UNIVERSIDAD SAN FRANCISCO DE QUITO

Vasoreactivity of the mesenteric artery in the chicken embryo: a study of the effects of chronic in ovo hypoxia in 15-day-old and 19-day old chicken embryo

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Tesis de grado presentada como requisito
para la obtención del título de Doctor en Medicina

Quito, noviembre del 2008

Dedication: I dedicate this work…

To myself, Christina, for taking the leap of faith and leaving everything I knew and was comfortable with and coming into a brand, new world… and not running away from it.

To my parents, for giving me their undying support through everything, for not overrunning me with their "I told you so's" when I make mistakes, for helping me grow as an individual.

To my brother, whom I hope to reunite with in friendship despite having him so near

To all the men in my life (please let's not make lists), whom, in tragedy and joy, always give me hope for love… and lust. Here's looking at you, kid. I mean you, Andrei.

To my friends, you were my family in Ecuador. You all have no idea.

Acknowledgements

I want to thank those crazy kids at AZM, for giving me a wonderful summer of fun and research experience!

Dr. Rob Moonen, I can find ways to thank you in a thousand languages. You know, without you, I would have graduated in the year 2020. Let's start with one phrase for now: Dank u wel!

Dr. Eduardo Villamor, for allowing me to work on his team in Maastricht and giving me the guidance I needed. And, of course, I must express my gratitude for lending me his lovely, pink bicycle, Lucy.

Let's give it up for God, y'all!

Resumen:

Teorías recientes se han desarollado sobre la disfunción endothelial como un factor causal en la patogénesis multifactorial en enterocolitis necrotizante (ECN). Hipoxia crónica, siendo uno de los patologías prenatales más comunes, se asocial con un riesgo augmentado de ECN y se ha demostrado en estudios previos a alterar la vasoreactividad de las arterias. ***Objetivos:*** A probar la hipótesis de que la hipoxia crónica pede causar la disfunción endothelial en la arteria mesentérica (AM) en embriones de pollo de 15 y 19 días. ***Metódos:*** Analizamos la vasoreactividad a los agonistas de contracción y relajación en el AM de embriones de pollo de 15 y 19 días (tiempo de incubación es 21 días) que han sido expuesto a O_2 a 15% desde el día 0. ***Resultados:*** La hipoxia crónica disminuye la masa corporal de embriones de 19-días. La respuesta a agonistas de contracción mejora con maduración basada en edad. Relajación independiente del endotelio es aumentada en la AM hipóxica especialmente en la pre-contracción con norepinefrina. Relajación dependiente del endotelio no es afectado por la hipoxia y no demuestra cambios significativos con la edad (P<0.05). ***Conclusiones:*** Exposición a la hipoxia crónica no causa la disfunción endotelial en la AM ni hubo cambios significativos relacionados al desarrollo del embrión. El papel del factor hiperpolarizante derivado del endotelio en la relajación dependiente del endotelio es enfatizado en la vasodilatación por acetilcolina con pre-contracción por norepinefrina. Se necesitará más estudios para investigar los effectos crónicos de hipoxia para confirmación de nuestros resultados.

Abstract:

Background: Recent theories have been developed on endothelial dysfunction as being an inciting factor in the multifactorial pathogenesis of necrotizing enterocolitis (NEC). Chronic hypoxia, one of the most common prenatal insults, also has an increased risk of NEC and has shown in prior studies to cause altered vasoreactivity of the arteries. ***Objectives:*** To test the hypothesis that chronic hypoxia causes endothelial dysfunction in the mesenteric artery (MA) of 15- and 19-day chicken embryos. ***Methods:*** We analyzed the vasoreactivity to contractile and relaxant agonists in the MA of 15-day and 19-day chicken embryos (incubation time is 21 days) that have been exposed to 15% O_2 since day 0. ***Results:*** Chronic hypoxia reduces body mass of 19-day embryos. Response to contractile agonists improves with age-dependent maturation. Endothelium-independent relaxation is increased in hypoxic MA. Endothelium-dependent relaxation is not reduced in hypoxic MA but shows increased response with precontraction to norepinephrine over K+ solution. ***Conclusions:*** Exposure to chronic hypoxia did not cause endothelium dysfunction in the mesenteric artery nor were there significant changes related to development. The role of endothelium-derived hyperpolarizing factor in endothelium-dependent relaxation is highlighted in acetylcholine-induced relaxation with precontraction to norepinephrine. Further investigations about chronic hypoxic effects are warranted.

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1. Introduction/Overview:

Necrotizing enterocolitis (NEC) is one of the most serious emergencies that a neonate can face. In the Western world, the incidence of NEC can reach up to 1 to 3 cases per 1000 live births and a higher incidence of 3-7% among preterm and low birth weight infants with the requirement of surgical intervention in severe cases and overall mortality from 20% to up to 50% (22). Although NEC has been studied for many years, but the etiology remains elusive. However, many different hypotheses about the pathogenesis are described in the last years. It is now believed that interaction of multiple factors and the overall inflammatory effect on the susceptible host cause mucosal injury. The most common risk factors associated with the condition are prematurity, low birth weight, enteral feeding, and periods of birth asphyxia among others; however, low gestational age and low birthweight are the most significant, associated with the prevalence of 10% of cases. With the advancements in successful management of preterm and low birth weight infants, the better survival rate resulting in an increased of number of at-risk neonates severs as a reason for the high incidence of NEC today.

As mentioned before, the pathogenesis of NEC has included many hypotheses. The diving hypothesis was one of the first and, eventually, the standard explanation in the previous decades. In diving mammals, the blood circulation as the mammal descends is rerouted to the more vital organs, including the brain and the heart. Accordingly, asphyxia of the neonate causes a decrease in intestinal blood flow through sustained adrenergic stimulation and associated damage to the intestinal mucosa. One of the more famous

demonstrations supporting the "diving reflex" was one conducted by Touloukian and his team, in which they were able to reproduce mucosal injury similar to NEC in asphyxiated piglets in the 1970´s (5). However, there have been a series of studies questioning the validity of hypothesis. Prematurity and not asphyxia is the most consistent risk factor for NEC. In a case-controlled study by Wilson et al. (1), it was shown that the episodes of hypoxia are at same frequency in both infants with or without NEC. In the infants with NEC, other manifestations that are associated with asphyxia, including acute tubular necrosis and intraventricular hemorrhage, have failed to show correlation. Also, NEC rarely does occur in the first neonatal week, so acute asphyxia as a causative factor is unlikely (2). Lastly, the autoregulatory escape mechanism prevents low blood flow and high resistance in the intestinal vasculature for more than a few minutes, which was demonstrated in a study conducted by Nowicki et al. in piglets (3,4). Because of the growing number of arguments, this theory has been discarded as the principal mechanism but may perhaps have a secondary role in pathogenesis.

The immaturity of several components of the gastrointestinal system has also been attributed to pathogenesis of NEC. . For example, motility and digestion has been shown to be underdeveloped in preterm infants and could contribute to the intestinal damage because of the prolonged transit time and the accumulation of luminal contents. Intestinal motility maturation occurs within the third trimester; consequently, the premature gut system is more disorganized although it can improve with enteral feedings (12). Other factors besides prematurity that can also hinder gut motility include fetal hypoxia and perinatal asphyxia, which can lead to consequent intrauterine growth retardation and

reduced postnatal intestinal motility (14, 15). And so, the accumulation of partially digested nutrients within the immature gut will allow a favorable environment for bacteria to flourish. Due to the problem of dysmotility, there will be a reduced bacterial clearance within the intestine, which can lead to overgrowth; the extended exposure of bacterial antigens may induce inflammatory response (12). Suboptimal digestion and absorption are also encountered in the preterm infant. The digestion of substances within the lumen can be considered as a innate line of defense against pathogens and other noxious elements. When digestion is impaired, the intestines are more vulnerable to the entry of pathogenic factors that can cause mucosal injury (12).

The immaturity of the epithelial barrier of the intestine in both the structural and biochemical aspects may also cause problems that lead to NEC. The intestinal lining is made up of tight junctions that maintain the connection between the epithelial cells and are key for regulation of absorption and secretion. However, according to Lebenthal and Lebenthal, the barrier is only fully effective later in the third trimester of gestation at at least 26 weeks (16). Any factors that con disrupt the integrity of the structural barrier, including epithelial repair, will lead to hyperpermeability of the intestine and allow for injury to occur, i.e. bacterial translocation. As reviewed by Lin et al., there is an increased incidence of intestinal hyperpermeability associated with preterm infants, especially higher with those with NEC; furthermore, there is even implication that apoptosis or necrosis of intestinal cells in an already delicate immature gut could be the first step of the cascade in the pathogenesis, as demonstrated with subsequent loss of epithelia in resections of gut (13). Also, since the intestine of a preterm infant can not selectively

control its functions of fluid secretion and nutrient absorption, it may be unable to wash out the toxins and pathogens in the lumen as part of its innate mechanism of defense. The intestinal barrier is also regulated by endogenous substances such as nitric oxide (NO), prostaglandins and epidermal growth factor (EGF). Prostaglandins help increase the resistance of tight junctions within the epithelial lining of the intestines, preventing hyperpermeability (13); according to Warner and Warner, EGF is important in the repair of cells and has been shown to be decreased in experimental models with NEC (13,17-18). Nitric oxide seems to have a dual role in the function of the intestinal barrier depending on the amount present. At low levels, NO is useful in vasodilation and maintaining an adequate mucosal bloodflow and, consequently, integrity. When at elevated, sustained levels, NO produced by enterocytes has a deleterious effect on the intestinal epithelium, causing injury and disruption through membrane oxidation and induction of apoptosis of the cells (13, 17)

Paneth cells, which are located in the intestinal crypts, are specialized enterocytes that secrete antimicrobial peptides, as also with the typical absorptive enterocytes, in addition to other substances, including lysosymes and phospholipases. The defensins and cathelicidins are the main antimicrobial peptides secreted and are particularly useful in determing the presence of commensal and pathogenic flora within the intestine; Paneth cells predominately secrete α-defensins, while the epithelial gut cells secrete β-defensins. The levels of the enteric defensins increase with gestational age and so will be relatively low in the preterm infants; Paneth cells have shown irregular patterns of expression in their defensins with active secretion and have a possible pro-inflammatory role in NEC

patients with pathologic findings of resected specimens (19). As another secondary reinforcement of the epithelial integrity, goblet cells secrete a thick mucin layer, which also aids trapping bacteria to be cleared out and enzymes to promote digestion. Within the mucin layer is IgA, an important adaptive defense mechanism against pathogenic bacteria in the gut through blocking their adherence to epithelium; thus, goblet cells in the preterm infant would not be able to produce an adequate mucin layer and would have reduced IgA levels, making the intestine more susceptible to bacterial translocation and mucosal injury (17).

There has been increasing evidence in studies conducted that intestinal bacteria, pathogenic and commensal, may have a role in the pathogenesis of NEC. In utero, the fetal intestine is bacteria-free and only exposed to sterile amniotic fluid; afterwards, the typical breastfed infant with have its gut rapidly colonized by normal gut flora, mainly bifidobacteria. Coincidently, there are no reported cases of NEC during this fetal period, and most cases present from the second neonatal week and onward (12-13, 17, 20). Also, the segments of intestine most commonly affected in NEC are the ileum and the colon, which happen to have the highest bacterial load (20). The implication of bacteria in the development of NEC is also demonstrated by occasional outbreaks and the isolation of the pathogens, including Clostridium, Klebsiella Staphylococci, E. coli and rotavirus; such outbreaks have improved with better general preventive measures to reduce infection (13, 20). Commensal flora, on the other hand, provide a natural protection of the intestine by several mechanisms: creating a physical barrier that prevents pathogenic bacterial adherence, sustaining a low pH from fermentation of substrates within the gut,

maintaining homeostasis in regards to pro- and anti-inflammatory pathways, and regulating on a molecular level the general functions of the intestine (12-13, 17, 20).

In reviewing the many pathogenic bacteria that have been associated with NEC, no single pathogen has been identified despite a long-standing association with the disease. However, there have been studies, notably by de la Cochetiere et al in 2004, that abnormal and early colonization of Clostridium perfringens has been associated with an increased incidence of NEC (12-13,17, 20-21). On the contrary, it seems that the disruption of the process of normal floral colonization of the intestine with abnormal bacteria in the neonatal intestine has a more predominant role in the development of NEC. The normal gut flora of a breastfed, healthy infant consists of bifidobacteria and lactobacilli, gram positive organisms; the preterm infant that has been in a NICU has less gram positive flora and more species of Enterobacter, Enterococci, Staphylococci and Clostridium as demonstrated by fecal samples (17). The preterm neonate has a higher susceptibility to improper and pathogenic colonization; because of his/her fragility and immaturity, the premature infant may not the immediate enteral feedings with breastmilk, may necessitate broad-spectrum antibiotics for other diseases and resides in a fairly sterile environment but with the ever-present exposure to nosocomial bacteria. Once the more pathogenic bacteria are present and colonizing within the intestine, the aforementioned dysmotility and stasis among with other factors promote bacterial overgrowth, leading to translocation to extraintestinal sites and injury and inflammation of the gut that can cause NEC. Despite this seeming straightfoward cascade of events ending in sepsis and necrosis of the intestine, only up to 48% of the cases of NEC present with positive blood cultures; thus,

the role of bacteria in the pathogenesis may as well be one the many factors that can contribute to the development of NEC (12). Its importance in comparison, however, may be of more significance. In a study conducted by Musemeche et al., experimental rat models with germ-free intestines were used in which three pathogenic factors of ischemia, substrate and bacteria were compared in induced NEC; bacterial colonization was the most important in intestinal necrosis (12, 22).

