of GTN in therapeutics, relatively little is known about the possibility that GTN may induce embryotoxicity. It has been suggested that NO and the peroxynitrite pathway may contribute to the embryotoxicity of some teratogens (Kasapinovic et al., 2004). *In ovo* exposure of

Hamilton Hamburger (HH) stage 9 Japanese quail embryos to GTN increased mortality, decreased embryo growth, and increased the incidence of embryo defects; these included a decrease in craniofacial, mesencephalon and eye sizes (Bardai et al., 2011). *Ex ovo* exposure of quail embryos to GTN increased ocular malformations; these malformations were associated with a GTN concentration-dependent increase in nitrated proteins (Bardai et al., 2011). This increase in the presence of nitrated proteins indicated that the embryo has the ability to biotransform GTN, releasing NO_2^- and/or NO and forming peroxynitrate. However, how the quail embryo metabolizes GTN is not known.

The enzymes implicated in the metabolism of GTN are mitochondrial aldehyde dehydrogenase (ALDH2) (Chen et al., 2002) and the cytosolic or microsomal glutathione S-transferases (GST; EC 2.5.1.18) (Lau and Benet, 1990). High concentrations of the GSTs are present in the cytoplasm; indeed GSTs may constitute up to 10% of the cytosolic proteins (Board, 2007). The first and rate-limiting step in the release of NO_2^- from GTN is catalyzed by cytosolic GSTs (Kozlov et al., 2003); therefore, GSTs represent an important source of enzymatic GTN metabolizing activity and NO_2^- production (Lau et al., 1990). While GSTs are found in all species, seven classes of cytosolic GST are recognized in mammalian species and are designated alpha, mu, pi, sigma, theta, omega, and zeta based on their functional and structural properties (Hayes et al., 2005). Several hepatic glutathione S-transferases isozymes, including the alpha and mu forms, are capable of denitrating GTN into glyceryl dinitrate (GDN) and NO_2^- (Singhal et al., 1996); these GSTs are also present in avian species (Bardai et al., 2006; Dai et al., 1996; Hsieh et al., 1999). The ability of the GSTs to metabolize GTN is dependent upon the presence of glutathione (γ -glutamyl-cysteinyl-glycine or GSH); the reaction is thought to proceed as follows: $\text{GTN} + 2\text{GSH} \rightarrow \text{NO}_2^- + \text{GSSG}$ (glutathione disulfide) (Needleman, 1976). In addition to their role in xenobiotic metabolism, GSTs are present in the squid and octopus eye as crystallins, where they play a structural role (Tomarev et al., 1991). In addition to its role as a cofactor in the GST reaction, GSH constitutes the largest pool (up to 10 mM) of low molecular weight thiols in cells (Pallardó et al., 2009). Therefore, GSH plays a central role in controlling cellular thiol/disulfide redox state; the ratio of reduced $[\text{GSH}]^2$ to oxidized $[\text{GSSG}]$ (the disulfide form) provides one estimate of redox status (Nkabyo et al., 2002). Proteins that are critical for embryo development contain redox-sensitive thiols, such as cysteine (Jones, 2008). These redox sensitive thiols control DNA replication, DNA repair, chromatin structure, transcription factor DNA binding, and cell proliferation (Chiu and Dawes, 2012). The proper functioning of redox sensitive thiols is dependent upon a finely tuned balance between their reduced and oxidized forms. Dysregulation of the redox equilibrium can severely hamper embryo

development (Wells et al., 2005).

Quail embryos are an excellent model system for research because they develop *in ovo* and, therefore, contributions from maternal metabolism are absent (Padgett and Ivey, 1959).

Furthermore, these embryos can be cultured *ex ovo*, allowing an examination of the effects of exposing specific areas of the embryos at one stage of development (Chapman et al., 2001). We hypothesize that the quail embryo contains a GST-type enzyme(s) capable of metabolizing GTN, using glutathione (GSH) as a required co-substrate. Furthermore, we postulate that GST-catalyzed metabolic activation of GTN plays a role in mediating its embryotoxicity in quail embryos. To test these hypotheses, we determined how the quail embryo metabolizes GTN and whether manipulation of this pathway protects against the embryotoxicity of GTN.

2. Materials and methods

2.1. Chemicals and reagents

Nitroglycerin (GTN, CAS 118-96-7; 99.9% purity) in a corn oil solution (0.2 g/mL) was supplied by General Dynamics Ordnance and Tactical Systems - Canada (Valleyfield, QC, Canada). Analytical reference standards 1,2-glyceryl dinitrate (1,2- GDN), 1,3-GDN, 1-glyceryl mononitrate (GMN) and 2-GMN were purchased from Cerilliant (Round Rock, TX, USA). Acetonitrile and acetone (high performance liquid chromatography, HPLC grade) were obtained from EM Science (Darmstadt, Germany). Reduced GSH, 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (EA) and SNAP (S-nitroso-N-acetyl-D,L-penicillamine, CAS 67776-06-1) were purchased from Sigma Chemicals (Oakville, ON, Canada). Sephadex G-25 was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals and reagents were of the highest grades of purity available and were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA) and Anachemia (Milwaukee, WI, USA).

2.2. Experimental animals

Fertilized Japanese quail (*Coturnix coturnix japonica*) eggs were obtained from a local breeder (Couvoir Simetin, Mirabel, QC, Canada). Eggs were incubated horizontally at 20 °C for 2 h about their long axis, and were then placed in a forced draft incubator (Brinsea Octagon, Titusville, FL, USA) at 37.7 ± 0.5 °C (average $\pm$ SD) and 55-60% relative humidity, for 3 d before embryo harvest. For the isolation of embryonic eyes, eggs were incubated for 5 d, and the whole eye was removed by dissection using a pair of fine scissors. For the *N*-acetylcysteine (NAC) rescue

experiments, embryos were treated and analyzed as previously described (Bardai et al., 2011) with the following modifications. Eggs were incubated until HH stage 9, at which point they were first pre-treated *in ovo* with NAC (10 mM) dissolved in phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) (Buffer A) or buffer alone; after 3 h they were injected with GTN (440 μ M) or saline.

