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Carbon monoxide affects early cardiac development in an avian model

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Abstract

Introduction: Carbon monoxide (CO) is a toxic gas that can be lethal in large doses and may also cause physiological damage in lower doses. Epidemiological studies suggest that CO in lower doses over time may impact on embryo development, in particular cardiac development, however other studies have not observed this association.

Methods: Here, we exposed chick embryos *in ovo* to CO at three different concentrations (3, 9, 18 ppm) plus air control (4 protocols in total) for the first 9 days of development, at which point we assessed egg and embryo weight, ankle length, developmental stage, heart weight, ventricular wall thickness, ventricular-septal thickness and atrial wall thickness.

Results: We found that heart weight was reduced for the low and moderate exposures compared to air, that atrial wall and ventricular wall thickness was increased for the moderate and high exposures compared to air and that ventricular septal thickness was increased for low, moderate and high exposures compared to air. Ventricular wall thickness was also significantly positively correlated with absolute CO exposures across all protocols.

Conclusions: This intervention study thus suggests that CO even at very low levels may have a significant impact on cardiac development.

KEYWORDS

air pollution, avian model, carbon monoxide, cardiac development, ventricular morphology

1 | INTRODUCTION

Carbon monoxide (CO) is a toxic gas without odor, color or taste (Penney et al., 2010). While produced endogenously, primarily through heme degradation (Stevenson et al., 2001), exogenous CO exposure in higher doses can be lethal. Hemoglobin (Hb) has a markedly higher affinity for CO than for oxygen, and carboxyhaemoglobin (COHb) is readily formed. Importantly, as CO remains bound and if exposures to the gas persists, COHb continues to rise at the increasing expense of oxyhaemoglobin (OHb) (Penney et al., 2010). CO also shifts the OHb dissociation curve and causes reduced release of oxygen to the tissues (Longo, 1977; Townsend & Maynard, 2002). Together, this can lead to severe hypoxia and death. Toxic mechanisms beyond hypoxia may also contribute to further morbidity, as symptoms can persist or appear even after COHb levels return to normal (S R Thom et al., 1995).

Pregnant women are at an increased risk for CO poisoning due to increased endogenous CO production

during gestation (Smollin & Olson, 2008). Acute CO poisoning during pregnancy is associated with premature delivery and spontaneous abortion, with pregnancy outcome likely dependent on severity of maternal poisoning and foetal age (Smollin & Olson, 2008). Foetal death may occur at nonlethal maternal carbon monoxide exposures (Longo, 1977).

While it is generally acknowledged that CO poisoning can cause severe damage and death, much less is known about low-level exposure. CO exposures of 6 ppm and lower can impact on vascular function (Bendell et al., 2020) and epidemiological studies report associations between maternal CO exposure and ventricular septal defects in the foetus (Dadvand et al., 2011a; Ritz et al., 2002; Zhang et al., 2016) at levels as low as ~1 ppm (Dadvand et al., 2011a). Other studies, however, have failed to replicate these findings (Chen et al., 2014). As foetal COHb will, under steady-state conditions, be 10%–15% higher than maternal COHb (Longo, 1977), the foetus may be particularly at risk during longer-term exposures.

CO exposure is difficult to study experimentally in humans. The level of CO that is ethical to deliver is limited both in terms of duration and amount. While some work has been done using low-level CO interventions in humans, for example Bendell et al. (2020), such studies would not be feasible in pregnant women. The chick is a common model for developmental research, as the embryo is easily accessible *in ovo* with progressive organ development which is highly conserved with mammals. It is also a good model for CO research, as CO responses in the chick resembles mammalian ones (Stupfel et al., 1982). Also, at Hamburger-Hamilton stage 35 (embryonic day (D)9) the chick embryo heart, with its four chambers, bears a closer structural resemblance to the human heart than other non-mammalian model organisms (Wittig & Munsterberg, 2016). The gaseous environment in which eggs are incubated can be easily controlled, thus further cementing its usefulness as a model for CO research. The chick embryo heart is fully formed at 10 days of development (Vilches-Moure, 2019). The aim of the current study was to interrogate the impact of low-level CO exposure on early development in the chick embryo, particularly focusing on cardiac development.

2 | METHODS

Fertilized Bovan Brown chicken eggs (Gallus gallus) (Henry Stewart and Co., Norfolk, UK) were used for all experiments. All experiments were performed according to relevant regulatory standards. Eggs were incubated from D0 to D9 of development (a total of 10 days) in an incubator (Thermo Scientific) at 37 ± 0.1°C. The eggs were separated in custom-made air-tight incubation boxes inside the main incubator, each containing a separate 25 mL glass container with ultra-pure water to maintain air humidity. Each box was equipped with two ports, closable with IV 3-way tap valves, used for adjustment of internal gas composition. Throughout the incubation period, each incubation box held either air (control) or CO at the following concentrations: 3, 9 and 18 ppm. The two lower levels were chosen for their similarity with current WHO guidelines for air quality exposure (3.2 ppm; WHO, 2021) and exposure over 8 h (8.1 ppm; WHO, 2000). The higher level was chosen as double the WHO 8-h exposure limit. The levels were dubbed "low," "moderate," and "high." The experiment was run on four separate batches of eggs, and the eggs in each batch was divided randomly and equally into the three CO conditions and Air control.

