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Impaired Cerebral Angiogenesis in the Fetal Lamb Model of Persistent Pulmonary Hypertension

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Abstract

Background—Persistent pulmonary hypertension of the newborn (PPHN) is associated with increased risk of neuro-developmental impairments. Whether relative fetal hypoxia during evolution of PPHN renders the fetal brain vulnerable to perinatal brain injury remains unclear. We hypothesized that in utero ductal constriction, which induces PPHN also impairs cerebral angiogenesis.

Methods—Fetal lambs with PPHN induced by prenatal ligation of the ductus arteriosus were compared to gestation matched twin controls. Freshly collected or fixed brain specimens were analyzed by immunohistochemistry, Western blot analysis, and RT-PCR.

Results—Cortical capillary density was decreased in PPHN lambs compared to controls (Glut-1, Isolectin B-4 and Factor VIII, n=6, p<0.05). Hypoxia inducible factor-1 α (HIF-1 α) and vascular endothelial growth factor (VEGF) protein levels were decreased in cortical cell lysates of PPHN lambs. PPHN increased angiopoietin-1 (Ang-1) and tyrosine-protein kinase receptor (Tie-2) protein expression while angiopoietin-2 (Ang-2) protein levels were decreased (n=6, p<0.05). PPHN did not change mRNA levels of these proteins significantly (n=6).

Conclusions—PPHN decreased cortical capillary density in fetal lamb brain. PPHN decreased the expression of proteins involved in angiogenesis. These findings suggest that PPHN is associated with impaired cortical angiogenesis.

Keywords

persistent pulmonary hypertension in the newborn (PPHN); cerebral angiogenesis; HIF-1 α ; VEGF; sheep

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1. Introduction

Persistent pulmonary hypertension of the newborn (PPHN) predisposes infants to long-term neurodevelopmental impairment^{1,2}. Up to 25% of infants that survive PPHN have neurodevelopmental impairments, including moderate-severe cerebral palsy and Bayley Mental and Psychomotor developmental indices below 70, despite advances in intensive care support¹. The onset of PPHN occurs during a time of robust cerebral angiogenesis in the newborn and this process is highly sensitive to changes in blood flow and oxygen levels^{3,4}. The clinical course of infants with PPHN suggests that their adaptation to systemic hypoxemia is impaired. It is unknown if the fetal brain undergoes appropriate vascular remodeling in the presence of PPHN and alterations in oxygen supply *in utero*. Our study investigated the adaptive mechanisms of cerebrovascular remodeling to PPHN in the fetal lamb brain.

PPHN occurs when the pulmonary vascular resistance fails to decrease at birth⁵. The affected infant fails to establish adequate oxygenation during postnatal life and may develop multi-organ dysfunction⁶. Hypoxemia in these infants is due to right to left extra-pulmonary shunting of blood as a result of supra-systemic pulmonary artery pressures. In utero pulmonary hypertension from ductal constriction leads to impaired development of pulmonary vasculature and decreased blood flow from right ventricle to the aortic arch, which may lead to altered cerebral blood flow. Previous studies suggested that altered autoregulation of cerebral blood flow in PPHN may cause hypoxic brain injury and contribute to cognitive deficits in this condition². Studies that evaluated school-aged outcomes in PPHN survivors showed that these outcomes were not significantly improved, despite advances in neonatal intensive care, suggesting that prenatal factors may also predispose these infants to postnatal impairments⁷. Hypoxemia and decreased cerebral blood flow in utero during evolution of PPHN may confer vulnerability to neurodevelopmental impairment.

The cerebral cortex undergoes vascular remodeling in order to preserve oxygen and nutrient supply needed to support neuronal function and metabolic needs. Understanding the mechanisms that facilitate this adaptation may help understand fetal brain adaptation to impaired oxygen states. Our group has shown that fetal lambs exposed to *in utero* PPHN are relatively hypoxemic compared to controls at birth⁸ and that maladaptation to changes in PaO₂ in PPHN in the lung occurs^{9,10}. Sheep have been used extensively to investigate many aspects of CNS homeostasis^{11,12}. The development of the ovine brain at birth is similar to that of the human infant with respect to completion of neurogenesis, cerebral sulcation, and detection of the cortical component of somatosensory evoked potentials^{13,14}. For these reasons, the sheep model is ideal to test the hypothesis that cerebral angiogenesis is impaired in the lamb cerebral cortex, as it is in the lung^{9,10}.