2.3. *Ex ovo embryo cultures*

Following incubation at 37.7 ± 0.5 °C and 55-60% relative humidity, HH 9 embryos (from 29-33h post-fertilization) were processed as previously described (Chapman et al., 2001). Briefly, the yolk and the albumen over the blastoderm were removed in a glass Petri dish. A square of filter paper with a hole punched in the middle was placed over the embryo. After cutting through the vitelline membranes, the filter paper with the attached blastoderm was placed ventral side up in a Petri dish with an agar-albumen substrate. Any remaining yolk adhering to the filter paper was gently washed away. For GTN exposure, a GTN solution (5.7 μ M in saline) or saline (control) was applied directly to the embryos using a pipette. Control and GTN-treated embryos (n = 3 embryos/group) were collected after 0, 0.5, 1, and 3 h to monitor GTN, nitrite, total GSH, and NADP/NADPH ratios. These data were also used to analyze the Pearson product-moment correlation coefficient

2.4. *GTN determinations*

For GTN determinations, 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 (λ = 230 nm) and a temperature control module was used. A Supelcosil LC-CN (250 $\times$ 4.6 mm, 5 μ m particles, Supelco, Bellefonte, PA, USA) 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/min for 8 min. A linear gradient was then run (30% to 65%, v/v) over 12 min. Following this, the solvent ratio was returned to initial isocratic conditions over 5 min. These initial conditions were then held for another 5 min. The sample volume injected was 50 μ L with a 25-min run time. The limit of quantification was 0.05 mg/L. Relative standard deviation 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.

2.5. *Measurements of total GSH concentrations and NADP⁺/NADPH ratios*

Total GSH was quantified by its reaction with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid (DTNB), according to the enzymatic method first reported by Tietze (1969), using a 96-well plate

kit (Oxford Biomedical Research, Rochester Hills, MI). NADP⁺, NADPH, and the ratio of NADP⁺/NADPH were assessed using a quantitation kit (K347-100, Biovision, Mountain View, CA, USA), according to the manufacturer's instructions. Briefly, NADP(H) was quantified using the enzymatic recycling method that reads total NADP(H). The samples were then heated to 60 °C for 30 min to decompose NADP(+). The reactions were performed in a micro-plate and read at 450 nm. This method specifically recognizes NADP(H) and not NAD(H).

2.6. *Electrochemical measurements of nitrite and NO*

Nitrite was analyzed by reduction to nitric oxide prior to detection with an Apollo 4000 Free Radical Analyzer (World Precision Instruments, Sarasota, FL, USA). Potassium iodide (0.1 mol/L) was used to reduce nitrite in the presence of sulfuric acid (0.1 mol/L). Calibration was carried out by adding different amounts of a standard potassium nitrite solution to the iodide solution and measuring the corresponding current change.

NO concentrations in quail embryo extracts following treatment with GTN dissolved in corn oil (440 µM) were measured by using a NO-specific sensor with a 2-mm-diameter tip (ISO-NOP sensor; World Precision Instruments) connected to an Apollo 4000 Free Radical Analyzer (World Precision Instruments), according to manufacturer's instructions. In brief, the NO sensor was calibrated prior to the experiment by using SNAP (0.09 mM) dissolved in PBS + EDTA in combination with a catalyst, copper sulfate, to generate known amounts of NO in solution. A similar calibration curve was also prepared using SNAP in combination with crude embryo homogenate. A calibration curve was constructed by plotting the signal output (pA) vs. concentration (nM) of SNAP. The estimated limit of detection for NO was 100 nM. For the experiment, a reaction mixture was prepared in a 10-mL glass vial that contained 5-mL PBS and 250 µL of crude embryo homogenate. The previously calibrated NO sensor was introduced into the homogenate and allowed to stabilize. Once a steady baseline had been achieved, 20 µL of GTN dissolved in corn oil was added.

2.7. *Determination of GTN biotransformation products with LC-MS*

A Bruker bench-top ion trap mass analyzer attached to a Hewlett Packard 1100 Series HPLC system equipped with a DAD detector was used. Partially purified GST and GTN incubation mixtures were injected into a 5-mm pore size Zorbax[™] SB-C18 capillary column (0.5 mm inner diameter × 150 mm length; Agilent, Mississauga, Ontario, Canada) at 25 °C. The solvent system was composed of a CH₃CN/H₂O gradient (30 to 70% v/v) at a flow rate of 12 mL per min. For mass

analysis, the negative electrospray ionization mode was used. The mass spectra of nitrate esters were characterized by the absence of the deprotonated molecular ions $[M-H]^-$ and the presence of the characteristic mass ion fragments m/z 62 (NO_3) and m/z 46 (NO_2). The mass range was scanned from 40 to 650 Da. The mass fragmentation data obtained were compared to the fragmentation data of pure 1,2- and 1,3-GDN standards.

2.8. Sample preparation and GST enzyme purification

Quail embryos (72 h old; $n=120$) were washed with ice-cold isotonic saline, pooled to final wet mass of 40 g and rapidly homogenized in 50 mL of ice-cold Buffer A using a Polytron tissue grinder (Brinkmann Instruments, Westbury, NY, USA). The homogenate was centrifuged at 10 000 g for 30 min, the supernatant was collected and further centrifuged at 100 000 g for 60 min. The 100 000 g supernatant (containing cytosol) was filtered through glass wool and used for further purification. All enzyme purification procedures, including fast protein liquid chromatography (FPLC[®]) (Amersham Pharmacia Biotech, Uppsala, Sweden), were performed as previously described (Bardai et al., 2006). Briefly, protein purification columns were pre-equilibrated in Buffer A (described above). A 50-mL aliquot of cytosol was then applied to a Pharmacia XK-50 desalting column. The desalted crude cytosolic fraction was passed three times through a XK-16 column containing 15 mL of GSH Sepharose at 1 mL/min. Following each pass of crude cytosol, the column was washed extensively with Buffer A, and the bound GST was eluted with Buffer B (50 mM Tris-HCl, pH 8.0, containing 10 mM GSH). Protein was monitored at 280 nm and the fractions with GST activity (i.e., those with high 1-chloro 2,4 dinitrobenzene (CDNB) conjugating activity, as described below) were collected. Buffer B was then exchanged by Buffer A, and the protein was concentrated using Amicon centrifugal filter units (15 mL; 10 000 MW cut-off) and stored at -80 °C until further use. Protein concentration was determined with a bicinchoninic acid protein assay kit from Pierce Chemical Company (Rockford, IL, USA) using bovine serum albumin (BSA) as the standard. Purification of total GST from embryonic eye was carried out in the same manner.

2.9. Analysis of enzyme-catalyzed GTN metabolism

Enzyme-catalyzed GTN biotransformation assays for *in vitro* embryo homogenates were run in triplicate using 6-mL glass septum-sealed vials, flushed with argon to create anaerobic conditions, with constant agitation in the dark at 37 °C for 2 h. Each 1 mL sample contained: GTN (30 μ mol), cytosol (5 mg protein) or enzyme preparation (0.50 mg protein), GSH (100 μ M), enzyme inhibitor, either ethacrynic acid (200 μ M), an α,β -unsaturated ketone that inhibits the enzyme by binding to

the cysteinyl residue in the active site (Cameron et al., 1995; Bryant et al., 2011), or diphenyliodonium chloride (100 μ M), an inhibitor of flavoenzymes (Bhushan et al., 2003) and Buffer A. Controls were prepared by omitting enzyme or GSH from the reaction mixture. The reaction was stopped with the addition of 1 mL acetonitrile, vortexed, and placed in the dark at 4 °C for 1 h to allow for protein precipitation. The solution was subsequently filtered through a 0.45 μ m membrane. GTN was measured by HPLC, as described below. Activity of the enzymatic biotransformation of GTN was expressed as micromoles nitrite per min per mg protein.