Gas exposure was conducted as follows: CO gas was sampled from a pre-mixed cylinder (1000 ppm CO in air, BOC) into a non-pressurized reservoir (2 L anesthetic bag), from which a pre-determined amount was drawn and introduced to the incubators via the ports using a 20 mL syringe. The amount of gas required was calculated based on the size of the sealed individual incubators (each box had a volume of 2.7 L). To test that CO levels in the incubators were reached and maintained throughout the experiments, gas levels were measured through one of the incubator ports every 1–2 days of incubation (TPI 716 flue gas analyzer, Test Products International Europe Ltd, West Sussex, UK). Every 2–3 days, this was also followed by a venting of the incubators and re-administration of gases to avoid longitudinal drift in oxygen and carbon dioxide content. Also at this point, ultra-pure water levels were assessed and replenished if necessary.

On D9 (after 10 days of development), eggs were removed from the incubator and weighed. Embryos were removed and assessed for viability (presence of heartbeat) immediately prior to termination by decapitation. Figure 1 shows a chick embryo, a schematic of the hindlimb and an image of the D9 heart. All nonviable embryos were excluded from further analysis. Viable embryos were harvested, removing any yolk/extraembryonic membranes. The following measurements were obtained prior to histological processing:

- *Weight*. Weight was measured using a standard laboratory analytical balance. Full embryo weight was assessed immediately following termination. The heart was then carefully excised, blotted dry and weighed.
- *Staging*. Development and morphogenesis were visually assessed using the Hamburger-Hamilton Staging Series (Hamburger & Hamilton, 1992; Martinsen, 2005) as a reference guide. For a subset of embryos, the

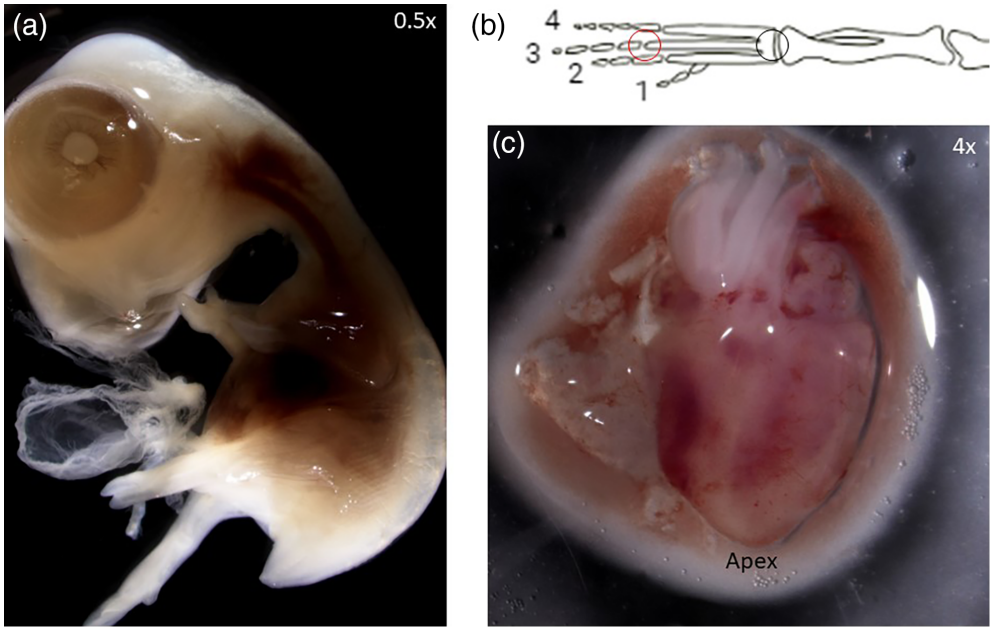

FIGURE 1 Chick embryo images. (a) whole embryo; (b) schematic of chick hindlimb, digits are numbered 1–4, red circle indicates the phalangeal-tarsometataral joint, black circle indicates anterior end of tarsometatarsus. Toe and ankle measurements were taken from the tip (posterior) of the third digit to each of the indicated sites; (c) chick heart at D9.

assigned stage was confirmed by a second researcher, as a quality control.

- *Size*. Embryo size was assessed for each viable embryo as a measure of development. Full length was measured from the top of the skull frontal bone to the posterior end of the pelvic bones, toe length (third toe) from the phalangeal-tarsometatarsal joint to the posterior end of the (third) toe, and ankle length from the anterior end of the tarsometatarsus—ankle joint—to the tip of the posterior end of the (third) toe (Figure 1). All measurements were conducted by positioning the embryo and limbs on a grid surface under a stereoscope.

