The Chicken or The Egg? Sildenafil Therapy for Fetal Cardiovascular Dysfunction During Hypoxic Development: Studies in The Chick Embryo

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Chronic hypoxia in the gestating fetus is a common occurrence which invariably leads to a variety of adverse events such as premature delivery, pulmonary hypertension, intrauterine growth restriction (IUGR) and fetal/adult cardiovascular dysfunction. The pathology is most commonly recognised as a secondary outcome of preeclampsia or placentar insufficiency, both of which appear to be closely related to obesity (Huang *et al.*, 2014), with rising prevalence rates worldwide. Despite this, few effective therapeutic regimes are in place for the protection of both fetal growth and cardiovascular development in the setting of IUGR.

While sildenafil is currently widely recognised for its utility in neonatal care as a pulmonary arterial hypertension therapy, application of the phosphodiesterase (PDE) 5 inhibitor prenatally in chronic fetal hypoxia remains in its infancy. A trilogy of multicentre clinical trials is scheduled to conclude within the next year, with the ultimate purpose of confirming the efficacy of sildenafil in improving mortality rates and reducing serious adverse events in early onset IUGR with dismal prognosis (Ganzevoort *et al.*, 2014).

Prior knowledge implicated the ability of sildenafil to enhance placental perfusion as the central mechanism of action in which the drug combats both IUGR and the subsequent cardiovascular dysfunctions observed in new-borns and adults who were exposed to chronic hypoxia during gestation. However, Itani *et al*. (2017) set out in teasing apart this interaction to elucidate whether the

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cardiovascular effects observed were solely as a secondary consequence of augmented placental perfusion, or if sildenafil itself might impact directly upon the fetal heart and vasculature.

Indeed, the chick embryo serves as a near ideal model for this purpose, as there is complete disconnect from the maternal system, allowing for quantification of fetus-specific effects in isolation. The authors built upon previous research in the area by inducing a hypoxic state in the fetus prior to initiation of intervention, at a developmental stage equivalent to when IUGR is generally identifiable in humans, rather than commencement of both concurrently (Itani *et al.*, 2016). The results demonstrated a reduced cardiac oxidative stress and improved vascular function in hypoxic embryos in response to sildenafil intervention. Broadly speaking, Itani and colleagues utilized an appropriate *in ovo* model of embryonal development to outline a novel role for sildenafil in the prevention of hypoxia-induced cardiovascular dysfunction.

Chick embryos were exposed to hypoxic conditions from day 1 until day 19 where they were sacrificed by decapitation. Treatment with sildenafil began at day 13 of incubation, which was equivalent to 25 weeks of gestation in human pregnancy, a period when fetal growth restriction is reliably diagnosed. This was a significant advantage of this study design, as previous studies began therapy at the onset of hypoxia, not giving the fetus sufficient time to adequately develop hypoxic growth restriction. Therefore, administering sildenafil therapy following established fetal hypoxia allowed for a greater translational study design. Dosing of sildenafil was determined based on data available from previous human and animal studies as no studies have currently assessed the dose-response relationship of sildenafil therapy on hypoxic growth restriction in the present model. It was suggested that a dose of 4mg/kg/day is clinically and scientifically relevant in humans, based on previous literature. Although, there seems to be insufficient evidence to definitively implicate this dose.

Itani and colleagues successfully created a chick embryo model of IUGR, evidenced by an increase in fetal hematocrit, asymmetric fetal growth, and increased oxidative stress in the heart and vasculature. They assessed IUGR in chick embryos grown in four different conditions: normoxic

(21% O2, N=11), hypoxic (14%, N=10), and normoxic (N=11) and hypoxic (N=10) with sildenafil therapy at day 13. When compared to the normoxic embryos, embryos grown in hypoxic conditions had decreases in heart, lung, liver, and kidney mass that were proportional to the decrease in body weight. Interestingly, brain weight did not follow the same trend and was proportionally larger when grown under hypoxic conditions. Hypoxic conditions increased the protein expression of pro-oxidant molecules 3- nitrotyrosine (3-NT) and 4-hydroxynonenal (4-NHE), while reducing the antioxidant molecules superoxide dismutase (SOD), catalase, and nitric oxide (NO) species. In addition, hypoxic embryos expressed significantly increased levels of PDE5 in the heart than the normoxic embryos. In the vasculature, hypoxic incubation reduced the sensitivity of vessels to acetylcholine-induced relaxation, due to NO-independent mechanisms. Moreover, treatment of the hypoxic embryo with sildenafil successfully attenuated increases in 4-NHE to normoxic-comparable levels, although the same effect was not observed with 3-NT in the heart. Finally, intervention prevented the increase in cardiac PDE5 expression, augmented expression of glutathione peroxidase, normalized cardiac NO bioavailability and restored peripheral vasculature endothelial function. The overall mechanisms through which sildenafil contributed to fetal cardiovascular protection in the model are outlined in Figure 1.