It has been deemed necessary to also mention the evidence that supports the nonspecific role of the immature innate immunity of the newborn. There seems to be disequilibrium in the fragile dichotomy of the pro-inflammatory and anti-inflammatory mechanisms of the neonate, whose immune system has just started to develop; the pathogenesis of NEC is likely to favor an aberration in the control of the pro-inflammatory events (12, 22). When there is cellular injury to the intestine that can be due to hypoxia, infection or other sources, the intestine responds and undergoes an acute inflammation, releasing inflammatory mediators and attracting inflammatory cells to the site of injury. Increased levels of inflammatory cytokines, such as interleukins and platelet-activating factor (PAF), has been associated with NEC (12,20, 22-25); for example, Caplan and colleagues have found a higher concentration of PAF in the stool samples of newborns with NEC, indicating increased production at a local level (25). Although the response against the harmful factors is supposed to protect the body, an unfortunate side effect of inflammation is the release of substances by the inflammatory cells, i.e. leukocytes secreting enzymes and oxidizing molecules, which can further damage the intestine. The resulting mucosal

injury will leave the intestine more susceptible to invasion by the pathogens and will propagate further inflammation (12).

Other studies, including that of Nanthaker et al., have also indicated that the intestinal gut of a preterm neonate can create an exaggerated pro-inflammatory response to the antigenic components of pathogens in comparison to the gut of the term infant (26). This may be the result of an abnormal or an immature interaction between Pattern Recognition Receptors (PRRs) on the host intestinal cells and the Microbial Associated Molecular Patterns (MAMPs) on the bacteria (12, 22). This kind of bacterial-host interaction participates and contributes to the inflammatory cascade; apoptosis; the nonapoptotic pathway; and the NF-κB signaling pathway, which, as a transcription factor, has its own important role in the synthesis of mediators of the inflammatory cascade (12,20,22). One PRR protein in particular, the human Toll-like Receptor 4 (TR4), has been especially taken into consideration. The TLR4 is a receptor for the lipopolysaccharide, which is a cell wall component of Gram negative bacteria and is especially pathogenic; once the TLR4 is stimulated, one of the events that ensue is the activation of the pro-inflammatory NF-κB pathway (12). Human TLR4 is known to be expressed in the fetal intestinal cells and is much more abundant when compared to the human adult intestine, which shows strong implications for why NEC is a common disease of the pre-term neonate (27). Also, in another important study by Jilling et al., it was demonstrated that the experimental rat models have decreasing levels of TLR4 mRNA expressed when fed with breastmilk over time. More interestingly, these levels of TLR4 increase along with inducible NO synthase and cytokines with formula feedings, hypoxia and cold-stress; thus, the proliferation of

expressed TLR4 in the setting of cellular injury to the intestine may encourage the pro-inflammatory process that can lead to NEC (28).

Although there is much evidence for the overstimulation of pro-inflammatory mechanisms behind the causes of NEC, there has been data collected on the lack of regulatory responses that can also contribute to the disease. According to Claud and colleagues, it has been shown that in rat models that immature enterocytes have a decreased expression of the IκB proteins, which are key inhibitory proteins for the NF-κB pathway (29). The decreased inhibitory control, in turn, will cause an exaggerated inflammatory response to pathogens, and in this case to flagellated organisms such as E. coli and Salmonella (29). Conversely to insufficient feedback mechanisms that end overwhelming pro-inflammatory pathology of the intestine, it has also been implicated that an inadequate inflammatory response may also be just as damaging. As mentioned before, inflammation is the first response of the body to any injurious agent; if there is nothing to help fight off the pathogens, the consequences would be abnormal bacterial colonization and possible NEC (12). Also, neonates and, especially premature neonates, have a delayed and immature immune system with suboptimal function in cytokine production, efficacy of inflammatory cells, and antibody responsiveness (20). In a study done by Zeng and colleagues, they concluded that the pro-inflammatory pathways and apoptic pathways of intestinal epithelia are closely associated, experimenting with the interaction of bacterial protein component flagellin with toll-like receptor 5. When the pro-inflammatory pathways are dysfunctional, like in preterm neonates perhaps, the flagellin that usually activates nonapoptotic mediators will instead induce apoptosis (29). This can also support

what has been seen in animal models with histopathologic resections, in which apoptosis is not only abundant among the intestinal cells but may also be the first step in the development of NEC (13, 30). And so, neonatal inflammation as a possible cause of NEC seems to be more of an imbalance of pro-inflammatory and anti-inflammatory pathways and overall results.

Genetic abnormalities have also been implicated with the development of intestinal disease, such NEC. It has already been established that genetic mutations from mother or fetus can cause inflammatory pathway dysfunction and deviations from normal renin-angiotensin function can lead to preterm labor with resulting prematurity and intrauterine growth restriction, respectively; both of these results are also strong risk factors for NEC (13, 41). Although intestinal circulation will be reviewed later, it is important to note genetic variations that cause vascular abnormalities, of which two are worth mentioning. A recent study on genetic mutations for the allele encoding for the vascular endothelial growth factor shows that such mutation may also be a risk factor for NEC (42). There have been many studies conducted which demonstrated decreased levels of arginine in infants with NEC; arginine, being a precursor for enzyme nitric oxide synthase to produce the potent vasodilator, and its deficiency can also be linked to polymorphisms for the rate-limiting enzyme carbamoyl phosphate synthetase, which is a precursor for L-arginine in turn (43, 44). Moonen and colleagues have demonstrated that an increased incidence of NEC in premature infants is associated with an increased number of polymorphisms for carbamoyl phosphate synthetase (44). Because of the significance of inflammation in the presentation of NEC, it may be logically assumed that gene irregularities within the

inflammatory pathway may exist. Nevertheless, few reports have been written to confirm as such, and many more have shown no association of genetic mutations of inflammatory signaling with NEC (13, 45-46). This reinforces the idea that inflammation is more of a consequence of NEC instead of its inciting factor (13).

Aberration of the intestinal circulation of the neonate also has its own role within the pathogenesis of NEC although the importance of it is still debated. In the case of term infants with NEC, the disease appears during the first few days, and, more importantly, there have frequently been prior antecedents that compromise the intestinal blood supply, including intrauterine growth retardation, ischemic-hypoxic events, polycythemia, and congenital heart disease (1, 13, 31). Preterm infants, as stated previously, develop NEC later in the neonatal period, making an acute perinatal event that affect circulation more doubtful; NEC appears to have a stronger link to inflammatory pathway dysregulation and abnormalities in bacterial colonization of the gut, according to Neu et al (13, 32). Regardless, although the "diving reflex" has been disproven by multiple theories, it has been suggested by Reber et al. that the gut mucosal ischemia that was seen in asphyxiated piglets by Touloukian et al. was likely to have been caused by means of reperfusion damage (5,6). Moreover, when reviewing the cardiovascular physiology that maintains a healthy intestine, the regulation of the blood flow in bowel necrosis and inflammation still seems noteworthy. The intestine during the fetal period is relatively inactive until after birth and within the next few days, when it becomes the site for nutrient absorption and has a high rate of metabolism because of its postnatal activity. The intestinal growth and development is extensive with intestinal cell proliferation and innervation in pig models

especially by 10 days, as noted by Widdowson and colleagues in the 70's (33). In order to keep up with the metabolic and oxygen demands of the intestine, there has to be critical changes in the vasculature that nourishes it.

The modifications of the circulation from a fetal to a newborn state are logically thought to adapt to a now active intestine, and what has been ascertain in experimental animal models is that there is a considerable decrease in basal vascular resistance in the neonatal period (6, 12). It should be kept in mind, as Nowicki and colleagues have studied, that the connection between increased metabolic rate and proportional increased blood supply as not as strong as believed; and so, there are other factors that may also contribute to such changes in vasculature (6, 34). Regardless, the basal vascular resistance of the gut is regulated by the following three mechanisms. The production of endothelial NO is important for vasodilation. The myogenic response is due to increased pressure upon the vessel wall and, in turn, induces vasoconstriction. The release of endothelin-1 can cause both dilation and constriction depending on the receptor to which it binds to; however, the global effect of endothelin-1 is to provide vasoconstrictive tone (6,12). The decrease is basal vascular resistance during the neonatal period is also significant in comparison to that of an adult; it has been shown that the drop in resistance is due to a large amount of NO released within the intestinal circuit and dominates over the vasoconstrictor effects of endothelin-1 (4). As the infant ages, the resistance increases slowly until it reaches adult values due to changes in the size of the intestine and concurrent dimensions of the blood vessels; and the vascular tone will then mainly be regulated through adrenergic nerve stimulation, which was present but immature during the neonatal period (3,6). And so, any

deleterious event that can upset the normal vascular physiology that the neonatal intestine undergoes may be an underlying cause for NEC.

As a result, current evidence seem to support the theory of endothelial dysfunction in premature infants, and its novelty appear to show future promise as one of the many etiologic factors in the pathogenesis of NEC.. There are several matters that can be encountered during the neonatal period that can bring about the loss of endothelial function. Ischemia-reperfusion events, platelet activating factor release along with other inflammatory mediators, bacterial translocation, intestinal stasis and mucosal disruption have all been associated with endothelial dysfunction; however, damage due to ischemia-reperfusion seems to have the strongest association with endothelial dysfunction as experimented by Nowicki (12, 35). In addition, a neonate has even more difficulty to regulate the intestinal blood flow when under cardiovascular stress, and so the rise in oxygen consumption from a stressed infant is not compensated with sufficient blood supply (20). In turn, the hypoxia of tissues results in an increased production of endothelin-1 within the intestinal circulation and cause secondary localized ischemia from the vasoconstriction (4, 6). More importantly, the release of NO for vasodilation is also reduced due to the initial ischemia-reperfusion damage with oxygen free radicals to the endothelium and has an aggregated effect upon the already compromised bowel. Since the newborn intestine relies mainly on the NO to counterbalance endothelin-1 and the myogenic response, a decrease effect of this mechanism will end with an exaggerated vasoconstrictive response from the other two. Reduced nitric oxide synthase and reduced arterioloar production of NO, which are both indicative of endothelial dysfunction, has

been shown in the human infantile intestine with NEC (36). In conclusion, an imbalance of vasodilatory and vasoconstrictive forces may also be a contributory factor in NEC, but further studies are needed to elucidate the mechanism behind endothelial dysfunction, which may require new experimental animal models besides piglets (6)

2. Literature Review:

As discussed previously, the theory of endothelial dysfunction as a crucial antecedent in the development of NEC is a fairly new explanation which requires further investigation. Thus, the three mechanisms of maintaining vascular resistance should be reviewed in more detail with the importance of endothelial NO and its function highlighted. The endothelial cell form of nitric oxide synthase (ecNOS) constituitively produces NO through the reduction of L-arginine. NO, as a gas, diffuses quickly to the vascular smooth muscle and binds to soluble guanylate cyclase, which makes cGMP; the cGMP reduces the intracellular calcium (Ca^{2+}) and relaxation ensues (6). The myogenic response is intrinsic to the vascular smooth muscle without the influence of neural factors and consists of a contraction in response to a mechanostimulus. It still remains to be elucidated how the stimulus is transduced to a response, but it is certain that the myosin light chains are phosphorylated, and the motor unit of actin and myosin filaments begins contraction (6). Endothelin-1 is a protein also made in the endothelium; and it is able to bind to ET_A, causing vasoconstriction, and ET_B, causing NO-mediated vasodilation. It is believed, as explained by Reber et al., that there is much more endothelin in the newborn period due to the angiogenesis that must occur to keep up with intestinal growth; the overall tone mediated by endothelin is vasoconstrictive (6).