Microvascular remodeling occurs in the cerebral cortex in order to preserve tissue oxygen and energy supply for neuronal function in hypoxic states¹⁵. In the rodent model of chronic hypoxia, exposure to mild hypoxia results in systemic and central nervous system adaptations that allow acclimatization to changes in the microenvironment^{3,16-18}. The first structural changes to chronic hypoxia are seen at 4-7 days and continue for up to 3

weeks^{3,18}. The structural changes not only include increased capillary density, but also capillary length and diameter^{16,17}. Angiogenesis is a highly complex and coordinated process requiring multiple angiogenic and regulatory factors, receptors, and intracellular signaling pathways¹⁵. There appears to be at least two major pathways and likely a number of other redundant pathways responsible for brain angiogenesis: hypoxia-inducible factor-1 (HIF-1) dependent upregulation of vascular endothelial growth factor (VEGF) and HIF-1 independent, cyclooxygenase-2 (COX-2) dependent process with upregulation of angiopoietin-2 (Ang-2)¹⁵. Brain vascular remodeling in response to changes to oxygen availability depends on the balance between these two angiogenic pathways. We hypothesized that dysregulation of these intersecting pathways contribute to impaired cerebral angiogenesis in PPHN.

2. Material and Methods

2.1 Creation of PPHN model

This study was approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee (IACUC) and conformed to the current guidelines of NIH Care and Use of Laboratory Animals. PPHN was induced by fetal ductus arteriosus constriction at 128 ± 2 days gestation (term gestation ≈ 145 days) in twin gestation sheep as we described previously.^{8,19} Control fetal lambs received sham operation. After 8 days of ductal constriction, fetal lambs were delivered by Cesarean section, euthanized, weighed, gender determined, and the brain was quickly removed and weighed. One half of the brain was removed and frozen for protein analysis. The parietal lobe was sectioned, formalin-fixed, and paraffin-embedded for immunohistochemistry.

2.2 Immunohistochemistry and determination of capillary density

Brain samples from normotensive and hypertensive lambs from six separate experiments were fixed in 10% neutral-buffered formalin, processed, and embedded in paraffin. Coronal serial sections (5 μ m) were obtained and mounted on positively charged glass slides (Fisher). For isolectin B4, slides were hydrated with deionized water, antigen retrieval with citrate buffer (pH 6, Dako, Carpinteria, CA, s1699) at 90°C for 20 minutes was performed, and then slides were cooled in citrate buffer for 20 minutes. All slides were stained using a Dako Autostainer Plus using programmed steps for peroxidase blocking and incubation with biotinylated primary antibody (1:200, Vector, Burlingame, CA, b-1205) for 60 minutes in room temperature. Following incubation with antibody overnight at 4°C, slides were rinsed in TBST (Thermo Scientific, Fremont, CA, TA-999-TT), then Dako tertiary with Streptavidin HRP (1:300, Dako, P039701-2) and DAB application (Dako, K346811-2) was performed and the slides were counterstained with hematoxylin (Dako, s33093-2) followed by 0.1% Ammonium hydroxide solution. For Glut-1 and factor VIII a Leica Bond Max Immunostainer was used. Both antibodies required antigen retrieval using Leica HI antigen retrieval reagent (AR9961), with Glut-1 needing 10 minutes and Factor VIII, 20 minutes. Both antibodies were detected and visualized using Bond Polymer Refine Detection System (DS9800) with the addition of a DAB enhancer (AR9436), using the MOD F protocol/ software from Leica. An optimal concentration of 1:900 was determined for Glut-1 (Thermo-Fisher, MS-10637) and 1:100 for Factor VIII (Biocare, CP039). All slides were

counter-stained with hematoxylin and coverslipped using a synthetic mounting media. Omission of the primary antibody served as negative control. Quantification analysis was performed by a researcher blinded to the treatment groups using a computer-assisted method with Visiomorph™ (Visiopharm, Denmark).

2.3 Quantitative Analysis of Image Data

Microscope slides of the histological staining were scanned at 40×, 20×, 10×, and 5× magnification using a Nanozoomer HT system (Hamamatsu, Japan) in the Pediatric BioBank & Analytical Tissue Core at the Children's Research Institute at the Medical College of Wisconsin. The resulting digital images were used for quantitative analysis using commercial image post-processing software (Microimager and Visiomorph, Visiopharm, Denmark). Brown, DAB-positive pixels, blue (Hematoxylin) pixels, and white (background) pixels were classified by the software user using linear Bayesian Classification. Counts and area of DAB- positive events (<5µm and >800µm were excluded) and area of Hematoxylin-positivity were determined and exported to an Excel worksheet, where the number of DAB-positive events (=micro vessels or capillaries) per square millimeter tissue was calculated as count DAB/sum of areas DAB and Hematoxylin.