2.10. Analysis of GST isozymes using reverse phase HPLC

Affinity-purified embryo and embryonic eye GSTs were concentrated to 1 μ g/ μ L and 100 μ L was combined with 1 μ L trifluoroacetic acid (TFA) prior to injection onto a 150 mm $\times$ 4.6 mm $\times$ 5 μ M C4 column (Grace Vydac, Hesperia, CA, USA) using acetonitrile containing 0.1% TFA and water containing 0.08% TFA as the mobile phase solvents. The column flow rate was 0.7 mL/min, and gradients were developed in 20% acetonitrile that was increased to 40% in 10 min, followed by a linear increase to 60% acetonitrile over 1 h. Peaks were detected using a Waters diode array detector UV–Vis detector (λ = 210, 214, and 280 nm) and integrated using Empower (Waters, Columbia, MD, USA) software. Eluted fractions containing the GST subunits were collected post-detection, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), silver stained, and identified by mass spectrometry.

2.11. Sodium dodecyl sulphate – polyacrylamide gel electrophoresis and mass spectrometry analysis of HPLC fractions

To confirm sample purity and determine molecular weights of the purified proteins, SDS–PAGE was done in 15% gels containing 0.1% SDS (Laemmli, 1970) using a BioRad Mini Protean II electrophoresis system. The loading volume was 10 μ L (containing 1 μ g protein) per well. Molecular weights were determined from R_f values of standard marker proteins (BioRad, Hercules, CA, USA), and protein bands were visualized using a silver stain. Gel analysis was carried out using the BioRad Quantity One Analysis software.

After resolution by SDS-PAGE, the HPLC fractions corresponding to GST isozymes were excised from the acrylamide gel and prepared for mass spectrometry. Bands were first minced into 1 mm³ gel pieces then transferred to a 96-well tray (each band was placed in separate well) and subjected to reduction, cysteine-alkylation, and in-gel tryptic digestion by automation using a MassPrep Workstation (Micromass, Manchester, UK), as previously described (Wasiak et al., 2002). Briefly,

the gel sections were washed twice with 50 mM ammonium bicarbonate (NH_4HCO_3), dehydrated in acetonitrile, and finally dried under vacuum. Proteins were in-gel digested by adding 25 μL of trypsin (6 ng/ μL in 50 mM NH_4HCO_3) and incubating for 30 min at room temperature followed by 4.5 h at 37 °C. To extract peptides, 30 μL of a mix containing 1% formic acid and 2% acetonitrile was added and incubated 30 min at room temperature. Gel sections were rinsed twice in 50 μL of 5% acetonitrile in 0.1% TFA, followed by a wash in 50% acetonitrile in 0.1% TFA. Supernatants from each wash were collected and combined with the initial protein digest. Samples were then dried to 10–20 μL total volume under vacuum and stored at –80 °C. Extracted peptides were then subjected to mass spectrometry analysis.

Samples were desalted and subsequently resolved on a 10 cm $\times$ 75 micron PicoFrit[®] column containing BioBasic[™] C18 packing (Supelco, Bellefonte, PA, USA). Peptides were eluted from the column with a 30 min gradient of 10-95% acetonitrile (v/v) containing 0.1% formic acid (v/v) at a flow rate of 20 nL/min using an Agilent (Mississauga, ON, Canada) 1100 series NanoHPLC system. Precursor ion selection for subsequent tandem ms fragmentation analysis was done as follows. Briefly, up to three doubly, triply or quadruply charged ions of intensity greater than 2×10^6 counts per second (cps) from each enhanced-ms survey scan were selected for passage into a collision cell. Collision-induced dissociation was facilitated by collision with nitrogen gas; fragment ions were trapped in Q3 and scanned. The peak listed data were then searched against a copy of the Universal Protein Resource (UniProt) data base (May 21, 2008) limited to the Galliformes taxonomy (taxon ID 8976) (9880 sequences; 3 554 051 residues).

2.12. Glutathione-S-transferase activity

The GST assays were done at room temperature using a 96-well microtiter plate reader (Biotek[™]; Molecular Devices Corp, Sunnyvale, CA, USA) set at an absorbance of 340 nm. Activity was measured using CDNB as the substrate, and reduced GSH as the cofactor. The CDNB (20 mM) was prepared in anhydrous ethanol. Fresh solutions of GSH (20 mM) were prepared daily in 100 mM sodium phosphate buffer (pH 6.5). Each reaction mixture (250 μL) contained: 100 mM phosphate buffer, 1 mM GSH, and 1 mM CDNB. The ethanol concentration was less than 5%. The increase in absorbance was recorded for 5 min to ensure that the reaction went to completion (steady state). Non-enzymatic conjugation of GSH with CDNB was subtracted from all assays by including a blank (buffer only) consisting of all the assay components except the protein preparation. One unit of GST activity was defined as the initial rate of one micromole of the product (S-2,4-dinitrobenzene-glutathione) formed per min measured at 340 nm using an extinction coefficient of

9.6 mM⁻¹ for the conjugate (Habig et al., 1974). Kinetic studies of the partially purified enzyme were done using varying concentrations of CDNB (from 0.02 to 5 mM) and GSH (from 0.07 to 5 mM); the apparent enzyme kinetic constants (app. $V_{\max}$ and app. K_m) were calculated according to the Michaelis-Menten equation. Specific activities were expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein.

2.13. Statistical analyses

Differences between the test and control samples were considered significant at $p \leq 0.05$ using the Student's t test for paired data or one-way ANOVA with Tukey's multiple comparisons test, where appropriate. The Pearson product-moment correlation coefficient was used to analyze the association between all studied parameters and considered significant at $p \leq 0.05$. Statistical analyses were done using GraphPad Prism version 5.0 for Macintosh, GraphPad Software (San Diego, CA, USA). The GST kinetic constants (K_m and $V_{\max}$) were determined by Lineweaver-Burke plots and were compared to the K_m and $V_{\max}$ constants generated by the Michaelis-Menten equation using Kaleidagraph (Reading, PA, USA).