For histological processing, Hamburger-Hamilton stage 33–35 hearts were fixed in formalin 10% for 24 h at room temperature, then processed for paraffin embedding (see supplement for protocol) in a Leica TP 1020 tissue processor. Tissues were embedded in Leica HistoCore Arcadia H. Hematoxylin and Eosin (H&E) staining were performed manually (see supplement for protocol) on 3 μm transversal plane sections obtained by the Leica RM2235 microtome.

2.1 | Histological assessments included

- *Ventricular wall thickness*. Ventricular wall thickness was measured across transverse plane sections from the outer surface to the inner surface of the myocardium at three different points for three different sections using cellSens Dimension (Olympus Life Science, Olympus, Tokyo) through an Olympus IX81 motorized inverted research microscope (Olympus Life Science). Ventricular wall thickness was averaged for each section for statistical comparison. Ventricular wall thickness measurements were independently re-assessed to verify findings.
- *Ventricular-septal thickness*. Ventricular-septal thickness was measured across the coronal plane sections towards the apex of the heart where the septum separates from the ventricular walls of the left and right ventricles. Measurements were taken at three equidistant points across three sections. Micrographs were taken from an Olympus BX60 microscope and analysis completed using ImageJ. Measurements were averaged for statistical comparison.
- *Atrial wall thickness*. Atrial wall thickness was measured across coronal plane sections from the outer surface to the inner surface of the atrial wall at three equidistant points perpendicular to the outer atrial wall across three different microscopic sections using ImageJ software from micrographs taken from an Olympus BX60 brightfield microscope (Olympus Life Science). Atrial wall thickness was averaged for each section for statistical comparison.

Atrial septum was not assessed as this structure does not fully close until hatching. All measurements (except septal thickness) were done on the left side of the heart.

Students' *T*-tests (one-tailed) were used to compare control and CO exposure levels (Microsoft Excel, Microsoft Corporation, WA, USA). As three comparisons were made for each measurement, corrections for multiple comparisons (Bonferroni method) meant that outcomes needed to meet a threshold of $p < .0167$ to be considered significant at an alpha of $p < .05$. For atrial wall thickness, a secondary further three comparisons were made

to fully describe the data, and Bonferroni corrections for this measure thus needed to meet a threshold of $p < .008$. All p-values are reported as is, but only those meeting the adjusted threshold reported as significant. Correlation between individual CO levels (directly measured, thus accounting for experimental fluctuations in CO) and the end-point measurement ventricular wall thickness were assessed using a regression model (SPSS 24.0 [IBM SPSS Statistics for Windows, Armonk, NY, USA]).

3 | RESULTS

Measurements were initially taken from 44 viable embryos. Viability in the study was ~75% and there was no significant difference in viability between protocols. One embryo was excluded from analysis following an outlier analysis (underdeveloped embryo), four were used to optimize the histology protocol, and a further four embryos were excluded from analysis of heart weight and ventricular wall thickness due to failure to dissect complete tissues. This left 43 embryos for gross morphology analysis (including egg and embryo weight, stage, embryo, ankle and toe length). Of these, 35 were suitable for heart weight analysis, 35 were suitable for ventricular wall thickness analysis, 13 were suitable for atrial wall analysis and 14 were suitable for ventricular septum analysis. A further 3 embryos were incubated to supplement the atrial wall and ventricular septum analyses, bringing the total for these to $N = 16$ (atrial wall) and $N = 17$ (ventricular septum).

3.1 | CO values

CO values are presented in Table 1. There was a significant difference in measured CO levels between the low, moderate and high exposures, as expected (one-way ANOVA [$F(3,39) = 142.9$, $p < .0001$]), but not between experiments (one-way ANOVA [$F(5,37) = 2.088$, $p = .089$]).

3.2 | Egg weight, embryo staging and size

We observed no significant difference in egg weight or embryo weight between any of the CO exposures and air control, nor was there any significant difference in stage between any CO exposures and air control. A significant reduction in ankle length ($p = .014$) was found for moderate CO compared to air control. Data are presented in Table 1.

3.3 | Heart weight

We observed a significant reduction in heart weight for the moderate CO exposure compared to air control ($p = .013$, Figure 2). Average heart weight was 24 mg (±1 mg) for air control, 18 mg (±0.6 mg) for low CO exposure, 18 mg (±0.5 mg) for moderate CO exposure, and 21 mg (±0.4 mg) for high CO exposure.

3.4 | Histological measurements

Figure 3 shows typical example images of histological sections of heart tissue for conditions air (control), low, moderate and high CO exposures. Means and standard deviations for histological assessments are shown in Table 2.

A significant increase in ventricular wall thickness was found for the moderate ($p = .0005$) and high ($p = .005$) CO exposures compared to air (Figure 4).