As stated before, the production NO is important for the balance of vasodilatory and vasoconstrictive forces in the newborn endothelial intestine and not in older subjects. All three elements are especially responsive to the mechanostimulus of flow-dilation in young subjects; loss of the effect of NO causes an exaggerated response on the side of myogenic and endothelin mechanisms (47). NO also participates through other ways in maintaining vasodilation. Through NO interference of trimeric G- protein and receptor coupling, NO is able to decrease the binding affinity of angiotensin II and norepinephrine to their respective receptor. The end-result is an overactive vasoconstrictive response; these findings are only pertinent, once again, in the newborn intestine (6, 48). If there is endothelial damage that can cause dysfunction, the effect on the ET_B receptors that line the vasculature and which are coupled to NOS must also be considered (6). Reber and colleagues in her review emphasized the relevance of the endothelial dysfunction hypothesis because it reveals how the characteristics that would cause NEC participate in a physiologic regulation of the intestinal circulation and are distinctive in the newborn period. Also, the damage caused in the endothelium involves etiologic factors pertinent to the many of the mechanisms of NEC pathogenesis previously described. Morever, the pathology of NEC starts as localized damage to the mucosa that in hours becomes generalized; and as such, the vascular dysfunction may start in the smallest vessels and also generalize to the larger ones (6).

The chicks in the experimental group were incubated under chronic hypoxic conditions in order to create test subjects analogous to neonates with intratuterine growth retardation

due to similar disease conditions. As mentioned previously, the common risk factors for the development of NEC are prematurity and low birth weight; however, a few studies have been conducted on the addition of intrauterine growth restriction to a preterm neonate and the incidence NEC. In general, the premature, small-for-gestational-age, or SGA, infant will have a higher incidence of morbidity and mortality than a preterm infant with an appropriate birth weight for gestational age (8). More specifically, the gastrointestinal pathology that includes anatomic, physiologic and metabolic aberrations is typical in cases of IUGR infants. Consequently, the incidence of NEC is increased in IUGR preterm infants in comparison to appropriate for gestational age, or AGA, infants and even younger preterm infants with similar birth weight (9). Basing upon evidence of the role of vascular compromise in the pathogenesis of NEC, the insufficient oxygenated blood supply during the development of growth-restricted individuals would also affect, and perhaps more severely, the gastrointestinal system and, thus, contribute to an increased incidence of the condition. Other evidence has also supported the positive association of NEC in SGA individuals (11) In a study by Bernstein et al. in which almost 20,000 very low birth weight neonates were reviewed, IUGR was associated with a 1.27 times increased incidence of NEC (10). Thus, the experiment in the chicken model is to test and see if vasoreactivity is altered in premature subjects with growth retardation due to chronic hypoxia and its relevance to endothelial dysfunction; by simply incubating the chicken embryo in low oxygen conditions, it is possible to simulate a chronically hypoxic environment and obtain, as a consequence, intrauterine growth retardation (38).

The chicken embryo (*Gallus gallus*) as an experimental animal model has been employed successfully in the field of developmental biology because of its unique features. The investigation of cardiovascular physiology and pathology has particular relevance since the chicken embryo has a circulatory system similar to that of mammals. As stated in a letter by Ruijtenbeek, the chicken embryo has been used to study vascular tone; hemodynamic changes, including those that ensue after stressful events; and cardiovascular effects that result from genetic manipulation (37). The avian embryo has the choroallantoic membrane as its gas exchange mechanism, which is analogous to the placenta in mammalian embryos (39). What is distinctive in the transition to ex ovo life of the chicken is that the hatching takes place over the course of several days in contrast to the immediate change that from intrauterine to extrauterine life of mammals. Once the choroallantoic membrane cannot provide the oxygen required by the embryo around day 19, the chick carries out internal pipping; the beak enters the air cell of the egg, causing breathing (40). External pipping proceeds when the shell breaks, and the chick is able to breathe the air from the environment; the lungs develop adequately, and the hatching will occur (40). In the experiment at hand, it was important to use chick embryos that have not started the transition in order to reproduce a legitimate premature "birth" when handling the embryos. Otherwise, avian embryos are particularly useful in studying physiologic changes that occur in the ex ovo transition because of the prolonged process. Lastly, the chick embryo has an advantage over other models because, within the egg, the chick is not influenced by maternal physiopathologic factors during incubation. Also, studies that involve malnutrition and abnormalities in oxygen delivery like chronic hypoxia, which are

common etiologic factors in neonatal diseases, can be studied independently in the chick embryo (37-39).

There have been many studies on the vasoreactivity of arteries chicken embryos in the past, of which have shedded some light on endothelial dysfunction. The vascular reactivity of the intrapulmonary arteries was compared in 19-day and 21-day old chicken embryos, in other words, from in ovo to ex ovo life. The pulmonary arteries have increased contractions proportional to age to KCl, endothelin-1 and U-46619; on the contrary, the contractions induced by adrenergic or perivascular nerve stimulation were inversely proportional to age (39). The endothelium-dependent vasodilators were able to produce responses in the chicken embryos; there was failure to demonstrate significant differences of endothelium-dependent relaxation in the pulmonary arteries between the two age groups, which indicate a smooth transition to ex-ovo life (39). The study is useful in characterizing the transition of high vascular fetal tone to low vascular fetal tone in the pulmonary circuit, in which complications can assume when normal transition fails. Villamor and colleagues researched the vasoreactivity of pulmonary arteries when the chicken embryo is subjected to chronic hypoxia. Decreased contractions of the hypoxic pulmonary artery through all mechanisms were found, including excitation-contraction, adrenergic stimulation, and pharmacomechanical coupling. In contrast, no marked changes in sensitivity to the endothelium-dependent and –independent vasodilators were detected in the pulmonary artery (53). Other reports have been published on vasoreactivity of even more premature chick embryos at day 15 but on the contractile ability of ductus arterious to various agents. It was noted that the ductus arteriosus of the 15-day-old

embryo, in comparison to day 19 and 21, has the ability to contract through means of excitation-contraction and pharmacomechanical coupling, which included KCl, 4-aminopyridine, ET-1 and U-46619 (52). Nonetheless, the 15-day old embryo did not respond to O_2 nor adrenergic substances, demonstrating later development of responses. These results have possible implications in PDA in premature infants.

Experiments on peripheral artery vasoreactivity have also been conducted. On the topic of sympathetic innervation in the peripheral arteries, it has been noted that chicken embryos that were incubated in chronically hypoxic conditions at 15% O_2 have increased density of sympathetic fibers and increased concentration of NE in the femoral arteries at day 19 of incubation. Concurrently, the hypoxic chicken embryonic arteries produced a lesser response of contraction to the NE; but this was corrected with the addition of cocaine, which stimulates release of NE from nerve terminals (49). In the same study by Villamor et al. on chronic hypoxia and its effects on the pulmonary artery, Villamor also noted that the femoral artery of the 19-day hypoxic embryo was less responsive to the relaxant consequences of the endothelium-dependent acetycholine (53). In another study performed by Ruijtenbeek and colleagues, the effects of protein malnutrition and chronic hypoxia on vasoreactivity, particularly endothelial function, of the femoral arteries in 19-day-old chicken embryos (50). Neither stressor had an effect on the endothelium-independent relaxation of sodium nitruprusside; however, the arteries subjected to chronic hypoxia did have a reduced relaxation response to the endothelium-dependent acetylcholine. The relaxation response of the normoxic and hypoxic chicken embryo groups were also conducted in the presence of the NOS inhibitor L-NAME, and the

difference in response was no longer significant. Therefore, chronic hypoxia seems to be associated with endothelial dysfunction of the peripheral arteries with a reduced production of nitric oxide (50). Endothelial dysfunction, demonstrated by a decreased relaxation to acetycholine, may also be observed weeks after hatching and could have a role in cardiovascular abnormalities later in life (51).

The study conducted was a comparison of vasoreactivity of the mesenteric artery of non-internally pipped chicken embryos incubated in hypoxic conditions to embryos incubated in normoxic conditions at 15 and 19 day of the 21-day incubation period. By testing the the vasoreactivity of the arteries before the completion of 21 days and hatching, we are able to create animal models representative of prematurity. Incubating the eggs in an environment of 15% oxygen since day 0 of the period helps form a pathologic state of chronic hypoxia for the experimental group. What was to be determined was whether or not chronic hypoxia, which is one of most common prenatal insults for the human infant, is associated with endothelium dysfunction in the mesenteric artery of a premature chicken model. Contractile responses to norepinephrine, potassium chloride, endothelin-1, and electric field stimulation were tested. Also, the relaxation responses to endothelium-dependent acetylcholine and endothelium-independent forskolin and sodium nitroprusside were also conducted. More specifically, the experiment shall assess differences in endothelium-dependent relaxation between the normoxic and hypoxic group and, thus, support the theory of endothelial dysfunction as a potential inciting factor in the pathogenesis of NEC

3. Methods:

The goal of the research is to investigate differences in vasoreactivity, which includes both contraction and relaxation, between the normoxic and hypoxic groups of chicken embryos at the 15-day and 19-day period of the 21-day incubation period .

Incubation of chicken embryos and vessel isolation.

Experiments were performed in accordance with Dutch law for animal experimentation. Fertilized eggs of White Leghorn chickens were incubated at 37.8°C, 21% O2, 45% humidity and rotated once per hour (Incubator model 25HS, Masalles Comercial, Spain). In the hypoxic group the incubation was in 15% O2 from day 0. Embryos incubated for 15 and 19 of the 21-day incubation period were studied. The 19-day were defined as non-internally-pipped embryos, as verified by candling. The embryos were taken out, immediately killed by decapitation, placed on the dorsal side on a petri-dish coated with silicon and a midline laparotomy and sternotomy were performed. With the aid of a dissecting microscope, the cranial mesenteric artery (MA) was carefully dissected free from surrounding tissue .

Recording of MA reactivity

Two stainless steel wires (diameter 40 μm) were inserted into the lumen of the MA, which was mounted as a ring segment between an isometric force transducer and a displacement device in a myograph (Danish Myo Technology A/S model 610M, Aarhus, Denmark). The myograph organ bath (5 mL vol) was filled with Krebs-Ringer bicarbonate (KRB, composition in mmol L^{-1}: NaCl, 118.5; KCl, 4.75; $MgSO_4$ •$7H_2O$, 1.2; KH_2PO_4, 1.2;

$NaHCO_3$, 25.0; $CaCl_2$, 2.5; glucose, 5.5.) buffer maintained at 39°C. MA rings were normalized to a resting pretension corresponding to an intraluminal pressure of 10 mmHg in the 15-day embryos and 20 mmHg in the 19-day embryos. During the mounting, stabilization, and the experiments, MA rings were maintained in KRB buffer aerated with 95% O_2/5% CO_2.

Contractile responses

Concentration-response curves to KCl (31.25–125 mM), norepinephrine (NE; 10 nM – 0.1 mM), and endothelin (ET)-1 (0.1 nM-0.1 μM) were constructed by increasing the organ chamber concentration of the drug, by cumulative increments after a steady-state response had been reached with each increment. When two or more agonists were studied in the same arterial preparation, the vessels were repeatedly washed and allowed to equilibrate for at least 30 min. If the tone did not recover to resting level, the vessels were discarded for further experiments. (Technical Services, Universiteit Maastricht, The Netherlands).

Relaxant responses

Relaxations induced by ACh (10 nM- 0.1 mM), ,the NO donor sodium nitroprusside (SNP, 10 nM – 0.1 mM) and the adenylate cyclase activator forskolin (10 nM – 10 μM) were studied in vessels contracted with NE (10 μM) or K+ (62.5 mM). When stable contractions were obtained, Ach, SNP or forskolin was added cumulatively to the bath until a maximal response was achieved. In some experiments and in order to analyze the involvement of NO, ACh-induced relaxations were studied in the presence of the NOS inhibitor N-omega-Nitro-L-arginine methyl ester (L-NAME, 0.1 mM) and SNP-induced

relaxations were studied in the presence of the soluble guanylate cyclase (sGC) inhibitor 1H [1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM).

Data analysis

Results are shown as means and SEM of measurements in n embryos. Contractile responses are expressed in terms of active wall tension (N/m). Relaxation responses are expressed in terms of active wall tension (N/m) and in terms of percentage (%) relaxation of precontraction. Sensitivity to the vasodilators and vasoconstrictors was determined for the mesenteric arteries by fitting individual data of concentration-response to a nonlinear regression curve and interpolating. Differences between mean values were assessed by one-way ANOVA followed by Bonferroni's post hoc t-test or unpaired and paired t-test where suitable. Differences were considered significant at a $P<0.05$. All analyses were performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

Drugs and solutions

Solutions containing different concentrations of K^+ were prepared by replacing part of the NaCl of the KRB buffer by an equimolar amount of KCl. Arterenol bitartrate (NE), ACh, SNP, L-NAME and forskolin were obtained from Sigma (St. Louis, MO); U-46619 was from Cayman Chemical (Ann Arbor, MI), and ODQ was from Tocris (Ballwin, MO).

