Examensarbete

Effects of the NO donors Sodium Nitroprusside and S-nitrosoglutathione on oxygen consumption and embryonic organ growth in the domestic broiler chicken, *Gallus gallus domesticus*.

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Examensarbetet utfört vid IFM Biologi
2010-06-01

LITH-IFM-G-EX--10/2326—SE
### Title

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### Abstract

Nitric oxide (NO) is an important chemical factor that controls vascular tone in the cardiovascular system. NO is a vasodilatory molecule that plays a role in blood pressure and blood flow regulation as well as vessel formation and tissue cell proliferation. NO influences the flow by which nutrients and other metabolites required for growth are transported to the tissues. The aim of this study was to investigate if NO, through mediation by the NO donors Sodium Nitroprusside (SNP) and S-Nitrosoglutathione (GSNO) affect growth and oxygen consumption of prenatal broiler chicken. The results indicate that, although the treatments did not have clear significant effects on the embryos or the organs examined, a slight delay in development can be observed in the GSNO treatment embryos. The study could not conclude, however, if this was due to effects of NO donors.

### Keywords

Hypotension, Nitric Oxide, NO donor, Oxygen consumption, Prenatal growth, S-Nitrosoglutathione, Sodium Nitroprusside, Vasodilation
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1. **Abstract**

Nitric oxide (NO) is an important chemical factor that controls vascular tone in the cardiovascular system. NO is a vasodilatory molecule that plays a role in blood pressure and blood flow regulation as well as vessel formation and tissue cell proliferation. NO influences the flow by which nutrients and other metabolites required for growth are transported to the tissues. The aim of this study was to investigate if NO, through mediation by the NO donors Sodium Nitroprusside (SNP) and S-Nitrosothiol (GSNO) affect growth and oxygen consumption of prenatal broiler chicken. The results indicate that, although the treatments did not have clear significant effects on the embryos or the organs examined, a slight delay in development can be observed in the GSNO treatment embryos. The study could not conclude, however, if this was due to effects of NO donors.

*Keywords: Hypotension, Nitric Oxide, NO donor, Oxygen consumption, Prenatal growth, S-Nitrosothiol, Sodium Nitroprusside, Vasodilation*

2. **List of abbreviations**

- CAM = Chorioallantoic membrane
- MAP = Mean Arterial Pressure
- C = Control
- NO = Nitric Oxide
- ED = Embryonic Development
- NP = Not Pipped
- EP = Externally Pipped
- PBS = Phosphate Saline Buffer
- GSNO = S-Nitrosothiol
- SNP = Sodium Nitroprusside
- GSNO-L = GSNO Low dose
- SNP-L = SNP Low dose
- GSNO-M = GSNO Medium dose
- SNP-M = SNP Medium dose
- GSNO-H = GSNO High dose
- SNP-H = SNP High dose
- IP = Internally Pipped

3. **Introduction**

In all vertebrate species, the first functional component that develops is the cardiovascular system which provides the cells and tissues of the organism with all the metabolites necessary for cellular and organ growth. Regulation of the cardiovascular system is controlled by extrinsic and intrinsic mechanisms which include the autonomic nervous system, endocrine system and other chemical factors that regulate cellular and muscular components of the system (Fritsche et al. 2000). The endothelium lining the blood vessels of the cardiovascular system is an important regulatory mechanism. In this mechanism, the endothelial cells produce paracrine vasoactive molecules that affect the smooth muscle tissues of the blood vessels.

One such vasoactive molecule, which will be of relevance for this study, is nitric oxide (NO) which has a relaxant and vasodilatory effect on the smooth muscle tissues causing the vessels to expand which reduces blood pressure and vascular resistance (Moncada and Higgs, 2006).

NO and vasodilation are important in developmental processes such as angiogenesis (vessel formation)(Gallo et al, 1998), and also in antiangiogenesis where NO donors
have been shown to inhibit angiogenesis in the chorioallantoic membrane (CAM) in developing chicken embryos (Pipili-Synetos et al. 1995, Pipili-Synetos, 1999).

The regulation of vascular growth is also affected by oxygen availability and metabolic rate of the tissues that it supplies. As metabolic rate increases the vascular growth tends to increase and vice versa. The vascular growth and metabolic supply is regulated by angiogenesis and vasodilation to meet the metabolic need of the tissues (Adair et al.1990).