3. Results

3.1. GTN, NO_2^- , total GSH, and NADP(H) concentrations after ex ovo treatment of embryos with GTN

A GTN/saline solution (5.7 μM) was applied to embryos *ex ovo* and GTN, NO_2^- , GSH, and NADP⁺/NADPH concentrations were determined over different time intervals (0, 0.5, 1, and 1.5 h). There was a significant decrease in residual GTN when analyzed by ANOVA at all three time points (Fig. 1). Tukey's multiple comparison tests showed significant decreases in GTN between 0.5 and 1.0 h ($p \leq 0.05$) and between 1.0 and 1.5 h ($p \leq 0.01$). The decrease in the concentrations of GTN remaining over time was associated with significant increases in both NO_2^- and the NADP⁺/NADPH ratio, and a decrease in total glutathione (GSH) when compared to paired controls treated with saline only (Fig. 2 A, B, and C). Tukey's post hoc test showed a significant increase in nitrite levels at 0.5 h ($p \leq 0.05$), 1 h ($p \leq 0.01$) and 3 h ($p \leq 0.001$), and in the NADP⁺/NADPH ratios at 1 h ($p \leq 0.001$) and 3 h ($p \leq 0.001$) when compared to their time-matched controls. Total GSH levels decreased significantly at 1 h and 3 h when compared to the time-matched controls. A Pearson product-moment correlation coefficient was calculated to assess the relationship between metabolism of GTN over time (Fig. 1) and its subsequent biological effects on NO_2^- , NADP(H), and GSH. There was a significant inverse relationship for both NO_2^- and NADP(H), and a

significant positive correlation for GTN and GSH (Fig 2 A, B, C inset). These data demonstrate that the embryo can metabolize GTN *ex ovo* by a denitration pathway, releasing nitrite; this was accompanied by a decrease in GSH and an increase in the ratio of NADP⁺/NADPH compared to time matched controls.

3.2. Enzyme-catalyzed GTN biotransformation

To further characterize the metabolism of GTN by embryos, time course studies were carried out using HPLC analyses of crude whole embryo cytosol to identify the GTN biotransformation products. Approximately 97% of the initial 30 μ mol GTN added to the 1 mL samples was metabolized after 4 h of incubation (Fig. 3A). The rate of GTN disappearance was 0.060 ± 0.001 nmol/min/mg protein based on the linear descending phase of GTN concentrations, i.e., from 0 to 1.5 h incubation. In control samples, the GTN concentration in the samples containing buffer only (i.e., with no cytosol added) showed little to no change in GTN concentrations, indicating the absence of detectable abiotic transformation. The cytosol-dependent decrease in GTN concentration (Fig. 3A) was accompanied by increases in the formation of nitrite (NO₂⁻) and the denitrated metabolites, 1,2- or 1,3-GDN (Fig. 3B) that were maximal at 4 h, and decreased until the end of the 24 h study. The increase in nitrite levels and decrease in GTN levels were stoichiometric in the first 2 h (1:1). However, after 2 h the increase in nitrite was not stoichiometric when compared to the decrease in GTN, indicating that further denitration of the 1,2-GDN or 1,3-GDN metabolites was occurring. At 4 h incubation, the amount of nitrite began to decrease (Fig. 3B), suggesting that the nitrite formed earlier was being sequestered by proteins. These *in vitro* data confirm our *ex ovo* data, and indicate that GTN is metabolized by quail embryo cytosol, as evidenced by the decrease in GTN concentrations and concomitant formation of GTN metabolites.

To identify the key enzymes involved in the metabolism of GTN in quail embryos, GTN metabolism studies were carried out using samples of crude whole cytosol containing 5 mg/mL protein and GTN, in the presence or absence of 200 μ M GSH (Fig. 4). Ethacrynic acid (EA), a GST inhibitor (Cameron et al., 1995; Tew et al., 1997), was used as a biochemical tool to determine if quail embryo GSTs are involved in the *in vitro* biotransformation of GTN. The GTN concentrations remained constant in the Group 1 abiotic control samples. Group 1 samples contained GTN, GSH, and ethacrynic acid (EA, 100 μ M), and saline only. The purpose of this *in vitro* treatment group was to determine if GTN spontaneously decomposed or if it reacted abiotically with GSH or EA. These results indicate that the addition of GSH and EA did not react abiotically with GTN. Group 2 samples included cytosol and GTN (i.e., no GSH or EA were added). The addition of cytosol led to

a significant 67% decrease ($p \leq 0.001$) in GTN concentrations, from $28.4 \pm 0.15 \mu\text{M}$ (Group 1) to $9.3 \pm 0.4 \mu\text{M}$ (Group 2). The GTN concentration was decreased further when the whole cytosol was incubated with the addition of exogenous GSH ($200 \mu\text{M}$) (Group 3). The addition of EA to whole cytosol incubated with GTN led to a significant increase in residual GTN concentrations ($17.0 \pm 1.0 \mu\text{M}$; Group 4) compared to cytosol samples incubated without EA (Group 2). Thus, EA significantly decreased ($p \leq 0.05$) the biotransformation of GTN in the crude cytosol of quail embryos. Addition of the flavoprotein inhibitor diphenyliodonium chloride ($100 \mu\text{M}$) (Group 5) did not inhibit GTN metabolism (Group 2 vs. Group 4), suggesting that flavoproteins are not involved in the metabolism of GTN. It should be noted that the studies described above used crude whole cytosol containing soluble proteins and co-factors. Taken together, these data show that a crude cytosolic preparation from the quail embryo contains the enzymatic activity required to metabolize GTN. Inhibition with EA indicates that this activity may be a GST isozyme. The addition of exogenous GSH only increased the metabolism of GTN by 9%, signifying that GTN metabolism is in fact enzymatic, and not simply dependent on the presence of thiol-containing compounds.

3.3. Enzyme purification of quail embryo GSTs

GSTs were purified from preparations of whole embryo or embryo eye using GSH-affinity chromatography. Table 1 summarizes of the activity profiles, comparing the GST activity (reflected by the metabolism of CDNB) and GTN metabolism (reflected by NO_2^- production) at the different purification steps of quail embryo GSTs. In preparations of quail whole embryo, the GST specific activity (nmol/min/mg), using CDNB as the substrate, increased from 0.12 in the crude homogenate to 15.52 in the affinity chromatography purified samples (Table 1), resulting in 129-fold purification. Based on the NO_2^- production rate in the different fractions, the GTN removal rate remained similar in the crude (0.29 nmol/min/mg) and 10 000 g supernatant (0.26 nmol/min/mg), but increased in the 100 000 g cytosol fraction (0.48 nmol/min/mg). Notably, the largest increase in the NO_2^- production rate was observed in the affinity-purified fraction (1436 nmol/min/mg) of quail whole embryos. This represents a 4951- fold increase in GTN metabolizing activity.

In embryonic eye preparations, the specific activity of GST (nmol/min/mg), using CDNB as the substrate, remained the same in the first three fractions (0.01, 0.02, and 0.02) increased to 1.25 in the affinity chromatography fractions (Table 1), resulting in 125-fold purification. The GTN removal rate, based on NO_2^- production rate in the different fractions, increased at each subsequent purification step (0.49, 0.77, and 0.90 nmol/min/mg) with the largest increase (34.2 nmol/min/mg) observed in the affinity purification step. This represented a 70- fold increase in GTN metabolizing

activity.