All drugs were dissolved initially in distilled deionized water (except U46619 and ODQ in DMSO and forskolin in ethanol) to prepare adequate stock solutions and further dilutions were also made in deionized water.

4. Results:

Characteristics of Samples:

A total of 78 chicken embryos were used in the experiments with 42 pertaining to the 19-day-old chicken embryo test group and 36 pertaining to the 15-day-old chicken embryo test group**.** Within the 19-day-old chicken embryo group, 20 were exposed to hypoxic conditions, and 22 were exposed to normoxic conditions. Within the 15-day-old chicken embryo group, 24 were exposed to hypoxic conditions, and 12 were exposed to normoxic conditions. Exposure of chicken embryos to 15% O_2 from day 0 to day 15 of the incubation period did not result in a significantly reduced body mass when compared to 15-day-old embryos exposed to 21% O_2 from day 0 to day 15 (12.75 ± 0.3 vs. 13.22 ± 0.5 g; $P<0.05$) (See Fig. 1A) Conversely, the body mass of 19-day-old chicken embryos exposed to 15% O_2 from day 0 to day 19 did result in a significantly reduced body mass when compared to its control group (26.04 ± 0.7 vs. 29.89 ± 0.5 g; $P<0.05$) (See Fig. 1B).

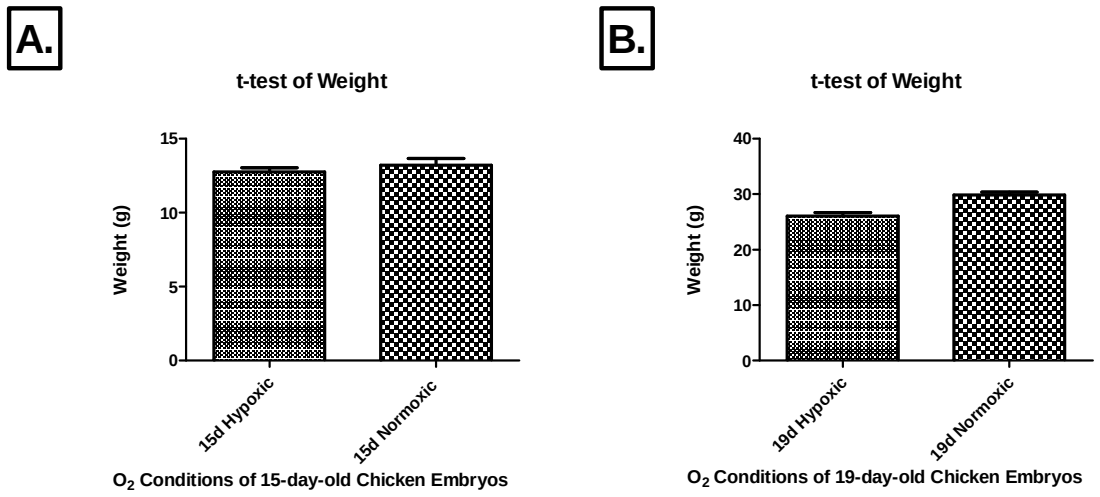


Figure 1. Effects of exposure to 15% O_2 on body mass. *A*: Hypoxic-induced changes in body mass in 15-day chicken embryos. Each bar represents the mean +/- SE of 24 hypoxic and 12 normoxic embryos. *B*:Hypoxic-induced changes in 19-day chicken embryos. Each bar represents the mean +/- SEof 20 hypoxic and 23 normoxic embryos.

Contractile responses in isolated mesenteric artery rings:

Isolated DA obtained from 15- and 19-day chicken embryos responded to depolarizing high-K+ solution (concentrations of 31.25mM to 125mM) with a tonic contraction and the amplitude of this response significantly increased with age in both hypoxic and normoxic groups (see figures 2A-2D). In the 15 days chicken embryos of the hypoxic group the contraction was significantly lower with the highest concentrations of K when compared with the normoxic group. In contrast, in the 19 days chicken embryos, there were no significant differences between the hypoxic and normoxic groups among all concentrations of K.

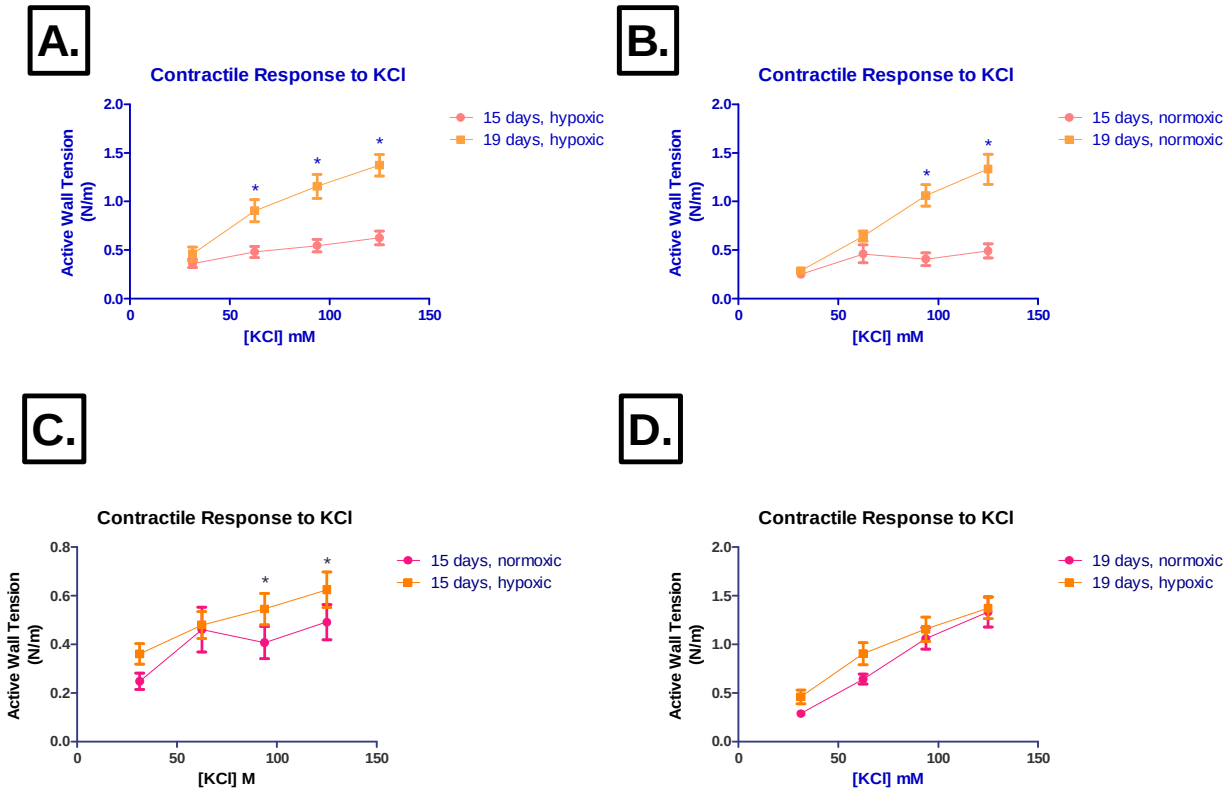
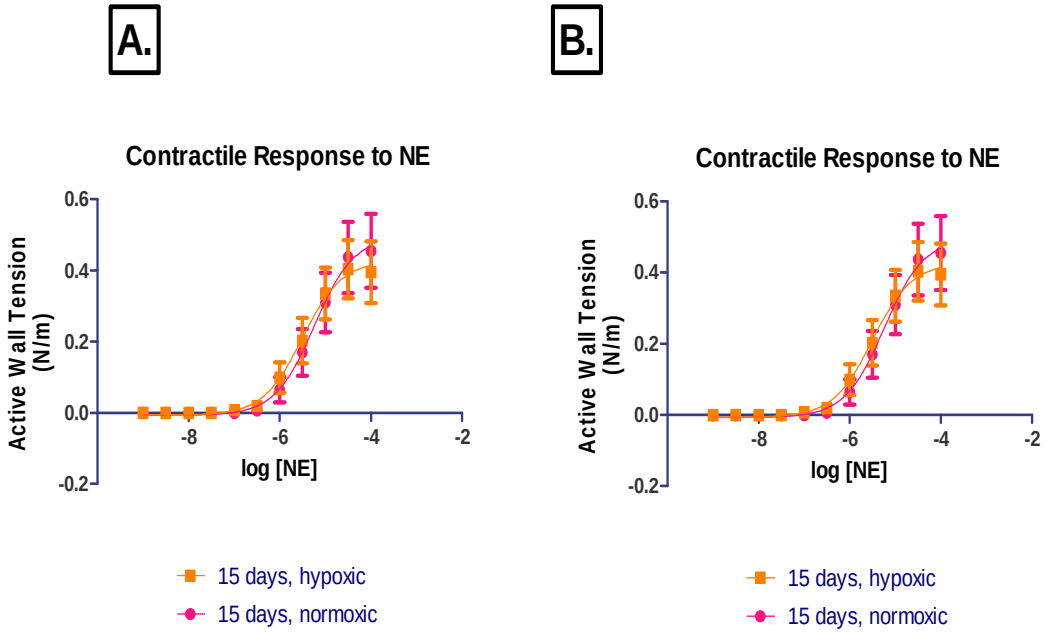


Figure 2.1. Concentration-dependent contractile effects of KCl in endothelium-intact mesenteric arteries of non-internally pipped 15-day and 19-day chicken embryos exposed to 15% O_2 or 21% O_2 of the 21-day incubation. A: Hypoxic, 15-day and 19-day embryos. B: Normoxic, 15-day and 19-day embryos. C: 15-day, normoxic vs. hypoxic. D: 19-day, normoxic vs. hypoxic. Each point or bar represents a mean +/- SE of n embryos. *P<0.05, **P<0.01

The nonselective adrenergic receptor agonist NE (see Fig. 3A-3D) caused a concentration-dependent contraction of the MA. The maximum response (Emax) induced by NE was not significantly different between the hypoxic and normoxic groups in both 15- and 19 days chicken embryos (normoxia 15 days: Emax=0.46 N/m, SD 0.25, n = 6; hypoxia 15 days: Emax=0.46 N/m, SD 0.25, n = 6 and normoxia 19 days: Emax=1.23 N/m, SD 0.61, n = 6; hypoxia 19-day: Emax=0.87 N/m, SD 0.76, n = 7). When comparing this contractile response according to age within the normoxic and hypoxic samples, there was only a

significant increase of Emax in the 19 days chicken embryos in the normoxic group (p = 0.02).