Several studies show that fetal growth is compromised by an inadequate supply of oxygen during development. Hypoxia has been shown to reduce VO\(_2\) and cause hypometabolism in chicken embryos (Mortola and Labbé, 2004). Chicken embryos exposed to hypoxia (15%) during early embryonic development shows reductions in growth and survival as well as reduction in cardiac ventricular function (Sharma et al, 2006).

Chicken eggs incubated in high altitude (PO\(_2\) = 100mm Hg) with a lower oxygen tension also impaired embryonic growth (Giussani et al, 2007) McCutcheon et al demonstrated in 1982 that the organ masses of the heart, liver an brain were smaller in hypoxic embryos (as cited by Azzam and Mortola, 2007) and there are reported effects on the growth pattern of the CAM in hypoxia in which the CAM increases in mass (Azzam and Mortola, 2007)

In studies on chicken-, turtle-, and alligator embryos (Metcalfe et al, 1984; Kam 1993, Crossley and Altimiras, 2004) it was reported that hypoxia reduces yolk assimilation and retards embryonic growth during hypoxic incubation, further evidence on the inhibiting effect of hypoxia on growth.

A study conducted on White Leghorns chicken embryos demonstrates that prenatal exposure to hypoxic conditions affect the growth of the fetal heart by biventricular cardiac hypertrophy (Villamor et al, 2003).

In contrast, Tintu et al demonstrated in 2009 that hypoxia induces cardiac deterioration, with reductions in ventricular wall mass and increased cardiomyocyte apoptosis, and an impairment of cardiac function (Tintu et al, 2009)

Hypoxia has also been shown to affect the development of the prenatal chick brain. It has been demonstrated that chicken embryos exposed to 14% oxygen concentrations during a 24 hour period resulted in a reduction in neuronal cell density and an increase in astrocyte density, which impaired cognitive function, in the chick brain (Rodricks et al, 2003). These studies indicate that an adequate amount of oxygen is required to initiate and maintain normal embryonic organ development of the prenatal fetus.

The developmental period of the broiler chicken, Gallus gallus domesticus, is 21 days. Until day 19 of embryonic development, the CAM constitutes the major respiratory organ of the developing fetus through which all oxygenation and gas diffusion of the fetus is mediated (Strick et al, 1991). The angiogenic vessel formation in the CAM starts at day 4 and ends at around day 11(Staton et al. 2008) and 12 (Azzam and Mortola, 2007) of embryonic development after which vascularization in the CAM is completed. When the CAM is unable to provide the growing embryo with sufficient oxygen, internal pipping (IP) and subsequently external pipping, occurs (Christensen et al, 2001). In this phase the fetus breaks pierce the CAM into the air space (IP) and finally breaks the eggshell (EP).
The yolk sac is the main nutritional supply of the developing avian embryo. As the fetus grows the yolk sac is assimilated and this corresponds to the metabolic efficiency of the embryo (Riedy et al, 1998, Romanoff, 1944)

This study investigates whether the administration of NO releasing substances, NO donors, affects the embryonic growth and oxygen consumption in prenatal broiler chickens. Relevant for this study is the short acting NO donor Sodium nitroprusside (SNP) and the long acting NO donor S-nitrosoglutathione (GSNO) which imposes vasodilatory effects on vascular tissues by the spontaneous release of NO. SNP is a short acting NO donor with a half life of 1-2 minutes (Friederich and Butterworth, 1995) and duration of effect of 1-10 minutes (Huan et al, 2010). SNP has been used as for medical purposes in treating conditions such as hypertension (elevated blood pressure) but its use can be compromised because of the parallel toxic effects because it also releases CN⁻ (Cyanide) demonstrated by Smith et al, 1974 (as cited by Friederich and Butterworth, 1995). SNP has also been shown to inhibit angiogenesis in an in vivo study conducted on the CAM of prenatal chickens (Pipili-Synetos et al, 1994, Pipili-Synetos et al, 1993).

GSNO has also been used for clinical purposes and it has been proven to be a potent vasodilator in both in vivo and in vitro studies (MacAllister et al, 1994., Sogo et al, 2000). GSNO has been demonstrated to be a long acting vasodilator, exerting long term effects on rat femoral arteries, in a study by Megson et al (as cited by Sogo et al, 2000).

It is hypothesized that, as vasodilation reduces mean arterial blood pressure (MAP) and thus decreases blood flow and metabolic supply to the tissues, the growth of embryonic tissues will be reduced.