To account for the differences observed in specific activities we carried out a series of kinetic studies on the embryo and embryonic eye GST-type enzyme fractions eluted from the affinity column. The calculated respective $V_{\max}$ ($\mu\text{M}/\text{min}$) and K_m (mM) values were significantly different ($p = 0.03$; $p < 0.001$) for whole embryo (30.0 and 0.20), and embryo eye (35.9 and 0.8) (Supplemental Fig. S1A, B). These data suggest that the enzymes responsible for GTN biotransformation are GSTs with differing specific and kinetic activities. SDS-PAGE analysis of proteins from embryonic eye (Fig. 5, lane A) or whole embryo (Fig. 5, lane B) purified using the GSH affinity column were within the 22–29 kDa range; this is consistent with the range of molecular masses reported for avian GSTs (24–30 kDa) (Bardai et al., 2006; Cameron et al., 1995; Hsieh et al., 1999). Thus, the enzymes purified from whole embryo and embryonic eye using the GSH affinity column are GST-type enzymes.

3.4. Time course of GTN biotransformation *in vitro*

GTN biotransformation studies were carried out using affinity purified whole embryo GST proteins. Metabolism of GTN did not occur or was negligible when incubated with buffer (PBS), cofactor (GSH) or EA alone (Fig. 6A). Metabolism of GTN occurred only when GTN was incubated in the presence of both purified enzyme and GSH (Fig. 6B), as evidenced by the complete disappearance of GTN after 15 min of incubation. The addition of EA inhibited GTN metabolism at 120 min incubation in samples containing partially purified GST and GSH, and gave results similar to studies using whole cytosol (Fig. 4). The incubation of GTN for 120 min in Buffer A with only GSH and no enzyme added, or with only enzyme (ENZ) and no GSH added, did not cause a significant disappearance of GTN compared to the time zero controls, or GTN samples incubated for 120 min without enzyme and GSH (Fig. 6A). Thus, GST partially purified from quail embryo can degrade GTN in the presence of GSH, confirming the studies with whole cytosol.

Time course studies were conducted using affinity purified GST to determine whether the observed metabolism of GTN *in vitro* was also accompanied by the formation of GTN metabolites, including NO_2^- , 1,2- or 1,3-GDN (Fig. 6B). As anticipated, the complete disappearance of GTN after 15 min incubation with partially purified GST was accompanied by an increase in nitrite levels ($31.5 \pm 4.7 \mu\text{M}$) and GTN metabolites ($24.0 \pm 2.5 \mu\text{M}$) when compared to time zero control. The nitrite levels continued to increase significantly ($p < 0.001$) ($44.9 \pm 0.5 \mu\text{M}$) from 30 to 60 min of incubation. However, during this time, the concentration of combined GTN metabolites ($21.7 \pm 0.4 \mu\text{M}$) was not significantly changed. These results indicate that the dinitrated metabolites (1,2- and 1,3-GDN)

may have continued to undergo further denitration to 1-glyceryl mononitrate (1-GMN), and 2-glyceryl mononitrate (2-GMN), metabolites that we were unable to resolve using our HPLC technique (Hashimoto and Kobayashi, 2003), leading to an increase in released nitrite, as shown in Fig 6. Finally, the addition of EA completely inhibited the ability of the enzyme to biotransform GTN. These data clearly show that GST is involved in the metabolism of GTN, and that GTN may undergo further metabolism to mono-denitrated products.

3.5. Biotransformation products identified with LC-MS

While HPLC data indicated the presence of GTN metabolites, it was not possible to distinguish between the 1,2- and 1,3-GDN metabolites. To identify the biotransformation products of GTN metabolism, we carried out LC-MS studies using purified embryo GST. Figure 7A is the total ion chromatogram (TIC) scanned from 40 to 650 Da. Three characteristic peaks (peak 1, 2, and 3, at 10.5, 11.5, and 21.5 min, respectively) were observed. Figures 7B, C, and D are the LC-MS data of the extracted ion chromatogram of Peaks 1, 2, and 3 detected at 62 Da. These peaks were identified as 1,3-GDN, 1,2-GDN and GTN, respectively, based on comparisons to the fragmentation patterns obtained using pure 1,3- and 1,2-GDN standards. The adduct ion masses $[M+Cl]^-$ m/z 262 and m/z 217 are the adduct ion masses of GTN and 1,2-GDN, respectively. The characteristic fragment m/z 47 (HNO_2) was not observed for 1,3-GDN. These characteristic fragmentation patterns were compared to fragmentation patterns of known control material.

3.6. NO assay

Since the quail embryo possesses the capacity to metabolize GTN to nitrite, and NO is a highly reactive pharmacological by-product of GTN metabolism, we sought to determine if the embryo further oxidizes the nitrite to NO. The addition of SNAP to crude embryo homogenates clearly showed that the probe was capable of detecting NO (Fig. 8) with a limit of detection of 1.1 nM NO. However, NO was not detected in embryo homogenates to which GTN was added, suggesting that HH 9 embryo homogenates may not be capable of further oxidizing nitrite to NO.

3.7. GST subunit analysis by reverse phase HPLC

Reverse phase HPLC (RP-HPLC) was used to separate the GST isozymes from the partially purified enzyme preparation of embryonic eye (Fig. 9A) and whole embryo (Fig. 9B) to determine which of the GST isozymes were capable of metabolizing GTN *in vitro*. The RP-HPLC separation of embryonic eye affinity purified proteins revealed the presence three major peaks (Peaks 2, 6, and

8), and seven minor peaks (Peaks 1, 3, 4, 5, 7, 9, and 10; Fig. 9A). The resolution of whole embryo fractions (Fig. 9B) revealed the presence of four major peaks (Peaks 2, 6, 8, and 9) and five minor peaks (Peaks 1, 3, 4, 5, and 7). When the individual collected fractions from the RP-HPLC separation from both embryonic eye and embryo were subjected to SDS-PAGE followed by silver staining, all visible proteins eluted in the molecular weight range consistent with GSTs (*i.e.*, 23–27 kDa, inset Fig 9A,B). This indicates that both the embryonic eye and embryo contain multiple GSTs that were resolved.

3.8. MS peptide sequencing and MALDI-TOF analyses

To determine the identity of the purified isozymes capable of metabolizing GTN from both the embryonic eye and embryo (Figs. 9A,B), we did BLAST analysis of the peptide sequences obtained from the MS analysis of the trypsin-digested peaks using the Swiss-Prot and NCBI databases. Protein peaks 2, 3, 4, 5, 7, and 10 of embryonic eye GST subunits (Fig. 9A), contained α -like GSTs, whereas peaks 6, 8, and 9 had μ -like GSTs. A similar analysis of whole embryo GST subunits (Fig. 9B) indicated that peaks 2, 3, 4, 6, 7, 8, and 9 contained α -like GSTs, whereas peaks 1 and 5 contained μ -like GSTs.