Overall the study was well designed and conducted with few limitations. As previously mentioned, given the dearth of previous literature, the dosing of sildenafil seemed appropriate; however, additional assessment with varying doses would have provided greater insight into its effects on the cardiovascular system. Additionally, although differences were detected, a larger sample size may have provided more power to detect differences between the treatment and control groups, perhaps regarding the pro-oxidant 3-NT and antioxidants, as fewer embryos were assessed for these markers. It may also be worth noting that embryos were not allowed to fully come to term, with termination at day 19 of 21, an aspect of the design which may indeed have hindered the authors in their ability to detect intervention-mediated alterations. Although all groups were sacrificed at the same time point, this limits the clinical translational capacity of the results as we remain ignorant to the effects of sildenafil in the terminal stages of uterine growth.

Delineating the specific mechanisms surrounding sildenafil-mediated attenuation of adverse cardiovascular and growth-restrictive events in a chronically hypoxic fetus has several important clinical implications. The importance of isolating the contributory roles of the fetus and placenta, respectively, lies in the potential for greater specificity of treatments upon recognition of IUGR. In addition, this study provides insight into potential bioschemical endpoints for fetal treatment of IUGR. By determining the mechanistic effect of sildenafil, the clinical dose may be able to be adjusted on a case-by-case basis, to achieve development of organ systems that give each fetus the best chance at survival. Since every fetus will not respond to treatment in the same fashion, this study will allow for a more guided therapeutic approach that optimizes the successful development of individual organs and organ systems without sacrificing peripheral systemic development.

As alluded to earlier, there is a lack of pharmacokinetic data on sildenafil therapy dosing in the chick model of IUGR. As a result, the authors herein applied a dose of 4 mg/kg/day, which has been previously reported as the upper limit in a clinical context. It is possible that a different dose may provide further insight into the mechanism of sildenafil treatment, which might alter the current therapeutic approach to managing IUGR in the clinical arena.

Although the chick embryo model was indeed the ideal model to determine the independent contribution of the fetus and placenta, it may be of interest to progress to a model with greater similarity to the human system. The authors have herein outlined several cardiac and vascular markers of sildenafil-mediated fetal health. These markers could be monitored in a porcine model of the disease state, which would allow for the addition of postnatal cardiovascular function assessment.

In human studies, sildenafil treatment was initiated as early as 25 weeks upon recognition of IUGR based on low predicted abdominal circumference and estimated fetal weight (von Dadelszen *et al.*, 2011). Given the 21-day gestation period of a chicken the initiation of treatment on day 13 is equivalent to week 25 in the human system and is therefore translationally appropriate, especially given the difficulty in estimating the fetal weight within a contained chicken egg. Therefore, the timeline for intervention in this study was in line with the earliest current recognition of IUGR

clinically. It may be of clinical value to determine whether earlier intervention would further improve the fetal microenvironment and subsequent outcomes in IUGR.

Future studies aimed at early interventions could translate to clinical therapeutic treatment in pregnancies at risk of developing IUGR, rather than initiation of treatment once the fetus is already exposed to a growth restrictive environment. Coupled with dose determination, the ideal timing of intervention could greatly contribute to the current clinical approach to IUGR management. This may ultimately result in improved outcomes as well as better understanding of sildenafil treatment mechanisms.

In summary, Itani and colleagues have outlined a putative role of sildenafil therapy in attenuation of hypoxia-induced fetal growth restriction. The authors describe a plethora of cardiovascular biochemical alterations in response to the intervention, including those involved in cardiac oxidative stress and vasculature relaxation (Figure 1). This study lays the foundation for further preclinical investigation and adds support to the trilogy of clinical trials currently underway which are investigating the utility of sildenafil in IUGR with dismal prognosis.