It is also hypothesized that the resulting reduction in MAP in the CAM and systemic circulation, induced by the NO donors, will affect the oxygen consumption of the embryos by a decrease in ventilation.

Since oxygen availability is a crucial component in organ development it is expected that a change in oxygen consumption will affect organ growth. Organs under examination in the study are the brain, heart, liver, lungs and kidneys along with determination of effects on embryonic mass and yolk mass.

4. Materials and methods

4.1 Animal subjects

The animal for the study (n=96) was the broiler strain Ross 308 of the domesticated broiler chicken, Gallus gallus domesticus. Fertilized eggs were acquired from a local hatchery (Lantmänne SweHatch AB, Väderstad, Sweden) and were kept in a refrigerator (18 °C) to inhibit embryonic development until start of incubation (ED0) (Fasenko, 2007). The eggs were automatically turned 90° every 4 hours along its long axis (Hova-bator automatic turner, Savannah, USA). At the time of start of embryonic development the eggs were weighed (Fisher Scientific, SG-601), labeled with date of incubation and egg mass and sorted by egg mass to create 7 incubation groups (4 groups N= 12 and 3 groups
N = 16) with a similar egg mass (59.6 ± 1.4 grams). The eggs were placed into an incubator (25 HS, Masalles commercial, Spain) under normoxic conditions (20.95% O₂) with a temperature of 37.8 °C and a relative humidity of 45%. The eggs were placed along their long axis, with the blunt side facing upwards. They were automatically tilted 90° every hour to ensure proper CAM development (Strick et al, 1991) and were monitored by candling to determine the developmental status of the embryos, dead and undeveloped embryos were removed from incubation.

The experimental procedures were carried out during ED13, 15 and 17 of embryonic development during which the injections were performed. VO₂ measurements (Section 4.6) started at ED18 and were completed at ED20 immediately followed by dissections of the embryos (Section 4.7). At this point of development the embryos normally are internally pipped (IP) or externally pipped (EP). The embryonic mass at day 13, 15 and 17 of development were estimated to be 7, 13 and 21 grams (Mortola and Awam, 2010). All experiments were approved by the local ethical committee of animal research (Dnr.25-10 & Dnr.26-10).

4.2 Treatments and dosages

As treatments the NO donors SNP and GSNO were used. A low (L), middle (M) and a high dose (H) of each substance was administered (SNP: 1, 10 & 100 mg kg⁻¹; GSNO: 0.3, 3 & 30 mg kg⁻¹). A control group was injected with the solvent used for the substances, in both cases sterile phosphate saline buffer solution, PBS (NaCl: 1.37 M; KCl: 26.8 mM; Na₂HPO₄: 101.4 mM; K₂HPO₄: 17.6 mM, pH = 7.4) (Q-BIOgene, MP, North America). 50 μl of treatment solution were administered into the air space of the test subjects (Section 4.5).

4.3 Preparation of Sodium Nitroprusside, SNP

A stock solution of SNP was created by dissolving the amount of SNP (Sigma-Aldrich®, Stockholm), corresponding to the high dose (SNP-H) treatments (70, 130 and 210 mg of ED13, 15 and 17, respectively) into 5 ml of PBS. The stock solution, corresponding to the high dose treatment (SNP-H), was used to prepare the middle and low dose treatment solutions by diluting 10x (SNP-M) and 100x (SNP-L). The control and SNP solutions were filtered with a sterile 0.45 μm Millex®-HV Syringe driven filter unit into a 1.5 ml eppendorf tube which were kept at a constant temperature of 38°C using a heat block prior to injection.

4.4 Preparation of S-Nitrosoglutathione, GSNO

A stock solution consisting of 25 mg of GSNO (Sigma-Aldrich®, Stockholm) dissolved in 500 μl N₂-equilibrated PBS was created. The equilibration was conducted by bubbling the PBS with nitrogen gas as a means to remove oxygen from the PBS due to the oxygen sensitive nature of GSNO. From the stock solution 12 aliquots, corresponding to the high dose (GSNO-H) treatments of each day of injection (Dose: 0.3, 3 and 30 mg kg⁻¹; Embryonic mass: 7, 13 and 21 grams on ED13, 15 and 17, respectively) were transferred
into 500 μl eppendorf tubes. From the stock the middle (GSNO-M) and low (GSNO-L) dose solutions were prepared by successive 10-fold dilutions. The preparations of GSNO required cool temperatures and low light conditions due to the temperature-, and light-sensitive nature of the substance (Bohlen et al, 2009). Therefore all preparations of the stock solution and aliquots were conducted in a cooling room (5°C) at low light intensity. The aliquots were stored at –20°C until preparations of the treatment solutions. The preparations of the treatment solutions were conducted on ice to inhibit any degradation of GSNO.