3.9. NAC supplementation experiments

Since we have shown that GSTs metabolize GTN in the quail embryo and that GSH is required, our next goal was to determine the consequences of supplementation with NAC on the embryotoxicity of GTN in quail embryos. GTN treatment increased the mortality of quail embryos (35%) compared to that observed after treatment with either vehicle (Control, 8%) or NAC (4%) alone (Table 2). A 46% decrease in mortality was observed when embryos were pre-treated with NAC prior to administration of GTN. All viable embryos were evaluated using a one-way between subjects ANOVA followed by Tukey's multiple comparisons test for embryo length. There was a significant effect of treatment on embryo length among the four treatment groups (Control, NAC, GTN, and NAC + GTN) (Table 2). Post hoc comparisons using Tukey's multiple comparison test showed a significant decrease in body length following treatment with GTN (Control *vs.* GTN; $p \leq 0.0001$ or NAC *vs.* GTN; $p \leq 0.0001$). Co-treatment of GTN-exposed embryos with NAC (GTN *vs.* NAC + GTN) resulted in a significant increase ($p \leq 0.001$) in body length. Finally, there was no significant difference between NAC and NAC + GTN treatment groups.

Because the sizes of craniofacial structures, such as the mesencephalon and the eye, were shown previously to be decreased significantly in embryos exposed to GTN 48-h post dose (Bardai et al.,

2011), we measured these parameters in the present experiment. A one-way ANOVA revealed a significant effect on embryo craniofacial, mesencephalon, and eye perimeter measurements (Table 3). Tukey's multiple comparisons test showed a significant decrease in craniofacial ($p \leq 0.001$), mesencephalon ($p \leq 0.001$) and eye ($p \leq 0.001$) perimeters between either the Control or NAC (10 mM) treated embryos and those exposed to GTN (440 μ M). Co-treatment of GTN-exposed embryos with NAC (GTN vs. NAC + GTN) resulted in a significant increase in craniofacial, mesencephalon and eye perimeter values ($p \leq 0.05$) towards control perimeters (Table 3). Together, these results indicate that NAC co-administration reduced the effects of GTN exposure on all parameters of GTN embryotoxicity.

4. Discussion

This study shows that the quail embryo metabolizes GTN. This action is observed *in vitro* and *ex ovo*, in the whole embryo and embryo eyes, and is catalyzed by alpha- and mu-type GSTs. During the metabolism of GTN, there is a decrease in total GSH concentrations and an increase in the ratio of NADP⁺/NADPH compared to time matched controls, suggesting that GTN affects these measures of redox status in the *ex ovo* developing embryo. The pre-supplementation of embryos with NAC offers a partial rescue from GTN treatment.

The first finding that the quail embryo metabolizes GTN, is supported by both *ex ovo* and *in vitro* experiments. Three hours following exposure to GTN we found that GTN levels decreased significantly in *ex ovo* treated embryos followed by a corresponding increase in nitrite in exposed embryos compared to vehicle treated embryos. The small decrease in GTN concentrations after 0.5 h that corresponds to a significant increase in NO₂⁻ can be explained stoichiometrically because for every molecule of GTN metabolized there is the release of three nitrites. Two major pathways have been proposed for the metabolism of GTN. The first is a "mechanism-based biotransformation" pathway that produces nitric oxide (NO) and contributes directly to vasodilation; the production of NO is accompanied by an excess of the 1,2-GDN metabolite relative to 1,3-GDN (Hashimoto and Kobayashi, 2003). The second has been termed a "clearance-based biotransformation or detoxification" since the NO₂⁻ that is produced has no apparent cardiovascular effect; in this pathway, equimolar amounts of both GTN metabolites are produced. In the LC/MS experiment, we observed the formation of equimolar quantities of 1,2- and 1,3- GDN and the presence of NO₂⁻, but not NO. These data are consistent with the, clearance-based pathway of GTN metabolism. The absence of NO was also observed in experiments using whole embryo homogenates. Thus, the GTN

metabolized by the quail embryo proceeds by a clearance-based biotransformation that results in the production of NO_2^- . Therefore, the increase in nitrated proteins we observed previously (Bardai et al., 2011) is mediated by a reaction of proteins with NO_2^- rather than an NO-mediated reaction. However, nitrite can be reduced to NO by nitrite reductase (Shiva et al., 2007). Whether nitrite reductase is present in the quail embryo or not is unknown.

The second finding that a GST- type enzyme metabolizes GTN, is supported by the *in vitro*, protein purification, and kinetic studies. As the cytosolic fraction of the whole embryo homogenate can metabolize GTN, we investigated the role of GSTs in this metabolic process. Ethacrynic acid (EA) is an unsaturated ketone that binds to the active site of GSTs, and can inhibit both human and rat GSTs of the α -, μ - and π -classes (Lau and Benet, 1992; Ploemen et al., 1990). This GST inhibitor significantly decreased GTN metabolism in quail embryo homogenates, suggesting that a GST type of enzyme was involved in the denitration reaction. As the addition of EA did not completely inhibit the metabolism of GTN *in vitro*, other enzymes may also be involved in the metabolism of GTN in the cytosolic fractions (Chen et al., 2002). However, GSTs are likely to play an important role in GTN metabolism. Our observations that EA significantly reduced GTN metabolism in crude cytosol and completely inhibited GTN metabolism in purified protein preparations indicate the presence a GST-type of enzyme. The fact that EA failed to completely inhibit GTN metabolism in the crude cytosol after 2h (Fig. 4), in contrast to the purified GST preparation (Fig. 6), can be explained based on the proposed mechanism of action of EA, whereby EA inhibits enzyme activity by binding to cysteinyl residues (Bryant et al., 2011). Therefore, in crude cytosol preparations, EA binds to the active site of GST and also reacts with other biomolecules that contain cysteinyl residues. Addition of excess EA may more specifically target the cysteinyl residues of the active sites in the purified GST preparation.

There is evidence in mammalian systems for the involvement of GSTs from the subcellular fractions of both the liver and vascular tissue in the metabolism of GTN (Kurz et al., 1993; Lau and Benet, 1990; Lau et al., 1992). Previously, we demonstrated that alpha- and mu-type GSTs present in the subcellular fractions of the adult quail liver were capable of metabolizing other nitro containing compounds (Bardai et al., 2006); here, for the first time, we show that in the quail embryo both the alpha- and mu-types of GSTs are capable of GTN denitration. In the 24 h time course study, the amount of free nitrite present decreased dramatically, suggesting that the nitrite was irreversibly bound to proteins. This is supported by our earlier observation that embryos treated with GTN *ex ovo* and examined 18 h later showed the presence of nitrated proteins (Bardai et al., 2011). This post-translational modification of proteins is a consequence of the formation of radical

nitrating species such as NO, a free radical that is generated as a consequence of GTN metabolism and is capable of causing developmental pathologies (Wells et al., 2005). We analyzed the cytosolic fraction to which GTN had been added for the presence of NO but were unable to detect NO in these assays (data not shown).