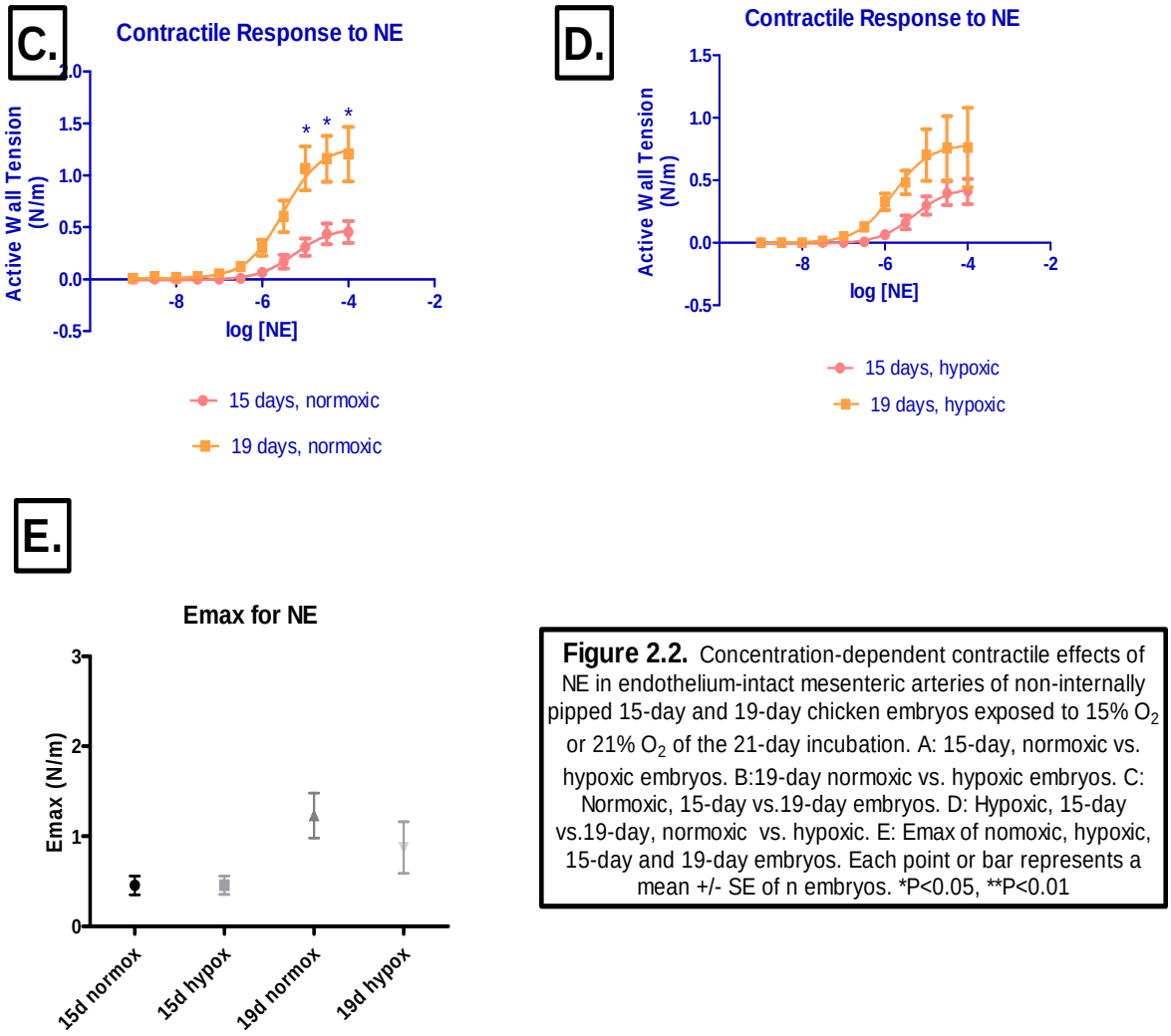
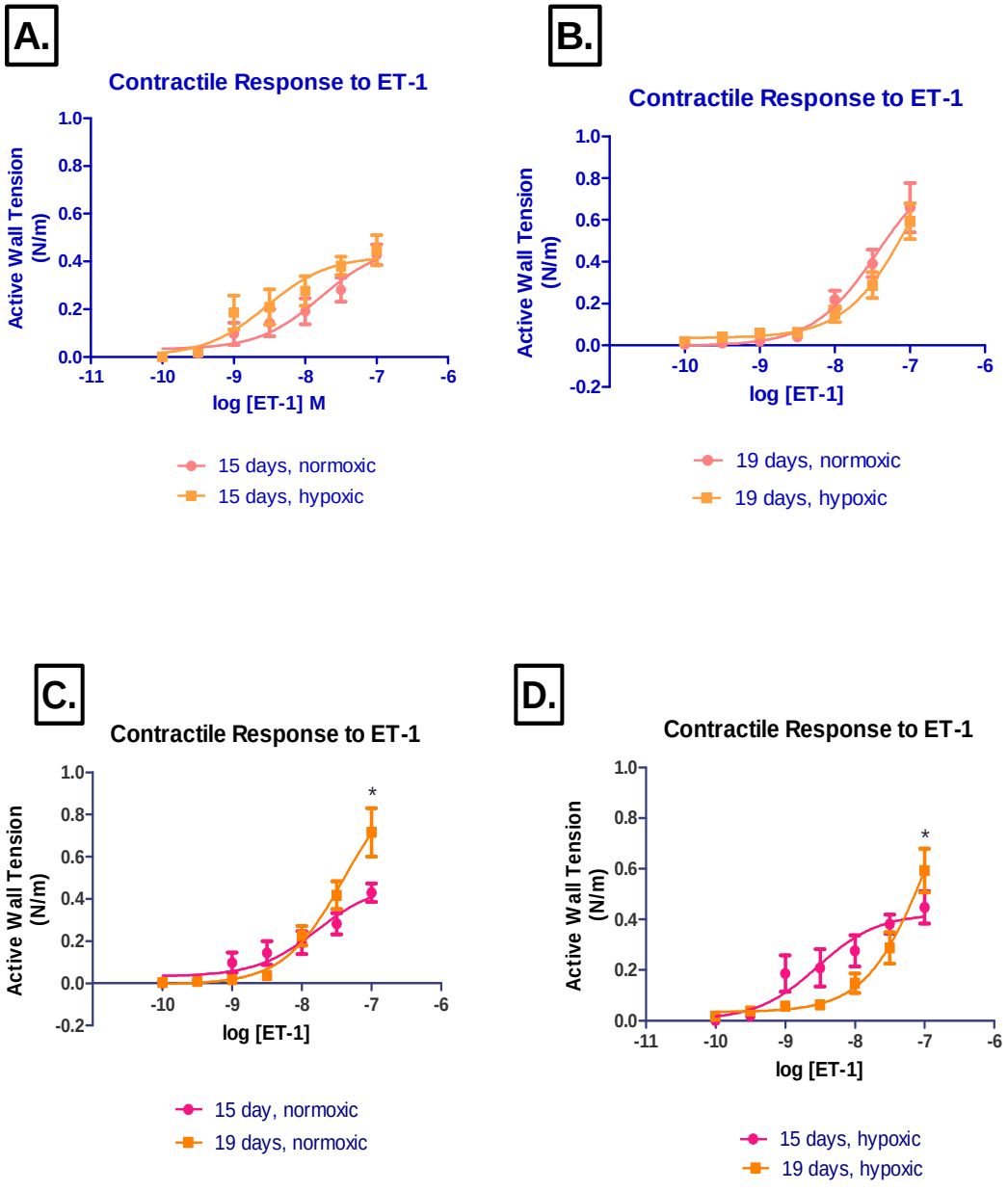


Figure 2.2. Concentration-dependent contractile effects of NE in endothelium-intact mesenteric arteries of non-internally pipped 15-day and 19-day chicken embryos exposed to 15% O_2 or 21% O_2 of the 21-day incubation. A: 15-day, normoxic vs. hypoxic embryos. B:19-day normoxic vs. hypoxic embryos. C: Normoxic, 15-day vs.19-day embryos. D: Hypoxic, 15-day vs.19-day, normoxic vs. hypoxic. E: Emax of nomoxic, hypoxic, 15-day and 19-day embryos. Each point or bar represents a mean +/- SE of n embryos. *P<0.05, **P<0.01

In another set of experiments, concentration-response curves to the polypeptide ET-1 (see figures 3A-3E) were performed. ET-1 also produced concentration-dependent contraction of the mesenteric artery (MA) in the 15- and 19-day chicken embryos. However, the Emax, or maximum response of contraction, induced by ET-1 was not significantly different between the hypoxic and normoxic groups in both 15- and 19 days chicken embryos (normoxia 15 days: Emax=0.55 N/m, SD= 0.27, *n* = 15; hypoxia 15 day: Emax=0.46 N/m, SD 0.17, *n* = 8) and normoxia 19 days: Emax=0.60 N/m , SD 0.35, *n* =

7, hypoxia 19 days: Emax=0.59 N/m, SD=0.29), *n* =11). When comparing this contractile response according to age within the normoxic and hypoxic samples, there was also no significant difference.



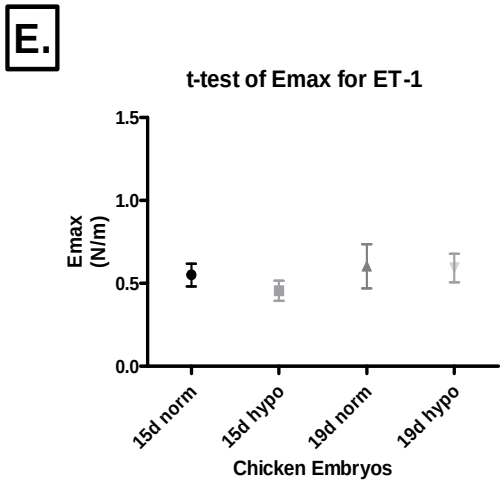


Figure 3. Concetration-dependent contractile effects of ET-1 in endothelium-intact mesenteric arteries of non-internally pipped 15-day and 19-day chicken embryos exposed to 15% O_2 or 21% O_2 of the 21-day incubation. A: 15-day, normoxic vs. hypoxic embryos. B:19-day normoxic vs. hypoxic embryos. C: Normoxic, 15-day vs.19-day embryos. D: Hypoxic, 15-day vs.19-day, normoxic vs. hypoxic. E: Emax of normoxic, hypoxic, 15-day and 19-day embryos. Each point or bar represents a mean +/- SE of n embryos. *P<0.05, **P<0.01

Relaxant responses in isolated mesenteric artery rings.

Figures 4A-4E illustrate the effect of ACh on MA contracted with NE or 62.5 mM K+. In endothelium intact 15 and 19-day MA, ACh evoked a biphasic response: low concentrations induced relaxation, while concentrations above 10 μM induced contraction. The maximum relaxation response to ACh was significantly higher (P<<0.01) in normoxic MAs contracted with NE (15-day: Emax = 72,7 %, SD 4.4, *n* = 4 and 19-day: 88.5 %, SD 17.2 , *n* = 8) than in MAs contracted with high K+ solution (15-day: Emax = 31.2 %, SD 16.7, *n* = 11 and 19-day: 44.4 %, SD 16.8, *n* = 12). In the hypoxic groups, the maximum relaxation response to ACh was not significantly higher in MAs contracted with NE (15-day: Emax = 50.9 %, SD 25.2, *n* = 4 and 19-day: 61.0 %, SD 45.24, *n* = 6) than in MAs contracted with high K+ solution (15-day: Emax = 28.6 %, SD 22.2, *n* = 15 and 19-day: 33.4 %, SD 21.6, *n* = 8).). There was no difference in maximum relaxation response between the normoxic and hypoxic groups in both the 15- and 19-days MA. In the comparison of normoxic and hypoxic groups of the 15-day MA, there was no

significant difference of sensitivity to the ACh with either contraction with NE or high K+ solution. Also the same comparison was drawn between the normoxic and hypoxic groups of the 19-day MA with both contractile agents, and no significant changes were found.

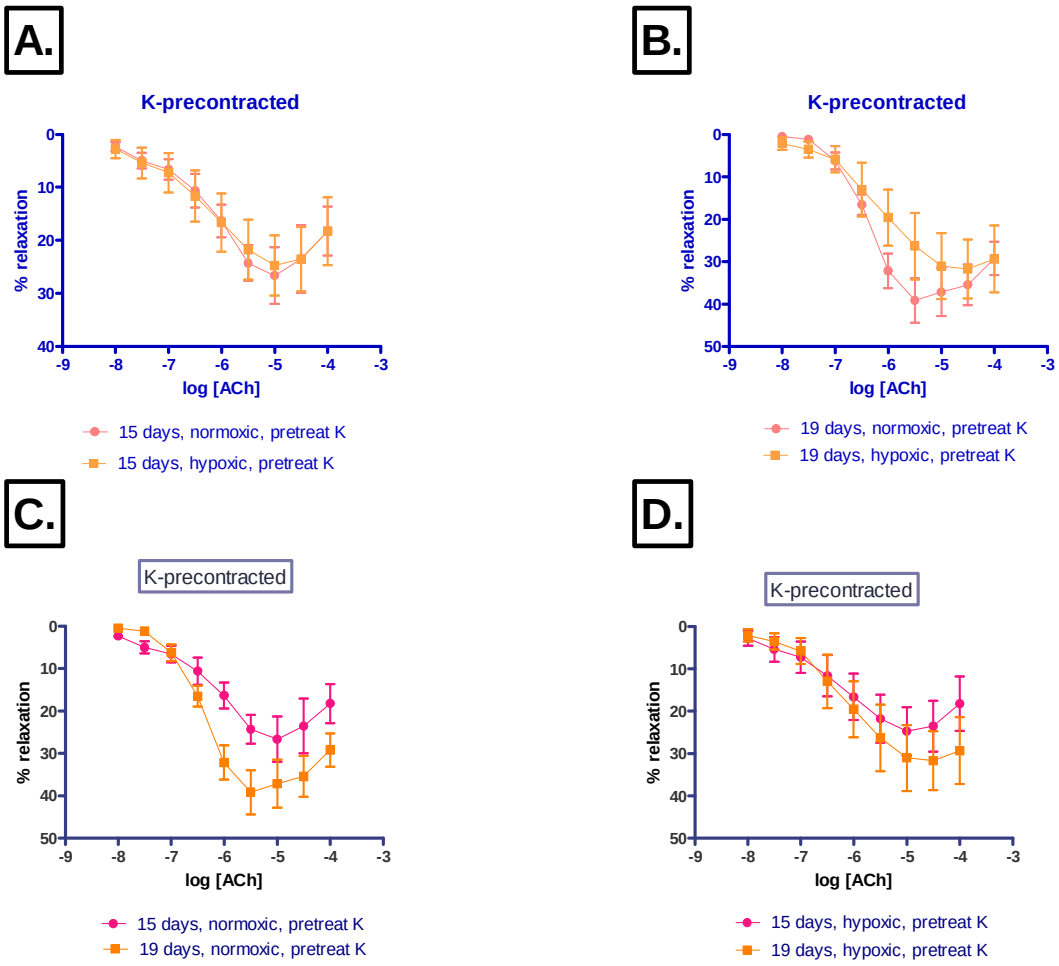


Figure 4.1 Concentration-dependent relaxant effects of ACh after precontraction with K in endothelium-intact mesenteric arteries of 15-day and 19-day chicken embryos exposed to 15% O_2 and 21% O_2 of the 21-day incubation period. A: 15-day, normoxic vs. hypoxic. B: 19-day, normoxic vs. hypoxic. C: Normoxic, 15-day vs. 19-day. D: Hypoxic, 15-day vs. 19-day.