4.5 Preparations of injection port and treatment administration

A small injection port was created in the blunt top of the eggs, were the air space is located, using a drill (Ø 1mm). Tests of the injection procedure indicates that the hole of the injection port needs to be larger than the size of the microsyringe needle (Ø 0.8 mm) to prevent any pressure buildup in the air space which can lead to ejection of the injected solution as the needle is being removed. The location of the injection port was carefully cleaned, disinfected and kept sterile using a cotton swab and 70 % ethanol prior to, during and after the drilling and injection. Injections were performed using an Ø 0.8mm micro syringe (250 μl, ILS Borosilicate glass 3.3). Following administration the injection port was sealed with paraffin wax to prevent any contaminants entering the air space.

The administration of SNP and GSNO was conducted during day 13, 15 and 17 of embryonic development. For an estimation of embryonic mass in these stages of development results from an earlier study were used (Mortola and Awam, 2010). The mean embryonic mass for 13, 15 and 17 day old embryos was estimated to be 7, 13 and 21 grams, respectively. A high, middle and a low dose of each substance and for each day of injection (SNP: 1, 10 and 100 mg kg⁻¹; GSNO: 0.3, 3 and 30 mg kg⁻¹) was created using PBS as solvent (section 2.2 and 2.3). 50 μl of treatment solution were administered onto the CAM of the air space through which the solutions were absorbed by the embryo.

4.6 Oxygen consumption measurements

For the measuring of embryonic oxygen consumption, VO₂, an open respirometry setup in a push mode configuration was utilized in which 7 eggs from each incubation group was tested in seven 48 hours runs. The system has been described elsewhere (Gräns and Altimiras, 2007). Briefly, the eggs were placed in 7 respirometry chambers (Ø 50mm, height 95 mm) equipped with air in-, and outlet tube connections (Tygon R3603, 3,2 x 4,8mm) through which the air flow ( 75 ml min⁻¹) in the chambers were regulated (FOX II analyzer, Sable Systems International, Las Vegas, USA). A chamber not containing an egg was used as a baseline chamber. Small fans (Papst, 25 x 25 mm, 3,2 m³ h⁻¹) located in the chamber lids provided adequate air mixing. The respirometry chamber was also equipped with thermocouples sensoring chamber temperature (TC-08, Pico® Technology Ltd). The experimental setup was placed in a modified incubator maintaining a temperature of 37.8 °C and a relative humidity of 45%. The air flow was shifted every 15 minutes (TR-RM8 Respirometer Multiplexer, Sable Systems International, Las vegas, USA) between the chambers. A 20 ml column containing desiccating substrate
(Indicating Drierite, anhydrous calcium sulfate and cobalt chloride, W.A.Hammond Drierite Comapnt Ltd.Xenia, USA) was used to remove water vapor from the air flow entering the Fox II analyzer. The Fox II analyzer and the Multiplexer were controlled by a Dell Optiplex GXA computer from which oxygen concentrations, flow, temperature and atmospheric pressure was monitored. Comparisons of the oxygen consumption between the experimental chambers containing eggs and a baseline chamber without an egg gives a measure of the oxygen consumption of the test subjects.

4.7 Dissections

At ED20 of embryonic development dissections of the embryos were performed. The embryos were picked out one at a time and were killed by decapitation followed by the dissection to remove the brain, heart, liver, lungs and kidneys. The embryo including the yolk sac, the yolk sac and the embryo excluding the yolk sac were weighed (Fisher Scientific, SG-601). The brain, heart, liver, lungs and the kidneys were weighed down too nearest hundreds of a microgram (Mettler AE 260, DeltaRange®). Prior to weighing the organs were carefully blotted to remove as much fluid as possible without damaging the organ tissue.