Reverse phase HPLC and tandem mass spectrometry of purified protein preparations clearly indicated the presence of alpha- and mu- type GSTs in both the whole embryo and embryo eye. Surprisingly, the results also showed that the kinetic profiles of GSTs in the quail embryo differed significantly from those purified from the eye. The specific activities obtained for the whole quail embryo GSTs were comparable to previous reports (Bardai et al., 2006; Dai et al., 1996). That we found similar ratios of CDNB/GTN specific activity in crude extracts and purified GSTs from both the whole embryo (0.03) and embryonic eye (0.01) indicates that all of the important GTN biotransformation activity was purified from the crude extract. However, differences in specific activity and K_m values indicate that there are different GST isozymes present in the whole embryo and embryonic eye extracts. It is possible that the GSTs present in the quail embryonic eye have a multifunctional role in addition to detoxification. Multifunctional GSTs in the squid eye lens (S-crystallins), similar in sequence to rat Gsta-1, have been described (Piatigorsky, 2003) and found to have to 40X lower activity with CDNB than GSTs purified from squid liver extracts (Wistow and Piatigorsky, 1987; Piatigorsky, 2003). This is similar to the difference in activity we observed between GST purified from whole embryo and embryo eye (Table 1). Proteins similar to chicken and human class alpha GSTs were reported to be abundant in the chicken corneal epithelium and stroma (Cuthbertson et al., 1992). The presence of both Gsta and Gstm isozymes in the embryo and embryonic eye, and their apparent differences in activity suggest that the quail embryonic eye may contain crystalline type GSTs. The role of crystallin GSTs in the quail embryo eye is not known. Our third finding that GTN metabolism by the embryo results in a change in embryo redox status, is supported by the *ex ovo* data showing that over a period of 3 h, concurrent with a decrease in GTN, there is a decrease in total GSH and an increase in the NADP⁺/NADPH ratio in treated embryos compared to their controls. NADPH is required as a cofactor for the regeneration of GSH from GSSG, catalyzed by glutathione reductase (Lu, 2009). In the quail embryo NADPH may be supplied by the pentose phosphate pathway and/or by two NADPH-producing cytosolic enzymes, namely NADP⁺-dependent malic enzyme and NADP⁺-dependent isocitrate dehydrogenase. Together, these data suggest that GTN exposure may disrupt the redox balance and/or the energy status in the developing embryo, leading to GSH depletion. GSH is a critical regulator of redox balance and is required for normal embryo development (Ufer et al., 2010; Yanes et al., 2010).

Interestingly, exposure to buthionine sulfoximine, an inhibitor of glutamate cysteine ligase, the rate limiting enzyme in the synthesis of GSH, results in the development of embryos with multiple developmental defects (Hales and Brown, 1991; Shi et al., 2000).

Our fourth finding is that supplementation with NAC offers a partial rescue to *in ovo* GTN treated. Pre-treatment with NAC, a precursor in the formation of GSH, decreased GTN-induced embryo mortality and growth retardation (Table 2,3). Previous studies have shown that the addition of NAC to the culture medium of gestation 9 day rat embryos diminished the growth retardation induced by high glucose concentrations added to mimic oxidative stress in diabetic pregnancies (Gäreskog and Wentzel, 2007). NAC may serve as a source of cysteine for the synthesis of GSH or may react directly with free radicals, serving as an antioxidant. It is also possible that NADPH depletion (Fig. 2) results in partial metabolic dysfunction of the pentose phosphate pathway, and that NAC may act by partially restoring energy homeostasis.

Our demonstration that the quail embryo can metabolize GTN, with the production of reactive nitrogen species and perturbations in cellular redox status, as assessed by total GSH and the ratio of NADP⁺/NADPH, suggests that more careful consideration is required when administering GTN during pregnancy. The possible involvement of other enzymatic systems capable of metabolizing GTN in the embryo needs to be further investigated to determine their role in mediating the developmental pathologies observed. Finally, GSTs in the embryonic eye differ in enzymatic activity from those in the whole embryo. It is not yet clear what role, if any, these GSTs within the eye may play in embryo development.

In summary, this study shows that the *ex ovo* quail embryo possesses both α - and μ -type GSTs that can metabolize GTN to produce nitrite. The metabolism of GTN by *ex ovo* quail embryo GSTs depletes total GSH and alters the ratio of NADP⁺ to NADPH, resulting in perturbation of the redox balance in the embryo. Because NAC administration provides partial protection to *in ovo* GTN treated embryos, we propose that this pathway plays a role in mediating the embryotoxicity of GTN.

Supplementary data description

Supplementary data (Supp. Fig 1) depict the kinetic constants (V_{max} and K_m) of purified GSTs obtained from whole embryo and embryo eye.

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Figure Legends

Fig. 1. Time course of the metabolism of glyceryl trinitrate (GTN, 5.7 μ M) by quail embryos *ex ovo* (mean $\pm$ SD; n= 3 embryos/group). Asterisks denote a significant difference (*p < 0.05; **p < 0.01, one-way ANOVA followed by a post hoc Tukey's test).

Fig. 2. Nitrite (A), total GSH (B) and the ratio of NADP⁺/NADPH (C) in quail embryos treated *ex*

ovo with saline (control, Ctrl) or glyceryl trinitrate (GTN, 5.7 μ M, treated) (mean $\pm$ SD; n= 3 embryos/group). Asterisks denote a significant difference from time-matched controls (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, one-way ANOVA followed by a post hoc Tukey's test). The insets show the Pearson correlation coefficient (r), and the statistical significance.

Fig. 3. *In vitro* time course studies of GTN (5.7 μ M) biotransformation in whole quail embryo cytosol. Symbols used in 3A (-◆- buffer; -●- whole quail embryo cytosol) indicate the concentration of remaining GTN; In 3B symbols (-▲- nitrite (NO₂⁻) (-■- 1,2- or 1,3-GDN) indicate the appearance of GTN metabolites. Incubation conditions: 37°C. Data are the means of triplicates; error bars represent SD.

Fig. 4. The enzyme dependent biotransformation of glyceryl trinitrate (GTN, 5.7 μ M) by quail embryo cytosol is inhibited by ethacrynic acid (EA, 100 μ M) but not diphenyliodonium chloride (DPI, 100 μ M). Bars represent the concentration of GTN remaining. Incubation conditions (at 37°C for 2 h under anaerobic conditions) are shown in the table below the graph. Data are the means of triplicates; error bars represent SD. Asterisks denote a significant difference (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, one-way ANOVA followed by a post hoc Tukey's test)

Fig. 5. SDS-PAGE of GSH affinity column purified quail embryonic eye and whole embryo cytosolic GTN degrading enzymes. The gel was silver stained. Lane A: eye proteins; Lane B: whole embryo proteins; Lane C: molecular weight markers (97 kDa Phosphorylase b, 66 kDa Serum albumin, 45 kDa Ovalbumin, 31 kDa Carbonic anhydrase, 21.5 kDa Trypsin, 14.4 kDa Lysozyme).