Furthermore, a significant positive linear correlation was found between the measured CO levels for each

TABLE 1 Weight and size measurements.

Measurement	Air (control)	CO, low	CO, moderate	CO, high
N	10	11	11	11
CO values (ppm)	0 (0)	3.4 (0.9)	9.0 (3.2)	18.2 (2.9)
Egg weight (g)	57.33 (3.28)	58.06 (2.78)	57.29 (2.40)	57.45 (2.97)
Embryo weight (g)	1.41 (0.27)	1.35 (0.23)	1.27 (0.19)	1.29 (0.22)
Stage (HH)	35 [35–35]	34 [33–36]	33.5 [33–35]	35 [33–35]
Embryo length (mm)	3.16 (0.34)	2.96 (0.17)	2.88 (0.27)	2.98 (0.24)
Ankle length (mm)	0.67 (0.11)	0.60 (0.08)	0.56 (0.08)*	0.60 (0.07)
Toe length (mm)	0.28 (0.04)	0.21 (0.11)	0.23 (0.07)	0.24 (0.05)

Note: Means and (SD). Numbers in parentheses are standard deviations for each measurement and protocol. Stage is given as median and [25th–75th quartile]. Statistical comparisons are between CO and air.
*$p < .05$, corrected for multiple comparisons.

batch and ventricular wall thickness ($R = 0.506$, $R^2 = 0.256$, adjusted $R^2 = 0.230$, $p = .004$, Figure 5). This analysis was conducted as there was an expected variation in actual (as opposed to target) CO levels in each protocol. A direct correlation analysis between individual CO levels and morphological changes is thus a powerful way of assessing dose-dependent relationships.

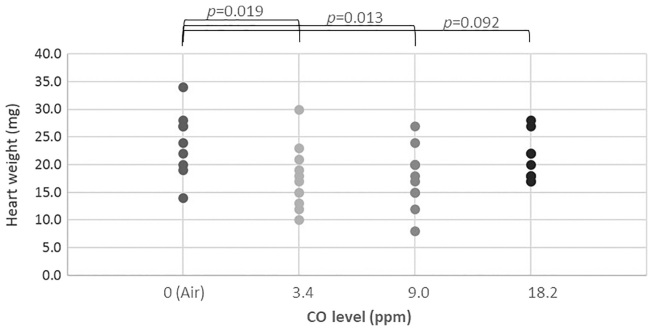

FIGURE 2 Embryo heart weight (blotted dry, mg). $N = 35$. Significant differences ($p < .05$) between air versus moderate CO exposure was found, but not between air versus low and air versus high exposures.

Based on the distribution in Figure 4, we also tested whether there was a U-shaped curvilinear relationship between heart weight and CO level. The trajectory showed a reduction in heart weight in the low and medium CO exposures compared to air control, and almost no difference in heart weight between the highest CO exposure and air control. We did not find a significant relationship between the measured CO levels for each batch (averages across each 10-day incubation period for each batch) and heart weight ($R = 0.308$, $R^2 = 0.095$, adjusted $R^2 = 0.046$, $p = .159$). However, there was a significant relationship between average CO