4.8 Statistical analyses

Data processing and calculations were conducted using the statistical tools in OpenOffice.org Calc and Microsoft Excel 2007. Statistical analyses were conducted using SPSS 17.0 (SPSS Inc.). For determination of statistical differences between treatments a general linear model univariate ANOVA was performed. In the test treatment was the fixed factor and as dependent variables the data from the dissections and VO2 measurements (see table 3). A Tukey post hoc multiple comparisons test was done for comparisons of the treatments.

5. Results

5.1 Test subjects, incubation and mortality

Of the 96 eggs that were put into incubation, 75 developed normally and were used for the experiments. 21 eggs (21.9%) did not develop properly or died during incubation and were discarded. The embryos exposed to the SNP-H treatment (n=6), and also one SNP-M embryo, died most probably due to the toxic nature of SNP. One GSNO-M embryo died during the VO2 test. One of the GSNO-H test subjects was severely underdeveloped and was discarded from statistical analysis. As for the control group (n=15) no individual was lost due to treatment or other effects during the experiment.
5.2 Embryonic mass

*Figure 1A* illustrates the mean embryonic masses of the treatment groups. The GSNO treatments show a slight reduction in embryonic mass compared to the control and the SNP treatments. The SNP-M treatment group shows a slight increase in embryonic mass compared to the control. The only statistical difference that exist is between the SNP-M and GSNO-M treatments (*p* < 0.05, *Table 3*) which correspond to a difference in mass of 12.9%.

![Graph](image)

*Figure 1. All values expressed as mean ± SD. A) Illustrates the embryonic masses of the treatments. There is a significant difference (*p*<0.05) between SNP-M and GSNO-M. B) Graph showing yolk masses of the different treatments. GSNO-M differs significantly (*p*<0.05) from the control.*

5.3 Yolk mass

*Figure 1B.* Depicts the mean masses of the yolk. It illustrates a slight increase in mass in the GSNO-M (17%), GSNO-H (13%) treatments as well as for SNP-L (10%) compared to the control group. A statistical analysis reveals that there is a significant difference (*p* < 0.05, *Table 3*) between the control and GSNO-M corresponding to a mass difference of 17.2%.

5.4 Organ mass and relative organ mass

Organ mass and relative organ mass (Organ mass / Embryonic mass) is expressed as means ± SD. No difference is observed either from the graphs (*Figure 2*) or from the statistical analysis (*p* > 0.05 for all variables tested, *Table 3*) of the mean organ masses or the relative organ masses.
Figure 2. Graphs showing the organ masses for the brain, heart, liver, lungs and kidneys. All values are expressed as means ± SD. No significant difference (p>0.05) exists between the treatments.

Figure 3. Illustrates the relative organ masses (Organ mass/ Embryonic mass) for the brain, heart, liver, lungs and kidneys. All values expressed as means ± SD. There is no statistical difference (p>0.05) between treatments.
5.5 Embryonic development and pipping status

*Table 1* lists the egg mass (means ± SD) of ED0 and ED20 as well as loss of mass, consisting mainly of water loss, at ED20 of embryonic development. It shows that the GSNO group eggs lost more water than the control and SNP treatment groups. There is a significant difference between GSNO-H and SNP-L (p < 0.05, Table 3) and between GSNO-L and SNP-L (p<0.05, Table 3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Egg mass, ED0</th>
<th>Egg mass, ED20</th>
<th>Water loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>59.8 ± 4.1</td>
<td>51.0 ± 3.4</td>
<td>13.8 ± 2.3</td>
</tr>
<tr>
<td>SNP-L</td>
<td>59.7 ± 3.3</td>
<td>51.6 ± 4.3</td>
<td>12.4 ± 2.6 *</td>
</tr>
<tr>
<td>SNP-M</td>
<td>60.8 ± 3.4</td>
<td>52.4 ± 2.7</td>
<td>13.3 ± 2.5</td>
</tr>
<tr>
<td>SNP-H</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>GSNO-L</td>
<td>58.4 ± 2.5</td>
<td>52.7 ± 2.0</td>
<td>15.0 ± 2.6 *</td>
</tr>
<tr>
<td>GSNO-M</td>
<td>58.7 ± 3.0</td>
<td>49.6 ± 2.4</td>
<td>14.3 ± 1.3 *</td>
</tr>
<tr>
<td>GSNO-H</td>
<td>59.3 ± 3.2</td>
<td>50.4 ± 2.6</td>
<td>14.0 ± 1.9</td>
</tr>
</tbody>
</table>

*Table 1*. Values of egg mass at ED0 and ED20 are expressed as means ± SD. Lists the egg masses at Day 0 and at Day 20 of incubation and water loss (%) during incubation. Treatment groups consist of embryos injected with the NO donors Sodium Nitroprusside (SNP) or S-nitrosoglutathione (GSNO) in low (L), middle (M) or high (H) doses. The control group was injected with sterile phosphate saline buffer (PBS). The embryos of the SNP-H group died due to treatment effects.