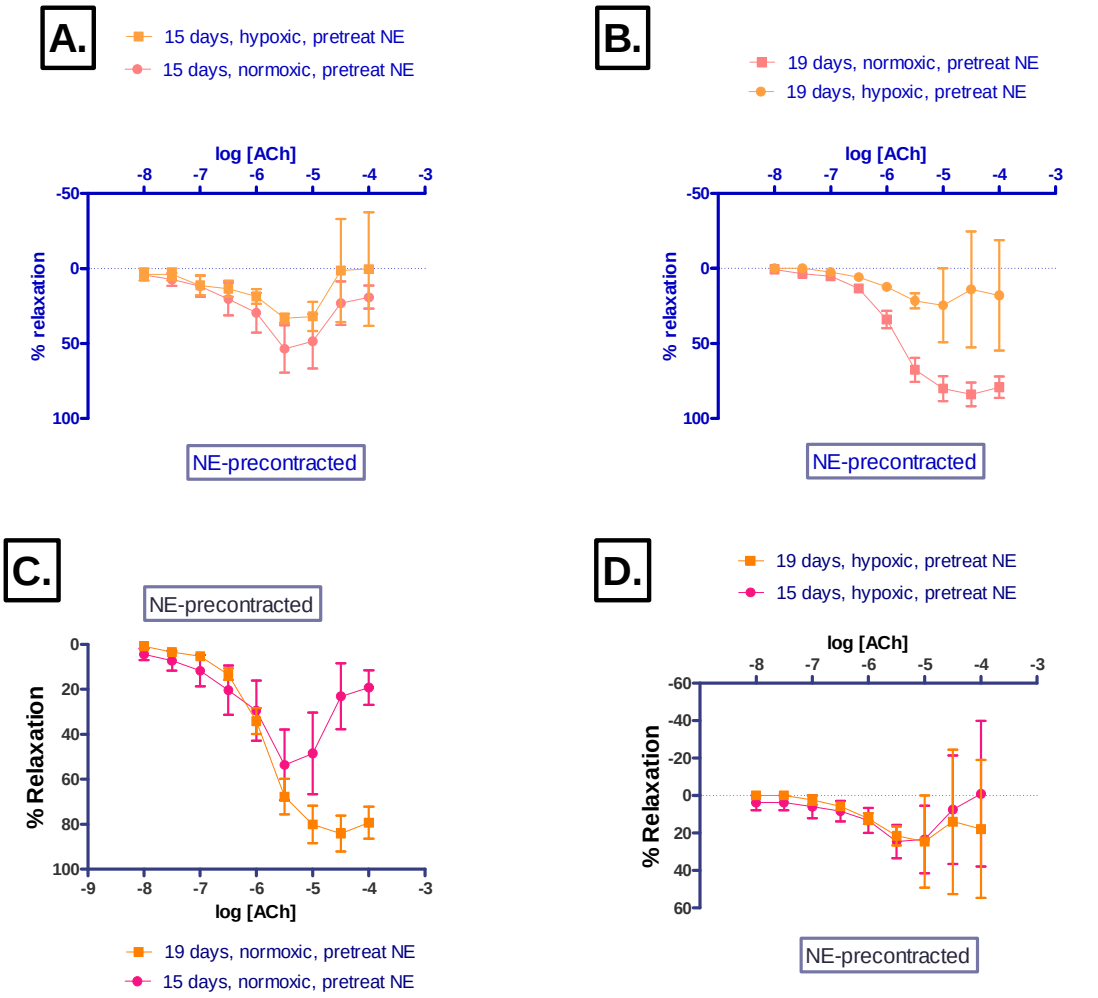


Figure 4.2 **Concentration-dependent relaxant effects of ACh after precontraction with NE in endothelium-intact mesenteric arteries of 15-day and 19-day chicken embryos exposed to 15% O_2 and 21% O_2 of the 21-day incubation period. A: 15-day, normoxic vs. hypoxic. B: 19-day, normoxic vs. hypoxic. C: Normoxic, 15-day vs. 19-day. D: Hypoxic, 15-day vs. 19-day.**

ACh-induced relaxation in the presence of the NOS inhibitor L-NAME (See figures 4.5-4.6) was only tested in 19-day normoxic K+ and NE-contracted MAs. The Ach-induced relaxation was not significantly decreased by L-NAME (K+ precontracted: Emax 39.1 %, SD 18.5, n = 11, P = 0.48 vs. control; NE-precontracted: Emax 63.5 %, SD 28.6, n = 7, P = 0.06 vs. control).

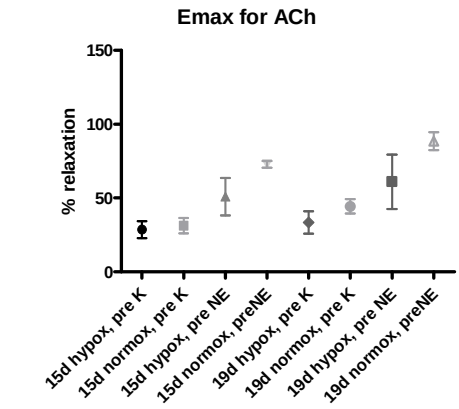
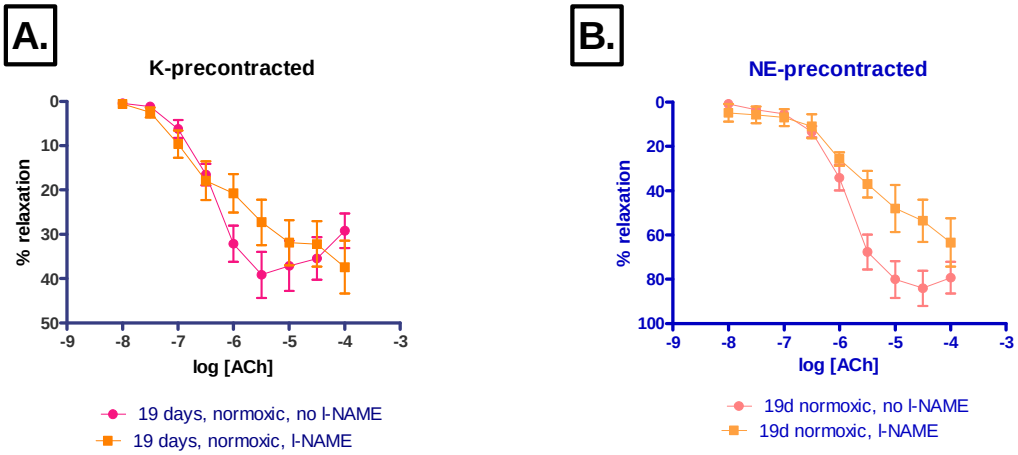


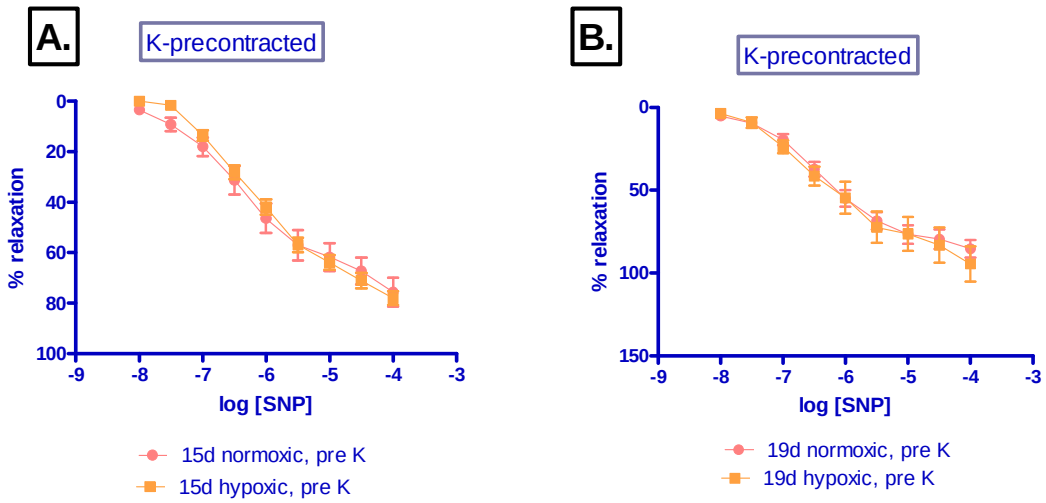
Figure 4.3 Emax for ACh in 15-day and 19-day embryos in normoxic and hypoxic groups

Figure 4.4 Concentration-dependent relaxant affects of ACh in the presence of L-NAME in 19-day normoxic embryos. A: 19-day normoxic, K-precontracted. B: 19-day normoxic, NE-precontracted.



Relaxant responses of the mesenteric artery to sodium nitroprusside, SNP, from 10^{-8} to 10^{-4} M were also performed and studied after a precontraction with KCl at 62.5mM or NE at 10^{-5} M. The NO-donor SNP also caused a concentration-dependent relaxation in the test and control groups of all ages. The maximum relaxation response to SNP was significantly higher ($P<<0.05$) in MAs contracted with NE (15-day normoxic: Emax = 136.6 %, SD 9.6, *n* = 4; 15-day hypoxic: Emax = 138.2 %, SD 18.0, *n* = 7; 19-day normoxic: 153.2 %, SD 18.5, *n* = 7; 19-day hypoxic: 122.1 %, SD 25.7, *n* = 7) than in MAs contracted with high K+ solution (15-day normoxic: Emax = 75,7 %, SD 16.0, *n* = 8; 15-day hypoxic: Emax = 78.1 %, SD 9.4, *n* = 12; 19-day normoxic: 85.5 %, SD 14.8, *n* =

8; 19-day hypoxic: 94.7 %, SD 30.1, *n* = 8). Except for the 19-day NE-precontracted MA (P = 0.02), there was no difference between the normoxic and hypoxic groups at the different ages. In both, NE-contracted and K+-contracted MA, the relaxation caused by SNP was significantly reduced in the presence of ODQ (data not shown).



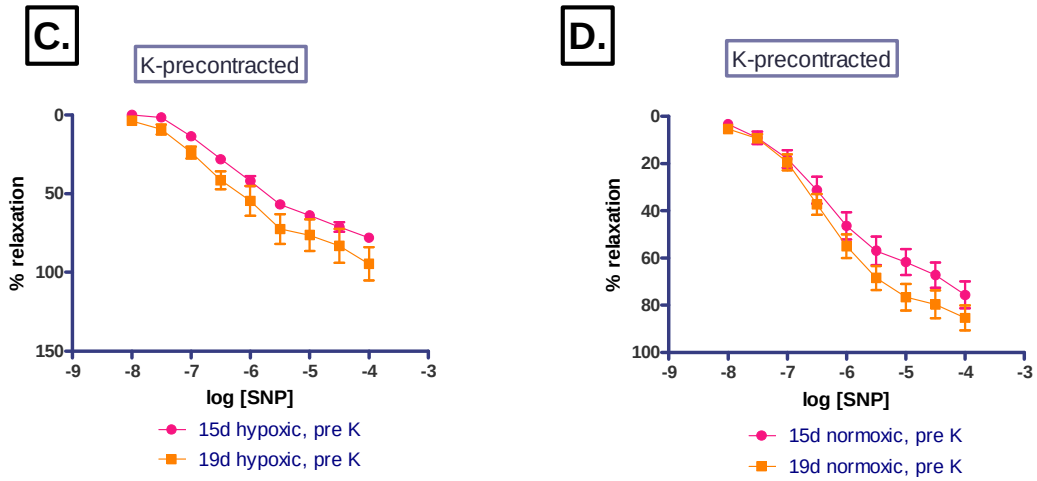
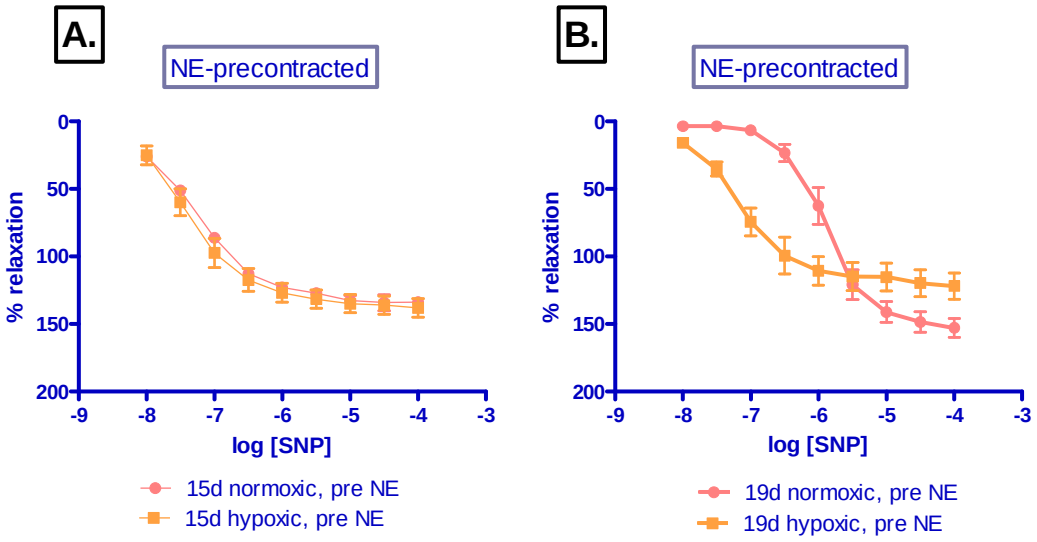


Figure 5.1 Concentration-dependent relaxant effects of SNP after precontraction with K in endothelium-intact mesenteric arteries of 15-day and 19-day chicken embryos exposed to 15% O_2 and 21% O_2 of the 21-day incubation period. A: 15-day, normoxic vs. hypoxic. B: 19-day, normoxic vs. hypoxic. C: Hypoxic, 15-day vs. 19-day. D: Normoxic, 15-day vs. 19-day.