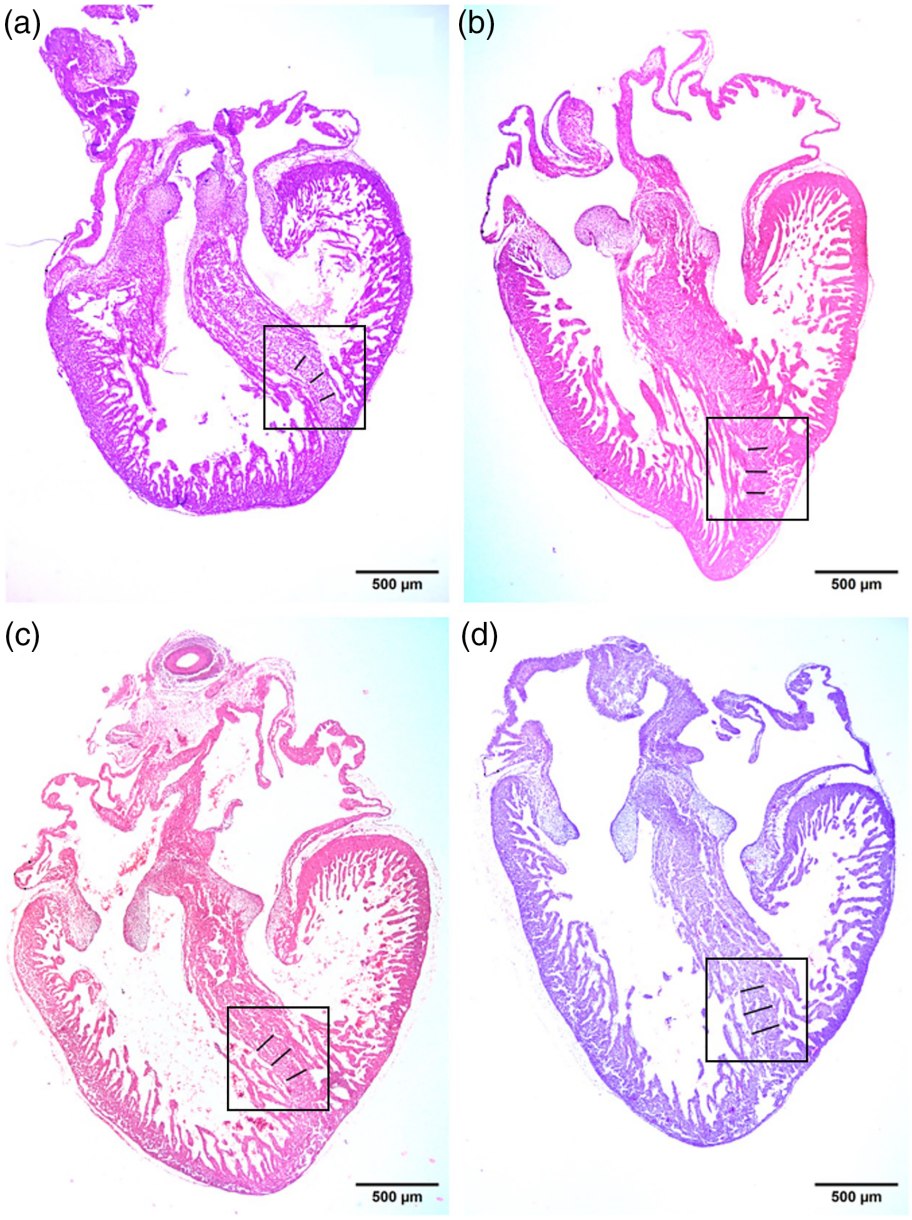

FIGURE 3 Histological sections of E9 heart tissue. Hematoxylin and eosin-stained coronal micrographs of embryonic day 9 hearts incubated in (a) air and, (b) low, (c) moderate and (d) high CO exposure levels. Sites of measurements (ventricular septum thickness) indicated for each section: black lines within black boxes.

TABLE 2 Histological measurements.

Measurement	Air (control)	CO, low	CO, moderate	CO, high
Ventricular wall thickness (μm), $N = 35$	397 (65)	460 (44)	519 (37)*	515 (57)*
Atrial wall thickness (μm), $N = 16$	11 (3)	11 (4)	16 (5)*	14 (4)*
Ventricular septal thickness (μm), $N = 17$	144 (49)	174 (34)*	203 (32)*	230 (41)*

Note: Means and (SD). Statistical comparisons are between CO and air.
*$p < .05$, corrected for multiple comparisons.

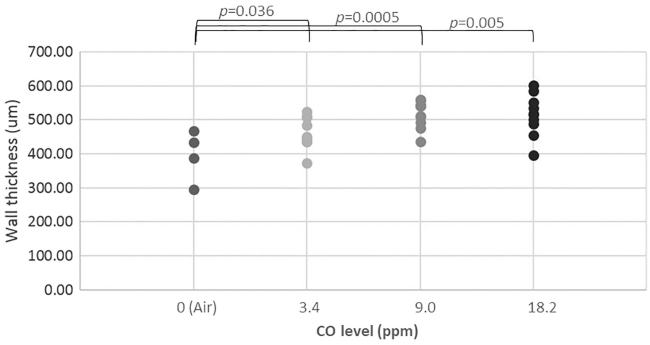

FIGURE 4 Embryo ventricular wall thickness (μm). $N = 35$. Significant differences ($p < .05$) between air versus moderate and air versus high CO exposures were found, but not between air versus low exposure.

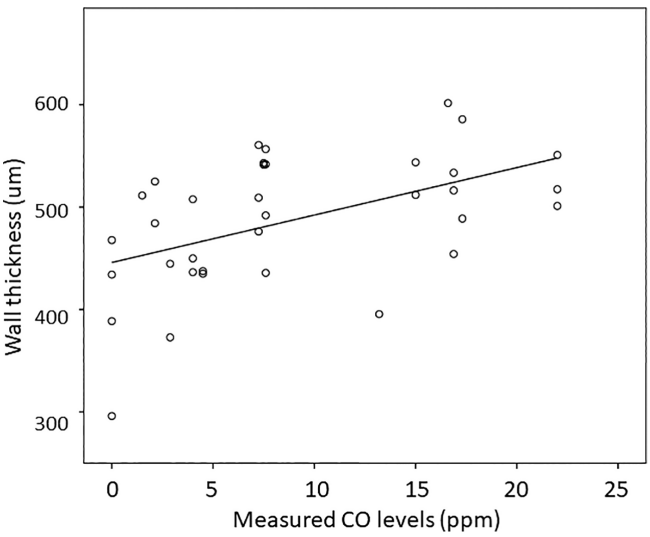

FIGURE 5 Ventricular wall thickness (μm) plotted against individual CO levels (ppm). $N = 35$. Linear regression line show slope of relationship ($R^2 = 0.256$, $p = .002$).

level across batches and heart weight ($R = 0.388$, $R^2 = 0.151$, adjusted $R^2 = 0.105$, $p = .048$).