Fig. 6. Time course of the biotransformation of GTN at 37°C under differing incubation conditions, as shown in the table below the graph. Data are the means of triplicates and error bars represent SD. PBS (phosphate buffered saline); ENZ (partially purified enzyme preparation; 0.5 mg protein); GSH (glutathione; 100 μ M); and EA (ethacrynic acid; 200 μ M). Asterisks denote a significant difference (*** $p < 0.001$, one-way ANOVA followed by a post hoc Tukey's test).

Fig. 7. Mass spectrometry of GTN and its metabolites. Total Ion Chromatograph (A) showing GTN (peak 3) and its intermediates 1,3-GDN (peak 1) and 1,2-GDN (peak 2). Extracted ion chromatograms of GTN (D) and its intermediates 1,3-GDN (B) and 1,2-GDN (C) were obtained using LC-MS. GTN was incubated for 20 min with GSH and affinity purified enzyme GST preparations from (72 h old; n=120) quail embryos.

Fig. 8. Analysis of quail embryonic eye and whole embryo GST subunits by reverse phased HPLC. 8A a representative chromatogram of embryonic eye affinity-purified GST separated by RP- HPLC,

reveals three major and at least seven minor protein peaks absorbing at 224 nm. Mass spectrometer identified peaks 2, 3, 4, 5, 7, and 10 as alpha-like; peaks 6, 8, and 9 are identified as mu-like. Inset shows SDS-PAGE silver staining of protein fractions eluted by HPLC. 8B, a representative chromatogram of whole embryo affinity-column purified GST separated by reverse phase HPLC, reveals four major and at least five minor protein peaks absorbing at 224 nm. Peaks 2, 3, 4, 6, 7, 8, and 9 are identified as alpha-like, while peak 1 and 5 contain mu-like isozymes. The inset shows SDS-PAGE silver staining of protein fractions eluted by HPLC with the molecular weight markers described in Fig. 5.

Fig. 9. *In vitro* NO production by H.H stage 9 quail embryos. Arrows indicate the addition of SNAP (-●-) and GTN (440 μ M) dissolved in corn oil (-■-, GTN-O) or PBS (-▲-, GTN-W) to whole embryo homogenates (250 μ L) in 5 ml PBS. The final concentration of NO (nM) detected in homogenates to which SNAP has been added is indicated.

Fig. S1. (Supplementary content).

Determination of the kinetic constants $V_{\max}$ and K_m of purified GST obtained from whole embryo (Fig. S1A) and embryo eye (Fig. S1B). The inset shows the Lineweaver-Burk substrate velocity data.

Table 1. Purification of quail embryo and embryonic eye glutathione *S*-transferases

Purification Steps	Total Protein (mg)	Total Activity (U)	GST Specific activity ^a	NO ₂ ⁻ Specific Activity ^b	Purification (-fold GST)	Purification (-fold NO ₂ ⁻)	Recovery ^c (%)
Quail – Embryo							
1. Crude Homogenate	500	6.0	0.12	0.29	1	1	
2. 10,000 × g supernatant	341	4.7	0.14	0.26	1.2	0.9	78
3. Cytosol	208	3.3	0.16	0.48	1.3	1.7	55
4. GST-Affinity Chromatography	0.04	0.58	15.52	1436	129	4951	9.7
Quail – Embryonic eye							
1. Crude Homogenate	120	1.38	0.01	0.49	1	1	
2. 10,000 × g supernatant	78	1.18	0.02	0.77	2	1.6	86
3. Cytosol	63	1.13	0.02	0.90	2	1.8	82
4. GST-Affinity Chromatography	0.24	0.29	1.25	34.2	125	70	21

^a Specific activity measured according to Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (U/mg protein).

^b Specific activity of NO₂⁻ production (nmol/min/mg protein).

^c % Recovery calculated as: 100X(Total activity of the purification step / Total Activity of the Crude Homogenate).

1

Table 2. Effects of *in ovo* NAC supplementation following GTN treatment of quail embryos.

Group ^a	n ^b	Mortality (%)	Crown Rump Length (mm) ^c
Control	30	8	14.2 ± 1.1
NAC (10 mM)	30	4	13.9 ± 1.1 ^d
GTN (440 µM)	30	35	11.5 ± 1.5 ^{e,f}
NAC (10 mM) + GTN (440 µM)	30	16	13.2 ± 1.7 ^g

2

3 ^a Injection volume = 20 µL. Abbreviations used: NAC (N-acetyl cysteine), GTN (glyceryl trinitrate)

4 ^b Number of eggs treated

5 ^c Mean ± SD

6 ^d Not significantly different from Ctrl and NAC + GTN

7 ^{e,f} Significantly different from Ctrl and NAC ($p \leq 0.0001$)

8 ^g Significantly different from GTN (440 µM) treatment alone ($p \leq 0.01$), using Tukey's multiple
9 comparison test

10

1

Table 3. Effects of *in ovo* NAC supplementation following GTN treatment on cranio-facial and mesencephalon perimeter and eye diameter.

Treatment ^a	Craniofacial perimeter (mm) ^b	Mesencephalon perimeter (mm) ^b	Eye diameter (mm) ^b
Control	8.3 ± 0.56	4.0 ± 0.29	3.0 ± 0.37
NAC (10 mM)	8.5 ± 0.72 ^{c,d}	3.9 ± 0.38 ^{c,e}	2.8 ± 0.39 ^{c,f}
GTN (440 µM)	7.0 ± 0.89 ^{g,h}	3.2 ± 0.36 ^{g,h}	2.2 ± 0.39 ^{g,h}
GTN +NAC	7.7 ± 0.96 ^{i,j}	3.6 ± 0.42 ^{i,j}	2.5 ± 0.49 ^{i,j}

2 ^a Injection volume = 20 µL. Abbreviations used: NAC (N-acetyl cysteine), GTN (glyceryl trinitrate)

3 ^b Mean ± SD, (N=30)

4 ^c Not significantly different from Ctrl

5 ^d Significantly different from NAC + GTN (** $p \leq 0.01$)

6 ^e Significantly different from NAC + GTN (* $p \leq 0.05$)

7 ^f Not significantly different from NAC + GTN

8 ^{g,h} Significantly different from either Ctrl or NAC treatment alone (*** $p \leq 0.001$) using Tukey's
9 post-hoc test

10 ^{i,j} Significantly different from GTN (* $p \leq 0.05$) and NAC treatment alone (* $p \leq 0.05$) using Tukey's
11 post-hoc test

A

B

A

B