*Table 2* illustrates the pipping status of the embryos at ED20 of embryonic development. It lists the number of internally pipped (IP), externally pipped (EP) and not pipped (NP) embryos. One GSNO-H embryo is listed as abnormally developed (AD) as it showed to be severely underdeveloped. The table shows a increase of pipped embryos in the GSNO (NP = 58.6%) group compared to the SNP (NP=15.4%) and control group (NP= 20%).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NP</th>
<th>IP</th>
<th>EP</th>
<th>AD</th>
<th>n</th>
<th>n (VO₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>SNP-L</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>SNP-M</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>SNP-H</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>GSNO-L</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>GSNO-M</td>
<td>7</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>GSNO-H</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Variable</td>
<td>p</td>
<td>ΔTreatments</td>
<td>Variable</td>
<td>p</td>
<td>ΔTreatments</td>
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</tr>
<tr>
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<td>-------------</td>
<td>-------------------</td>
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<tr>
<td>Embryonic mass</td>
<td>0.021</td>
<td>M ≠ M1</td>
<td>Embryonic mass</td>
<td>0.162</td>
<td>-</td>
<td></td>
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<tr>
<td>Yolk mass</td>
<td>0.095</td>
<td>-</td>
<td>Yolk mass</td>
<td>0.032</td>
<td>C ≠ M</td>
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<tr>
<td>MSVo2</td>
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<td>MSVo2</td>
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<tr>
<td>VO2/min</td>
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<td>-</td>
<td>VO2/min</td>
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<tr>
<td>Brain</td>
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<td>-</td>
<td>Brain</td>
<td>0.711</td>
<td>-</td>
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<tr>
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<td>Heart</td>
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<td></td>
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<tr>
<td>Liver</td>
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<td>-</td>
<td>Liver</td>
<td>0.230</td>
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<td></td>
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<tr>
<td>Lungs</td>
<td>0.954</td>
<td>-</td>
<td>Lungs</td>
<td>0.927</td>
<td>-</td>
<td></td>
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<tr>
<td>Kidneys</td>
<td>0.812</td>
<td>-</td>
<td>Kidneys</td>
<td>0.684</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Brain/EM</td>
<td>0.450</td>
<td>-</td>
<td>Brain/EM</td>
<td>0.312</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Heart/EM</td>
<td>0.126</td>
<td>-</td>
<td>Heart/EM</td>
<td>0.114</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Liver/EM</td>
<td>0.530</td>
<td>-</td>
<td>Liver/EM</td>
<td>0.861</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lungs/EM</td>
<td>0.276</td>
<td>-</td>
<td>Lungs/EM</td>
<td>0.510</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Kidneys/EM</td>
<td>0.550</td>
<td>-</td>
<td>Kidneys/EM</td>
<td>0.895</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Waterloss</td>
<td>0.046</td>
<td>L1 ≠ L(0.049); L1 ≠ H(0.028)</td>
<td>Waterloss</td>
<td>0.638</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Illustrates statistical differences between treatments analyzed by One-way ANOVA. Left table shows comparisons including all treatments (Control, SNP-, and GSNO group). Right table lists differences between the control and GSNO group. C = Control, L1 = SNP-L, M1 = SNP-M, L = GSNO-L, M = GSNO-M

5.6 Oxygen consumption and mass specific metabolic rate

Values from the oxygen consumption test are presented in Figure 4 as means ± SD. No significant (p > 0.05, Table 3) statistical difference can be found between the treatments in the VO$_2$ data.

![Figure 4](image-url)

Figure 4. A) Shows the masses specific metabolic rate (VO$_2$ h$^{-1}$/Embryonic mass) B) Illustrates the oxygen consumption for the treatments. All values expressed as means ± SD. No significant difference (p > 0.05) exists between treatment
6. Discussion

In this study it was hypothesized that a reduction in MAP induced by the NO donors SNP and GSNO would have an impact in embryonic organ growth and oxygen consumption in the prenatal chicken embryo.