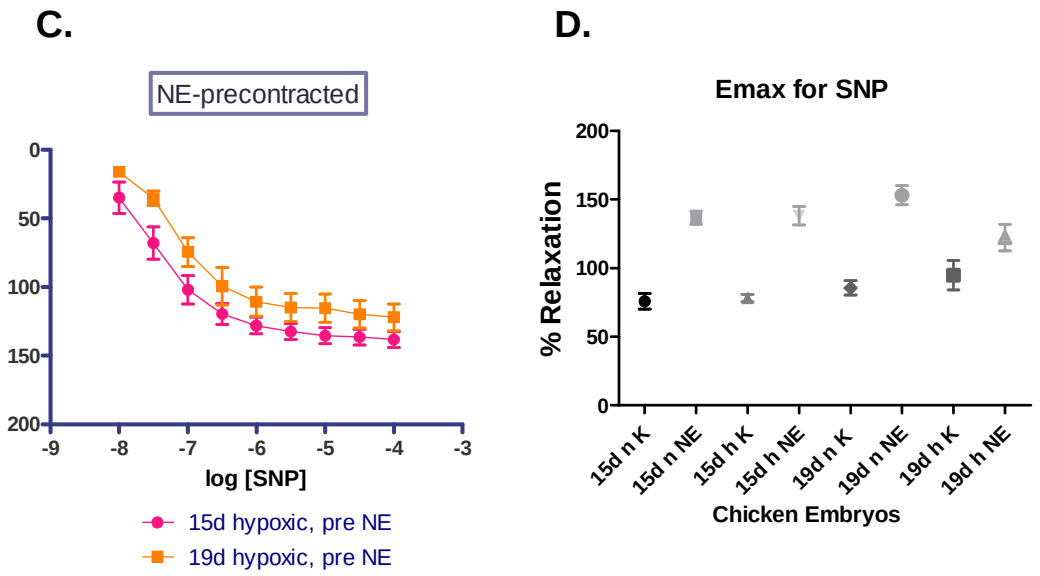


Figure 5.2 Concentration-dependent relaxant effects of SNP after precontraction with NE in endothelium-intact mesenteric arteries of 15-day and 19-day chicken embryos exposed to 15% O_2 and 21% O_2 of the 21-day incubation period. A: 15-day, normoxic vs. hypoxic. B: 19-day, normoxic vs. hypoxic. C: Hypoxic, 15-day vs. 19-day. D: Emax for SNP, normoxic, hypoxic, 15-day and 19-day, pre-K and pre-NE

The endothelium independent relaxation by the cyclic AMP pathway was studied by using Forskolin (FSK). In the 15-day hypoxic MA, the maximum relaxation produced by FSK after precontraction with NE was significantly increased compared with the K+-precontracted MA (NE: Emax=195.1%, SD=19.4, n=7; K+: Emax=88.0%, SD=6.5, n=4; P=0.003). In comparing responses of 15-and 19-day hypoxic chicken embryos with precontraction NE, there was a significant increased response in the 15-day hypoxic embryos with a value of P<<0.001 (19-day: Emax=115.1%, SD=4.07, n=12). No experiments were performed in the 15-day- old normoxic chicken embryos. In the 19-day normoxic MA there was no difference in maximum relaxation response to FSK (NE: Emax=114.9%, SD=8.7, n=8; K+: Emax=109.0%, SD=18.5, n=8).

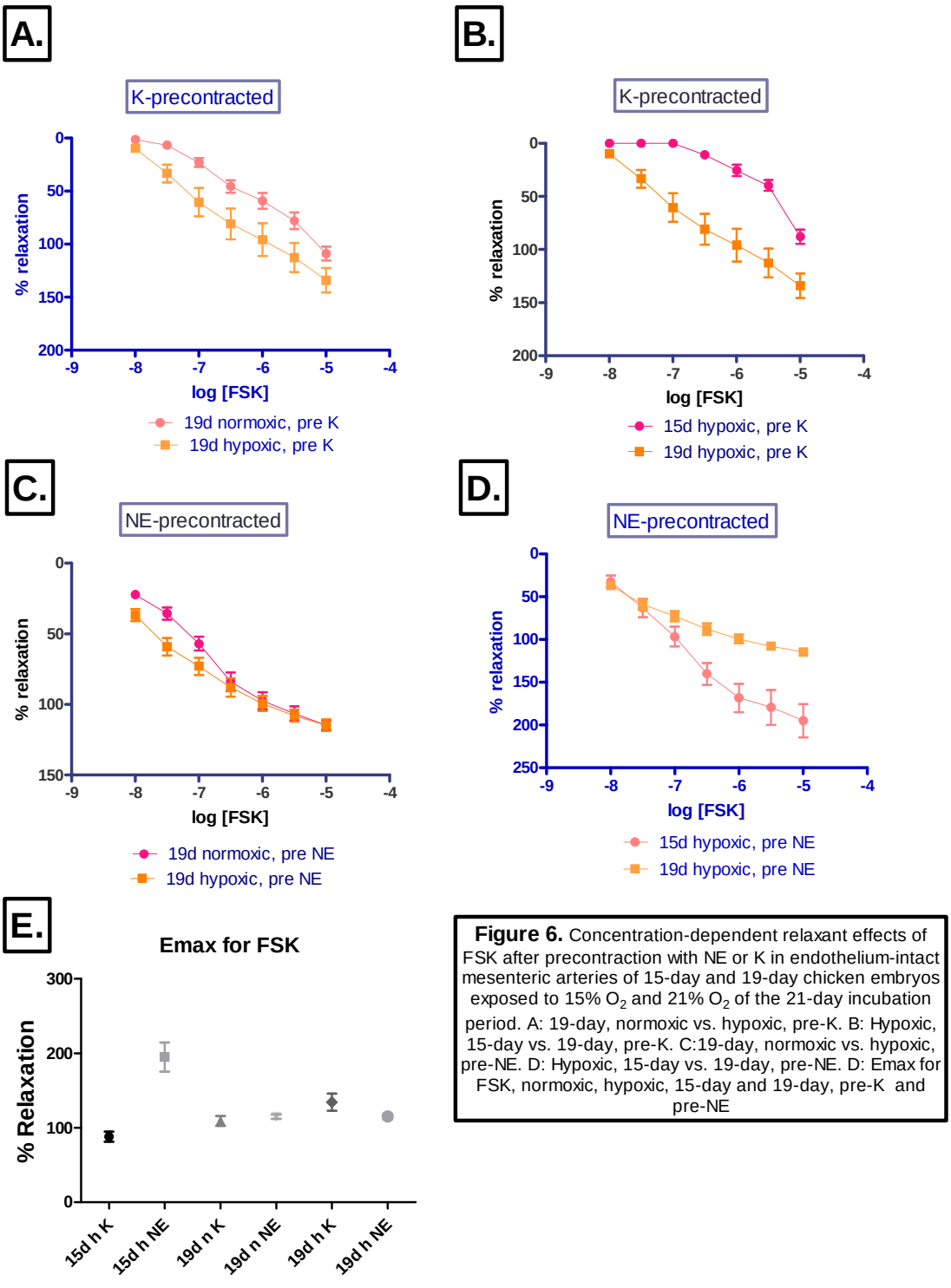


Figure 6. Concentration-dependent relaxant effects of FSK after precontraction with NE or K in endothelium-intact mesenteric arteries of 15-day and 19-day chicken embryos exposed to 15% O_2 and 21% O_2 of the 21-day incubation period. A: 19-day, normoxic vs. hypoxic, pre-K. B: Hypoxic, 15-day vs. 19-day, pre-K. C:19-day, normoxic vs. hypoxic, pre-NE. D: Hypoxic, 15-day vs. 19-day, pre-NE. D: Emax for FSK, normoxic, hypoxic, 15-day and 19-day, pre-K and pre-NE

5. Discussion:

Effects of chronic hypoxia on body mass.

We observed a significantly reduced body mass in the 19-day chicken embryo that was exposed to 15% O_2 during its entire incubation instead of 21% O_2. These findings were concordant to many studies done on the intrauterine growth delay in chicks that underwent chronic hypoxia at day 19 (50). As well, the reduced body mass at day 19 from our study may also be extrapolated and applied to body mass at hatch, as demonstrated by Miller and workers (54). On the other hand, no significant changes in body mass were discerned between the normoxic and hypoxic groups of the 15-day embryo. One may assume that perhaps the effects of growth imposed by the chronic hypoxia may not have a marked effect until later in development. Yet, a few studies have shown noticeable decreases in body mass in hypoxic chickens at day 15 and even in the first few days of incubation (54, 55).

Contractile properties.

In the present study, we observed that the response of the chicken MA to a receptor-independent (i.e. high-K+ solution) vasoconstrictor increased with gestational age in both hypoxic and normoxic groups. Such results were also seen in femoral and carotid arteries of the chicken embryo with increasing gestational age (64). It can be assumed that the excitation-contraction coupling improves and matures with age although the mechanism, with its contractile proteins and voltage channels, exists early in the incubation period. Better smooth muscle differentiation may elucidate a better contractile response with age.

Chronic hypoxia seemed to affect the ability of the 15-day mesenteric artery to contract as effectively as its normoxic counterpart when exposed to KCl solutions at higher concentrations; however, with an increase in age, the difference in response becomes insignificant. It is has been determined that the contraction induced by K+ solution is through membrane depolarization and influx of Ca^{+2} ; the influx of Ca^{+2} then stimulates the release of Ca^{2+} in the sarcoplasmic reticulum, which then bind to the Ca^{+2} receptor on the contractile proteins to induce vasoconstriction (58). As stated by Villamor et al., it may be suggested that the effects of chronic hypoxia may involve the ability of the membrane channel to depolarize with either abnormal resting membrane potentials, malfunction within the many pathways that involve Ca^{+2}, or weakened contractile proteins (53). In a study by Platoshyn et al about downregulation of K^+ voltage-channels in pulmonary arteries as a possible explanation for pulmonary hypertension, the exposure to chronic hypoxia does not seem to have an effect on the regulation of K^+ channels for resting membrane potential nor concomitant intracellular Ca^{+2} stores in the mesenteric arteries (59). And so, it may be suggested, that the reduction of contractile response from chronic hypoxia in the excitation-contraction is due to Ca^{+2} -independent mechanism. For example, as an adaptive mechanism to the chronic hypoxia, the reduced vasoreactivity of fetal blood vessels, as seen in mammals, may be due to alterations in the endothelium that cause an increase in NO and prostaglandin production (60).

We also examine the ability of the mesenteric artery to contract through increased doses of the adrenergic agonist, NE. What can be ascertained is that mesenteric artery does have the α-adrenergic system present early in develop as revealed with the contractions induced

in 15-day hypoxic and normoxic groups. Hypoxia in within this age group does not seem to impede adrenergic stimulation of the vessel, nor is the same observed within the 19-day age group of normoxic and hypoxic arteries. This is also supported by another study in which no changes in response to catecholamines were found in postnatal aortic rings of guinea pigs after chronic hypoxia exposure (61). Yet, in contrast to our findings, other studies conducted have shown differences in adrenergic response with normoxic and hypoxic group but with great variability. In one study by Auer and Ward, the aortic rings of rats demonstrated a blunted response to phenylephrine and KCl after prolonged exposure to hypoxia; more importantly, pulmonary arteries of chronically hypoxic chicken embryos have shown reduced contractile responses to NE (62, 53). On the other hand, an older study by Aoki demonstrated that the stressors for adrenergic responses were actually increased in the limbs of rats from the effects of chronic hypoxia (63). And so, perhaps, the effects of chronic hypoxia on adrenergic-induced contraction may vary according to animal model and artery.