Ganzevoort W, Alfirevic Z, von Dadelszen P, Kenny L, Papageorghiou A, van Wassenaer-Leemhuis A, Gluud C, Mol BW & Baker PN. (2014). STRIDER: Sildenafil Therapy In Dismal prognosis Early-onset intrauterine growth Restriction--a protocol for a systematic review with individual participant data and aggregate data meta-analysis and trial sequential analysis. *Syst Rev* **3,** 23.

Huang L, Liu J, Feng L, Chen Y, Zhang J & Wang W. (2014). Maternal prepregnancy obesity is associated with higher risk of placental pathological lesions. *Placenta* **35,** 563-569.

Itani N, Skeffington KL, Beck C & Giussani DA. (2017). Sildenafil therapy for fetal cardiovascular dysfunction during hypoxic development: studies in the chick embryo. *J Physiol* **595,** 1563-1573.

Itani N, Skeffington KL, Beck C, Niu Y & Giussani DA. (2016). Melatonin rescues cardiovascular dysfunction during hypoxic development in the chick embryo. *J Pineal Res* **60,** 16-26.

von Dadelszen P, Dwinnell S, Magee LA, Carleton BC, Gruslin A, Lee B, Lim KI, Liston RM, Miller SP, Rurak D, Sherlock RL, Skoll MA, Wareing MM, Baker PN, Research into Advanced Fetal D & Therapy G. (2011). Sildenafil citrate therapy for severe early-onset intrauterine growth restriction. *BJOG* **118,** 624-628.

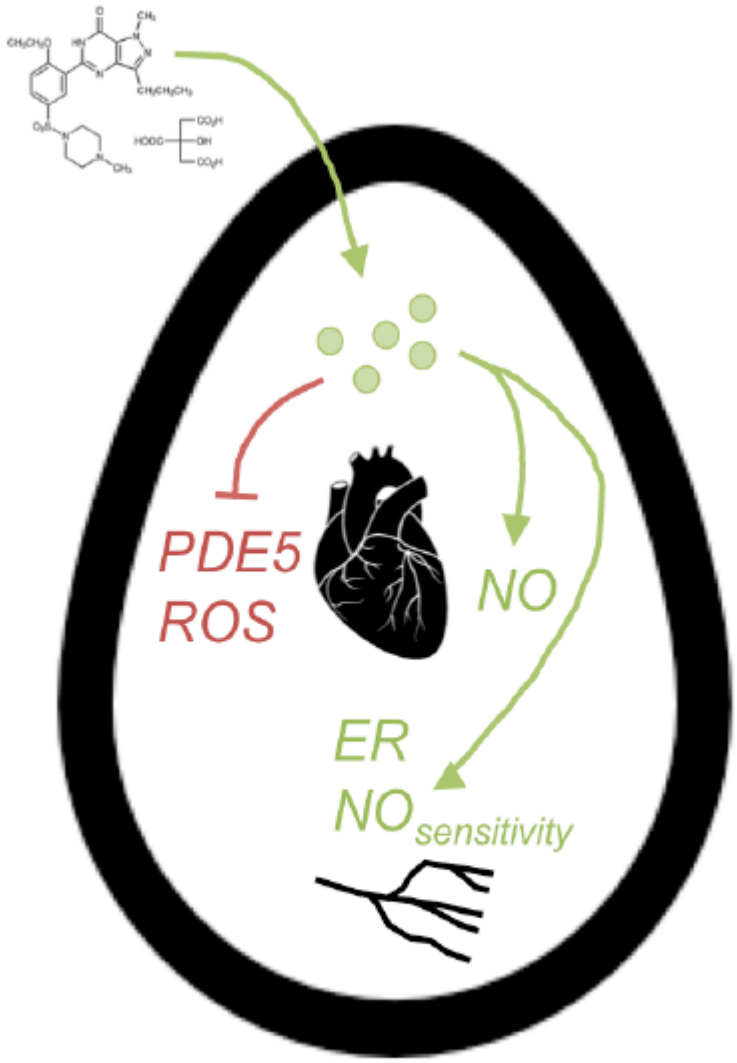

Figure 1. Sildenafil exerts cardioprotective effects in the hypoxic gestating chick embryo. Schematic depicting the mechanisms through which sildenafil impacts directly on foetal development, independently of effects on placental perfusion - as uncovered by Itani *et al. Phosphodiesterase type 5, PDE5; reactive oxygen species, ROS; nitric oxide, NO; endothelial relaxation, ER; nitric oxide sensitivity, NOsensitivity.*