6.1 Test subject mortalities

All of the test subjects in the SNP-H group and one SNP-M embryo died after the last treatment injection of SNP on day 17. The cause of this may be due to the toxicity of SNP, which during breakdown releases 5 cyanide ions (CN\(^{-1}\)) per molecule of SNP. The cyanide may upon release interact with methemoglobin which renders it nontoxic or it can be eliminated by thiosulfate in the liver. But at sufficiently high doses of cyanide relative to methemoglobin and thiosulfate, cyanide poisoning may be induced leading to tissue anoxia, due to the inactivation of cytochrome c oxidase and thus a inactivation of oxidative phosphorylation demonstrated by Hall et al., 1992 and Curry et al, 1991 (as cited by Friedrich and Butterworth, 1995). This can be a fatal condition if maintained.

The possibility that prolonged hypotension is the cause of death in the high dose embryos is not likely due to the short lasting vasodilatory effect of SNP (Friederich and Butterworth, 1995).

6.2 Pipping status, embryonic development and yolk assimilation

The only significant difference regarding embryonic mass between treatments was found between GSNO-M and SNP-M treatments which corresponded to a difference in mass of 12.9 %. Although no significant difference could be found between the other treatments, an indication of a difference could be seen when looking at the mean embryonic masses in Figure 1A, especially between the SNP and GSNO treatment groups. SNP-M increased in mean body mass relative to the control and the GSNO group, opposite to the effect observed in the GSNO group.

Regarding yolk mass there was a difference between the control and GSNO-M group (Figure 1B, Table 3). Yolk mass is related to embryonic mass as the yolk is the main nutritional supply for the embryo. As the embryo grows by nutrient assimilation from the yolk, the yolk mass is reduced which has been demonstrated in a study by Reidy et al. in 1998. The yolk assimilation is related to the metabolic efficiency of the embryo (Romanoff, 1944).

A reduction in yolk assimilation has been associated with embryonic development in hypoxia (Crossley and Altimiras, 2004) which corresponds to a decreasing metabolic efficiency due to the lack of oxygen. This may also be the case in the GSNO group treatments as a decrease in MAP would reduce oxygenation of the tissues and subsequently alter energy metabolism.

As expected the yolk mass of the GSNO-M is large which corresponds to the smaller embryonic mass of the embryo which may indicate a reduction in metabolic efficiency and growth in the GSNO-M group in comparison to the control.
The difference in embryonic -, and yolk mass between the SNP and GSNO treatment groups could be caused by the time of NO release by the two compounds. GSNO has been showed to, according to a study conducted by Megson et al (as cited by Sogo et al, 2000), exert vasodilation for several hours after administration. In comparison, SNP has a half-life of 1-2 minutes (Friederich and Butterworth, 1995) and a estimated duration of effect of 1-10 minutes (Huan et al, 2010) resulting in less NO release and thus less vasodilation over time compared to GSNO. A prolonged period of hypotension caused by vasodilation may account for the slight reduction in embryonic mass, due a reduction in metabolic flow provided by the yolk to the tissues, seen in the GSNO treatment embryos in comparison to SNP embryos. Also, a reduction in oxygenation by decreasing blood pressure in the CAM circulation could result in decreased oxygen supply to the systemic circulation which could lead to a reduction in tissue growth. A reduction on oxygen consumption is however not an observed effect in this study.

Differences in pipping status (Table 2) were observed in between treatments with higher number of NP in individuals in the GSNO treatment group. IP and EP is an indication of the onset of lung breathing and hatching as the oxygenation provided by the CAM no longer can provide the growing embryo with sufficient oxygen supply (Christensen et al, 2001). A delay in IP and subsequently EP can therefore be seen as a delay in development and hatching of the embryos. The pipping status of GSNO group may thus be an indication of a retardation of embryonic development, possibly due to GSNO treatment effects.

No significant difference could be found in between treatments regarding organ mass and relative organ mass. Hypoxic conditions have been shown to cause reductions in organ, yolk and embryonic mass in a number of studies (Azzam and Mortola, 2007, Crossley and Altimiras, 2004, Giussani et al, 2007, McCutcheon et al, 1982, Tintu et al, 2009, Villamor et al, 2003). In this study, however, it cannot be concluded that any severe reduction in oxygen diffusion and supply was induced by the administered NO donors. This explains the lack of differences between treatments in regard to organ masses.