Interestingly, in the present study, the increase in age demonstrates a more mature, significant response of contraction in the normoxic group but not in the hypoxic group. And so, the development of response to adrenergic agonists with age may be encumbered due to chronic hypoxic effects; once again, the delayed development may be due to altered NO production as an adaptive measure. The maturation of the neurohumoral mechanism to induce a stronger contraction was also seen in other peripheral arteries of the chicken embryo, highlighting the significance of age for effective adrenergic stimulation (64, 80). The concentrations of plasma catecholamines in chicken embryos are higher respective to

mammals and also reach their peak around day 19 before internal pipping (65-66). High concentrations of NE and increased sensitivity with a stronger contractile apparatus, more adrenergic receptors, better signaling pathways due to a more advanced gestation age may explain our findings in the normoxic chicks. The effects of hypoxia on catecholamines in the chicken embryo have been reviewed. Acute hypoxic causes an increase in the circulating catecholamines, i.e. NE, in the peripheral arteries (66). This illuminates the circulatory defense mechanism against stressor through cardiovascular redistribution of floor. Chronic hypoxia also has its own unique consequences on the chicken embryo with increased concentration of catecholamines and periaerterial innervation; however, the peripheral arteries are less sensitive to adrenergic stimulation perhaps through desensitization or increased NE uptake, which supports what was found in our study regarding the absence of neurohumoral maturation in the hypoxic group (49).

The contraction response to receptor-dependent agents was also recorded in our observations. The presence of contraction to endothelin-1 in 19-day and 15-day vessels in spite of normoxic or hypoxic conditions denotes the existence of receptor-dependent mechanisms early in embryonic development. Receptor-dependent contractions to ET-1 and thromoboxane A2 mimetic U-46619 were also induced in the ductus arteriosus in the 15-day chicken embryo, confirming that pharmacomechanical coupling appears early in gestation (52). Moreover, there was no significant change in sensitivity between the normoxic and hypoxic groups. In contrast, in a study of the effects of chronic hypoxia on vasoreactivity, the pulmonary artery of the 19-day hypoxic chicken embryo showed significant reduction in its ability to produce receptor-dependent contractions (53).

Another experiment that involved the pulmonary and femoral artery of adult rats demonstrated that chronic hypoxia produces time-dependent reduced contractions in the pulmonary artery, but that the femoral artery was unaffected (67).

Other functional studies on the vasoreactivity changes from hypoxia have varied results. Arteries that have been exposed to chronic intermittent hypoxia have shown increased contraction to endothelin-1 because of increased ET_A receptor expression; acute hypoxia has either no effect on mesenteric arteries (68-70). In light of these observations, it should be reiterated that the experiments were conducted in adult animals; knowing that endothelin-1 has a more significant role in the intestinal circulation, especially in the newborn period (6, 71). The physiology may be different in comparison and more studies of chronic hypoxia in newborn and fetal arteries are warranted. At the highest concentrations of endothelin-1 at 10^{-7} M, we were able to distinguish a significant age-dependent increase of the 19-day artery over the 15-day artery in both hypoxic and normoxic groups. The observations in the present study suggest the control of polypeptide endothelin on vascular tone is not affected by chronic hypoxia and may perhaps be age-dependent. The same age-dependent increases were recorded in pulmonary artery and ductus arteriosus of the chicken embryo, maintaining the idea that pharmacomechanical coupling improves with development (52-53).

Relaxant properties.

The use of acetylcholine as a endothelium-dependent vasodilator is due to its ability of raising intracellular Ca^{2+} in endothelial cells and, thus, stimulating NOS (56). And so, any

abnormalities of the relaxation response would be indicative of endothelium dysfunction. Acetylcholine was able to elicit relaxation responses in all four groups. In the normoxic groups in both 15- and 19-day Ma, ACh caused a significant higher maximum relaxation response in NE-precontracted MA compared with the K+-precontracted MA. This indicates the existence of an additional endothelial relaxing pathway that involves smooth muscle hyperpolarization, a non-characterized endothelial factor called EDHF. The larger relaxation induced by ACh in NE-precontracted MA was only partially reduced by L-NAME, which also alludes to other means of vasodilation.

There are many endothelium-dependent vasodilators that are responsible for vessel relaxation, including prostacyclin; NO; and the endothelium-dependent hyperpolarizing factor, EDHF, which has an important role in vasodilation especially in resistance vessels, like the mesenteric vasculature. The interplay of vasorelaxation with EDHF and endothelial NO has been confirmed in the mesenteric vasculature of the rabbit (76). However, in the setting of endothelial NO compromise, the value of EDHF remains to be elucidated. The ACh can induce the relaxation response through coactivation of K_{Ca} channels in the endothelium; and the hyperpolarization is transferred through gap junctions to reach the smooth muscle, characterizing the EDHF response (73). EDHF's action is mediated through the hyperpolarization of vascular smooth muscle, agents that target the Kv channels, like the K+ solution in our study, will reduce its effect (72). The concept is exemplified with the larger ACh-response in precontraction to NE and the partial, insignificant reduction of the response in the MA with the presence of L-NAME.

We were not able to observe significant differences in relaxation between normoxic and hypoxic groups with either precontractile agent in both age groups. These set of results may indicate that chronic hypoxia does not induce endothelial dysfunction in 15- and 19-day chicken embryo MA. In support of our study but in the setting of acute hypoxia, Shaul and Wells saw that the endothelial cells of mesenteric arteries in fetal sheep did not lead to a decrease in basal and stimulated NO production (81). In a different set of experiments of acute hypoxia on arteries of the chicken embryo, Ruijtenbeek and workers illustrate that ACh-induced relaxation was eliminated in the femoral artery (82). Furthermore, the observations by Villamor et al. also demonstrate how chronic hypoxia did reduce the ACh-relaxant response in femoral arteries; yet, no changes were seen in the pulmonary artery (53). Nonetheless, it seems that the decreased relaxation was not due to lack of response from vascular smooth muscle because of positive results obtained with SNP in these experiments, including our own. It has been thought that the amount of O_2 available is what regulates the amount of NO produced, which can limit vasodilation of arteries in animals exposed to hypoxic conditions (82-83). There have been a few studies executed that may explain why chronic hypoxia did not cause a diminished endothelium-dependent relaxation in our analysis. In one report on fetal carotid arteries exposed to chronic hypoxia in guinea pigs, Thompson et al. describe increased sythesis of NO and prostaglandins, inhibiting contraction and promoting vasodilation (60). The proposal was that local vascular changes occur in response to chronic hypoxia. In a different study, the up-regulation of NOS production was due to increased intracellular concentrations of Ca^{2+} in the endothelial cells, leading to a decreased sensitivity of the vascular smooth muscle to respond to Ca^{2+} and inhibiting constriction.

In neither the normoxic nor hypoxic group, no significant age-dependent changes were seen. In regards to the absence of age-dependent changes with ACh in our study, a prior analysis of vasoreactivity was carried out in peripheral arteries of chicken embryo; neither the carotid nor the femoral artery showed increased sensitivity to ACh with development in ages from 15-day to 21-day (64). In fact, when the endothelium-dependent response to ACh was examined in chicken embryos at earlier stages of incubation, days 13 and 17, there were still no significant differences in relaxation (80). The accumulation of these findings may suggest that the endothelium-dependent relaxation has already matured in very early stages of embryonic development. However, this may only apply to avian bird models since mammalian models have shown increased peripheral arterial sensitivity to ACh with age that continues even beyond the neonatal period (77-78). Further analysis with a wider range of age for the chicken embryo may be warranted.

Sodium nitroprusside is a NO donor and is, thus, an agent that can be used to produce endothelium-independent relaxation. Still, like the ACh-relaxation response of the normoxic groups, the curves produced by SNP after precontraction to NE were significantly greater than the ones produced after K+-precontraction in all groups except for the 19-day hypoxic group. Similar findings were described in pulmonary arteries of normoxic 19-day chicken embryos (39). To expound upon this, Rapoport et al. demonstrated that highly-depolarizing agents, such as K+ in our study, can inhibit cGMP synthesis, a required molecule for both ACh and SMP pathways for vasorelaxation (75).

As well, what we observed from the experiments conducted was that the MA is sensitive to SNP in both 15-day and 19-day test and control groups.

In addition, in general, the maximum relaxation response to SNP was significantly higher than the response to ACh. Therefore, the vascular smooth muscle has the capacity to react to donated NO and activate the soluble-guanylate cyclase/CGMP pathway for muscle relaxation, not taking into account of whether or not the endothelium has the ability to synthesize NO. A study concerning the vasoreactivity to ACh in 19-day embryos and 21-day externally pipped chicks showed comparable curves of relaxation with no significant difference; yet, the curves to SNP were significantly greater reaching 100% relaxation (39). In multiple studies on mammalian arteries, there have been reports that SNP induces a greater relaxation than ACh in fetal models; ACh response increases in newborn and adult models but SNP response stays the same (77-78). Thus, the endothelial cell may have to undergo maturational changes during postnatal development of the chicken. Curiously, the 15-day hypoxic groups with K+ and NE precontractions in our experiment have a greater relaxant sensitivity to NO than the corresponding normoxic controls with increased responses up to twofold. This was not seen in the 19-day chicks. In a similar study of chronic hypoxic effects on endothelium-independent relaxation, chronic hypoxia also did not seem to impair nor magnify relaxation due to SNP in 19-day chicken embryos (53). In contrast, acute hypoxia does sensitize and increase SNP-induced relaxation in peripheral arteries of the same 19-day chicken embryos (82). In another recent study on the DA of chicken embryos and relaxation responses, the DA was less sensitive to SNP with increasing age as it approaches closure. Villamor and colleagues have suggested the

possibility of down-regulation of soluble guanylate cyclase in the transition of the chicken to ex ovo life (79). The idea may have the same implications in MA sensitivity of the 15-day embryos as they advance in gestation, or the MA artery is more responsive to sGC in early stages of development; but reasons for the change are still unknown.

Forskolin functions as an endothelium-independent through the activation of adenyl cyclase to make cAMP. In turn, there are two proposed mechanisms for vascular smooth muscle relaxation with either decreased intracellular Ca^{2+} or decreased phosphorylation of myosin (57). Chronic hypoxia seems to heighten the sensitivity of the MA arteries to the endothelium-independent method of relaxation, as demonstrated in the 15-day hypoxic vessels that have precontracted with NE. Despite age, precontractile agents, or oxygen conditions, the other experimental and control groups had comparable curves of relaxation. In the study by conducted by Villamor and workers, the 19-day chicken embryos demonstrated no significant difference of endothelium-independent relaxation to forskolin when exposed to chronic hypoxia in either the femoral or the pulmonary artery (53). However, Priest et al. also saw an enhanced relaxation response to forskolin in small pulmonary arteries of the adult rat that have been under chronic hypoxic conditions in comparison to the control group; it has also been implicated, in the same study, that forskolin has an NO component because of attenuation of its response in the presence of L-NAME. Accordingly, it has been suggested that the increased response to forskolin is because of an up-regulation of adenylate cyclase or perhaps receptor density (74). Therefore, these findings may have the same implications in vasoreactive changes to forskolin of the 15-day chicken embryo that were obtained in the present study; also, the

significant relaxation was found in relation to the precontraction with NE over K+, which may contribute an added effect of EDHF in the response. However, why the response is heighted early in the incubation period and disappears with age remains unclear.

6. Conclusions

In the present study, we were not able to prove that chronic hypoxia causes endothelial dysfunction in the mesenteric arteries of 15-day and 19-day-old chicken embryos. Yet, we were able to observe how EDHF has a role in vasoregulation from the increased relaxation to ACh that ensued after NE-precontraction over K+-precontraction in the experimental groups and controls. The lack of change in the endothelium-dependent relaxation in spite of advancing gestational age also endorses the development of the ACh-relaxation mechanism in the early stages of incubation. SNP and FSK also show significantly heightened sensitivity in hypoxic groups, particularly the 15-day embryos; this may be due to an up-regulation of adenylate and guanylate cyclase and increased sensitivity in the more premature chicken embryo. NE, K+ and ET-1 all produced better responses proportional to age due to maturation of their own respective pathways. The exposure to chronic hypoxia reduced the sensivity to the contractile agonists; therefore, chronic hypoxia reduces vasoconstriction.

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