A significant increase in ventricular septal thickness was found for the low ($p = .002$), moderate ($p < .0001$) and high ($p < .0001$) CO exposures compared to air (Figure 6).

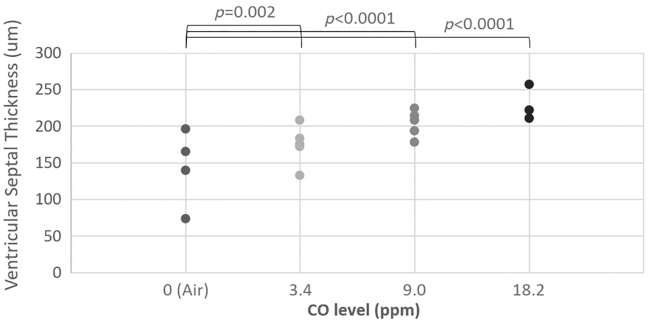

FIGURE 6 Ventricular septal thickness. $N = 17$. Significant differences ($p < .05$) were found between air versus low, air versus moderate, and air versus high CO exposures.

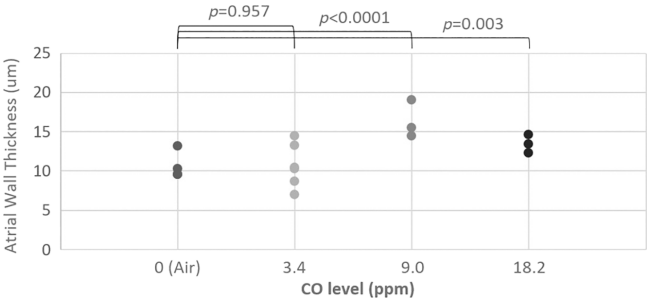

FIGURE 7 Embryo atrial wall thickness. $N = 16$. Significant differences ($p < .05$) were found between air versus moderate and air versus high CO exposure levels, but not between air versus low exposure.

A statistically significant difference in atrial wall thickness was found in the moderate ($p < .0001$) and high ($p = .003$) CO exposures compared to air (Figure 7).

Based on the average wall thickness being reduced for the high exposure compared to moderate exposure, a secondary analysis was conducted to assess whether this reduction was significant. For completion, the analysis compared low versus moderate, low versus high and moderate versus high exposures. Significant differences were found in the low versus moderate comparison ($p < .0001$), in the low versus high comparison ($p = .004$) but not in the moderate versus high comparison ($p = .02$).

4 | DISCUSSION

This study is, to our knowledge, the first to examine low-level CO exposure effects on cardiac morphology in the developing chick embryo. Here, we show that CO exposure impacts on cardiac development in the chick embryo.

We observe that there is a significant and dose-dependent impact on heart weight and structure (ventricular wall thickness), and that ventricular wall thickness correlates linearly with CO exposure. A similar correlation was not seen between CO dose and heart weight: heart weight was reduced with low and moderate exposures, but less so with the higher exposure, thus showing a curvilinear relationship. This could suggest that there is either a plateau in weight loss with higher doses, or that the weight loss might be offset by the increased wall/septum thickness. As we observed a change in atrial/ventricular wall thickness at moderate and high CO exposures, this remodeling might counteract the drop in heart weight, thus negating the heart weight change. However, further research is needed to confirm the mechanism underlying this observation and it remains unclear why heart weight was decreased at the low and moderate exposure levels of CO, but not at the highest exposure.

We observe a significant dose-dependent effect on ventricular-septal thickness. Furthermore, similar to the ventricular wall thickness, the atrial wall also showed a significant increase in thickness compared to air control for moderate and high CO exposures. It is, however, worth noting that there was no significant difference between moderate and high exposures for the atrial wall, which could mean that higher doses of CO do not have an increasing impact on atrial wall remodeling in the same way as it appears to have in the ventricular wall. Interestingly, we also observed an increase in ventricular-septal thickness, which was significant for all doses of CO. Air pollution has been found to cause ventricular septal defects in newborns as a result of maternal exposure to ambient levels CO and NO (Dadvand et al., 2011b). Our work demonstrates that the septum may be affected by CO in a dose-dependent manner from CO levels on par with ambient exposures through for example, pollution. This finding thus supports epidemiological findings in humans, indicating the vulnerability of this structure (Dadvand et al., 2011a; Ritz et al., 2002; Zhang et al., 2016), but cannot fully explain the etiology of such septal defects.