6.3 Oxygen consumption and water loss

There was no difference between treatments regarding oxygen consumption and mass specific metabolic rate. This is consistent with the findings that there was no major difference in growth between treatments. Previous studies on the vascularity on the CAM have shown that NO and NO donors have antiangiogenic effects on its vascularization (Pipili-Synetos et al, 1995, Pipili-Synetos, 1999). SNP applied directly on the membrane has been shown to have an inhibitory effect on the angiogenesis process in the CAM (Pipili-Synetos et al, 1994, Pipili-Synetos et al, 1993). A decrease in vessel formation in the CAM, and thus a decrease in oxygen diffusion area, would reasonably lead to a decrease in ventilation through the membrane and thus reduce oxygenation of the embryo. In this study the SNP was applied directly on the CAM in the air space. No significant change in oxygen uptake was observed in the SNP treatments which indicate that no angiogenic effect occurred.
A decrease in ventilation would also be the case if vasodilation resulted in a reduction in blood pressure in the CAM circulation. This does however not seem to be the case in this study as no apparent effect can be observed in oxygen consumption due to the treatment with SNP or GSNO.

In the case of water loss during embryonic development there was however a significant difference \( (p<0.05) \) in water loss between SNP-L and GSNO-L/GSNO-H. The GSNO treatment eggs lost more water than SNP-L. In the avian egg, gas conductance, including water vapor, is influenced by several factors, one being egg size and eggshell pore area which affect total gas diffusion area (Ar et al, 1974). Another factor is vessel density of the CAM which is influenced by oxygen availability and other angiogenic factors (Murray and Wilson, 2000). Any vasodilatory or angiogenic response occurring in the CAM and also in the systemic vasculature would theoretically lead to a greater area of diffusion and greater perfusion of gases in the tissues surrounding the pores of the egg shell. If the injection of GSNO on the air CAM induced vasodilation and/or angiogenesis it may account for the increase in water loss observed in the GSNO group.

6.4 Treatment preparations and injections

The expectations that GSNO and SNP would have significant effects on oxygen consumption and growth were not met. In evaluating why answers may be found in the experimental protocol and especially regarding treatment solutions and the injections. Preparations of GSNO were problematic due to the solubility of the substance (25 mg in 1 ml PBS). In the preparations of GSNO, half that volume (500 μl) was used to acquire the proper doses and amounts for the injection which, in theory, left half of the GSNO unsolved. This made it difficult to determine whether the prepared aliquots and thus the treatment solutions of GSNO did actually correspond to the actual doses planned for injection.

Other factors that may have influenced the results is the method of injections. First, there is no way of knowing how much of the substances that were absorbed by the embryo and in the case of GSNO, how much of the substance that was degraded before the absorption. An alternative method of administration is injecting the substances directly into the circulation to ensure that the actual doses are administered.

Second, as the half life and effect on NO release is limited for both substances, a continuous infusion method of administration might be a better option to ensure a prolonged effect of the NO donors.

Third, as the angiogenic process of vessel development is completed at day 11 of embryonic growth (Staton et al. 2008), the better option of administration might be to do the injection of NO donors between day 4 and 11 during which the vascular components of the CAM is under development.

6.5 Conclusions

There are no clear results which indicate that the NO donors SNP or GSNO have an effect on the growth patterns of the prenatal chick embryo from day 13 onwards of embryonic development. There is however, an indication that GSNO might affect
prenatal growth when looking at the embryonic mass, yolk assimilation, pipping status and water conductance, though nothing can be concluded from these observations that this is due to effects of GSNO. There is also no indication that the drugs used in this study have any major impact on oxygen consumption and metabolism in the prenatal chicken embryo.

For further investigation into the effect of NO on the growth of prenatal chicken embryos, a different protocol concerning the administration of NO donors might be used. For example, an infusion system that administers the drug during a prolonged time span which may result in a longer release and effect of NO. It would also be of use to examine more variables such as blood pressure and also to get an estimation of vasodilation, for example in the CAM, to better be able to evaluate the effects of NO donors in the chicken embryo.

7. Acknowledgements
   I would like to thank my supervisor Jordi Altimiras for the opportunity to do the study and for the valuable input and feedback provided during the project. I also want to thank the people in CADE laboratory for assistance with the study.

8. References