CO is known to influence physiology at low concentrations. For example, smokers are exposed to CO through cigarette smoke and typically have persisting elevated levels of CO of 6 ppm (exhaled air) or more (Middleton & Morice, 2000). An increase in exhaled CO to this level has been shown to reduce cerebrovascular reactivity and alter fMRI signal in healthy human non-smoking volunteers (Bendell et al., 2020). Similarly, low level exposure to CO in air pollution has been linked to a higher incidence of dementia (Chang et al., 2014), stroke (Hedblad et al., 2005; Maheswaran et al., 2005) and heart failure (Shah et al., 2013).

The foetus is particularly vulnerable to CO exposure. Carboxyhaemoglobin rises to higher maximal values that persist for longer in the foetus than in the mother (Aubard & Magne, 2000), and maternal exposure to CO, both chronic and acute is known to increase risk for several adverse developmental outcomes. Human epidemiological research links maternal CO exposure from air pollution and smoke to congenital heart defects (Dadvand et al., 2011a; Ritz et al., 2002; Zhang et al., 2016). However, air pollution and cigarette smoke are both a cocktail of toxic substances, and it is not possible to determine the exact contribution of CO on developmental issues with certainty without looking at CO exposure in isolation. The present study is the first to investigate impact of low-level CO on the developing embryo directly, that is, without the presence of other toxic gases or particles.

Congenital heart disease remains a common birth defect. Epidemiological studies continue to highlight the relationship between air pollutants and congenital cardiac disease, although there remains uncertainty as to how much each pollutant contributes. Our work supports epidemiological findings that the ventricular septum is vulnerable to CO, but also expands upon this, showing that CO exposure might also impact ventricular and atrial wall thickness. The implication of this finding remains unclear and should be further investigated, particularly as it is recognized that air pollution in general, including CO, is associated with higher risk of spontaneous abortion (Grippo et al., 2018).

Other studies have looked at chick development during exposures to much higher doses of CO. Baker and Tumasonis identified a "critical level" of CO concentration at 425 ppm for hatchability, at which point they observed no significant impact on egg weight, which concurs with our findings, nor congenital abnormalities, which were not formally assessed in our study (Baker & Tumasonis, 1972). The authors observed no effects when exposing eggs to <100 ppm, although no detailed investigation of cardiac development was done in the study (Baker & Tumasonis, 1972). Indeed, we observed no effect on viability for any of our exposure levels. In a subsequent study (Baker et al., 1973), the authors observed increases in hepatic oxidase enzymes in older embryos exposed to CO, which could represent an adaptation to hypoxia. It should, however, be noted that these levels of

CO are so high that direct comparisons with the current study are not feasible.

This study did not look at mechanisms, and so we cannot at present determine how CO causes the observed effect. Furthermore, CO is known to impact several important physiological pathways, meaning that there are several possible underlying mechanisms for the observed effects. In the next three paragraphs, some of these potential mechanisms will be discussed.

CO overexposure is typically associated with hypoxia (Penney et al., 2010), and organs with a high oxygen requirement, such as the heart and the brain, may be especially susceptible to the effects of CO through this mechanism (National Research Council, 2010; Townsend & Maynard, 2002). Decreased oxygen delivery due to CO exposure can be compensated to some degree by e.g. increases in cardiac output and extraction of oxygen, but these mechanisms may be insufficient to fully restore oxygen delivery to the tissues. Development is a dynamic process, in which the embryo shows a high degree of plasticity. It is possible that an attempted compensatory increase in myocardial contractility in our CO-exposed embryos may be linked to the observed dose-dependent increase in ventricular wall thickness. Indeed, hypoxia at 10% O_2 has been shown to cause hypertrophy in cultured neonatal rat cardiac myocytes, accompanied by an increase in hypoxia-inducible transcription factor-1 (specifically, HIF-1α) mRNA and protein levels (Chu et al., 2012). HIF-1α regulates the body's cellular and developmental response to hypoxia. Studies have shown that CO can increase protein levels of HIF-1α (Choi et al., 2010; Rosenberger et al., 2007) as well as its isoform HIF-2α (Wiesener et al., 2003), promoting vascular endothelial growth factor (VEGF, an angiogenic factor) production (Choi et al., 2010). It must, however, be noted that the above studies used non-gaseous CO-releasing molecules (Choi et al., 2010) or gaseous CO at levels that were orders of magnitude higher (1000 ppm) than those in the current study (Rosenberger et al., 2007; Wiesener et al., 2003). As level of HIF isoforms were not assessed in the present study, we can only speculate as to their involvement in our findings, and we cannot yet with certainty determine to which extent hypoxia (and/or modulation of HIF-1α or HIF-2α) is the driver of pathophysiology development. However, the lowest levels of CO exposure in the current study were below common ambient air exposures in for example, urban areas and may be too low to induce a strong enough hypoxic state to cause pathophysiology. Indeed, these levels were certainly below those of the studies described above. Furthermore, importantly, chick embryos are tolerant to hypoxic exposures, with viability remaining unchanged for oxygen levels down to 15% for D9 embryos (Onagbesan et al., 2007). Thus, while we certainly cannot rule out hypoxia as a mechanism in the present study, it seems unlikely that it is the primary driver for the morphological changes observed herein.

CO exposure may also lead to oxidative stress through the effects of nitric oxide (NO). It is known that CO can increase inducible NO synthase (iNOS) expression, and iNOS expression may mediate myocardial damage (Rose et al., 2017). Cardiac dysfunction may be caused by inhibition of oxidative phosphorylation and CO binding to myoglobin (Rose et al., 2017). In vitro work in rats has shown that CO levels as low as 10.5 ppm may significantly increase NO levels (Thom & Ischiropoulos, 1997), through the modulation of nitric oxide synthase (Thom, Bhopale, et al., 2004). Work in the same model organism shows that NO can generate peroxynitrite, a potent free radical and oxidant (Ischiropoulos et al., 1996). This has been further described in studies on CO poisoning: after CO poisoning, NO-derived oxidants are needed for neutrophil adherence to the brain endothelium (Thom et al., 2001) and platelet–neutrophil aggregates and increased plasma myeloperoxidase, a biomarker of inflammation, is found in patients (Thom et al., 2006). It has been suggested that an immunological cascade involving oxidative species may be an underlying factor in delayed neurological sequelae after CO poisoning (Thom, Fisher, et al., 2004). NO may also alter hemodynamic stress, through its function as a vasoactive molecule, and it is known that cardiac wall mass can be affected by haemodynamic stress (Patterson & Zhang, 2010). Indeed, similar levels of CO as in the current study has been shown to alter vascular function in humans (Bendell et al., 2020).

CO may react with heme-containing proteins other than Hb. Such targets include myoglobin and neuroglobin, NO synthase, NADPH oxidase, cytochrome c oxidase, cytochrome P450 and reactive oxygen species such as peroxidase (for an overview, see Wu & Wang (2005). These heme-containing proteins are important for several physiological functions, from mitochondrial respiration to signal transduction. For example, a study in mice have shown that cytochrome c release is impaired in newborn mice exposed to levels of CO comparable with those in the present study, and that this is associated with neurodevelopmental impairment (Cheng et al., 2012). The toxicity of CO to mitochondrial function has been shown in humans (Alonso et al., 2003). CO may cause mitochondrial dysfunction through binding to cytochrome oxidase, and it may release NO and form peroxynitrite, thus causing further inactivation of mitochondrial enzymes and endothelial damage. It is possible that this could be amplified by further immunological responses. In a recent review, Almeida et al (2015) suggest that CO

affects mitochondrial quality control machinery through reactive oxygen species signaling. Downstream effects of CO poisoning and the potential related mitochondrial dysfunction may also induce inflammatory signaling pathways (Rose et al., 2017) While some studies suggest that low doses of CO protects mitochondria from oxidative stress, the effect of CO on mitochondrial function is still poorly understood (Almeida et al., 2015).

While the above mechanisms may play a role in the impact of CO on embryo development, further study is needed to identify which of these mechanisms are involved in the morphological changes observed herein. Additionally, future work needs to take particular care to unpick the role of CO as a teratogen in its own right, or whether the downstream effects of hypoxia are the root cause. Also, in order to assess the impact of CO on health, it is important to know what dose produces which effect, and if this is uniform across the population. This is particularly important during pregnancy, as there is evidence to suggest that foetal tissues may be more vulnerable to hypoxia than maternal tissues. Indeed, CO levels that are not toxic to the mother may still be toxic to the developing foetus (Aubard & Magne, 2000; Farrow et al., 1990; Tomaszewski, 1999). There also remains the possibility that CO impact is not immediate, but rather that delayed sequelae may occur—as suggested in studies on the cognitive effects of CO poisoning (Choi et al., 2021; Jasper et al., 2005; Sönmez et al., 2018).

4.1 | Limitations

This study is the first to look at low-level CO impact on the heart in the developing chick embryo. While we observe an effect of CO on heart weight and the thickness of the ventricular wall, ventricular septum and atrial wall, the method of exposure means that CO levels did fluctuate throughout the 10-day exposure periods (Table 1). It should be noted, however, that exposure levels remained close to target, and we are thus confident that the current findings truly reflect low-level exposure effects. Future studies, however, should endeavor to minimize variations in ambient gas composition. Questions also remain about the underlying cause of this effect as well as impact on other cardiac metrics. Future studies might wish to include assessment of effects on other morphological markers, as well as interrogate the mechanism (s) underlying this impact.

5 | CONCLUSION

Low-level CO exposure *in ovo* for the first 10 days of gestation impacts cardiac development in the chick embryo, with a similar pattern observed for both ventricular wall development, atrial wall development and ventricular septum development. Further studies are needed to fully elucidate the impact of low-level CO on cardiac development.

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CONFLICT OF INTEREST STATEMENT

None of the authors have any financial and competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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