2.4 Preparation of Whole Cell Lysate

Frozen brain cortex samples from normotensive and hypertensive lambs from six separate experiments were dissected and homogenized in ice-cold lysis buffer (modified RIPA) containing 1% protease inhibitor cocktail (Roche, Nutley, NJ) and centrifuged to remove cell debris. Total protein concentrations of the homogenates were determined with a bicinchoninic acid protein assay (BCA, Pierce, Rockford, IL).

2.5 Western Blot Detection and Quantification of Protein

An aliquot of protein (30µg) was fractionated by SDS-PAGE electrophoresis and transferred onto PVDF membranes (0.2 micron, Bio-Rad Laboratories, Hercules, CA). Membranes were incubated with primary antibodies: HIF-1α (mouse monoclonal, 1:250, Invitrogen, Camarillo, CA), VEGF (mouse monoclonal, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA), COX-2 (rabbit polyclonal, Cayman Chemical, Ann Arbor, MI), Ang-1 (goat polyclonal, 1:500, Santa Cruz Biotechnology), Ang-2 (rabbit polyclonal, 1:500, abcam, Cambridge, MA), and Tie-2 (rabbit polyclonal, 1:200, Santa Cruz Biotechnology). Membranes were then blotted with HRP-conjugated secondary antibody (1:10,000) appropriate for each primary antibody. The membranes were exposed to Hyperfilm ECL (Phenix, Candler, NC) after treatment with SuperSignal West Pico (Pierce). Band intensity was analyzed with NIH ImageJ (US NIH, Bethesda, Maryland, USA, <http://imagej.nih.gov>). The final values represent an average of the densitometric values obtained from two to three different immunoblots. The densitometric values were presented as a ratio to the densitometric values of β-actin used as a housekeeping protein.

2.6 Quantification of mRNA Abundance

RNA was extracted from frozen tissue from normotensive and hypertensive lambs from six separate experiments using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad).

cDNA was synthesized from the extracted RNA using the iScript cDNA synthesis kit (Bio-Rad). The PCR primers were designed for sheep using Primer3 as previously described²⁰ and shown in Table 1. Real-time RT-PCR was performed using the iQ5 multicolor real-time PCR detection system (Bio-Rad). The PCR cycle was started at 95°C for 3 minutes followed by 40 cycles of 95°C for 10 s and then 58°C for 1 min. Melting temperatures were monitored for each pair of primers, and single-peak melting temperature was observed for all of the primer pairs. Relative mRNA level was calculated per equation previously described by Pfaffl²¹ for each gene of interest as 2^{-C_t} with control condition as 1 and the fold increase from this value for PPHN condition shown as mean $\pm$ 1 standard deviation from six separate experiments.

2.7 Statistical Analysis

Data were shown as mean $\pm$ 1 standard deviation. Normally distributed data between two groups was compared using a Student's t-test. Data that did not pass the normality test was analyzed with a Mann-Whitney U test. Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). In all cases, $p < 0.05$ was considered statistically significant.

3. Results

3.1 Effect of PPHN on microvessel density in cerebral cortex

Cerebral capillary density was determined by immunostaining for Glut-1, isolectin B4, and factor VIII immunostaining and counting the number of positively stained capillaries per unit area (N/mm^2). Quantitative analysis showed that the capillary density in the cerebral cortex was significantly decreased ($p < 0.05$) in the PPHN fetal lambs compared to gestation matched controls by all three staining techniques. (Control vs PPHN, $n=6$, $p < 0.05$, Figure 1).

3.2 Effect of PPHN on key angiogenic protein expression

HIF-1 α (120 kDa) level was significantly lower in cortical cell lysates of PPHN lambs compared to twin matched controls ($n=6$, $p < 0.05$, Figure 2A). VEGF (42 kDa) protein levels were also lower in cortical cell lysates of PPHN lambs compared to twin matched controls, consistent with lower HIF-1 α levels in PPHN ($n=6$, $p < 0.05$, Figure 2B). Ang-1 (75 kDa) and Tie-2 (125 kDa) protein levels in cortical cell lysates of PPHN samples were significantly increased compared to gestation-matched twin controls ($n=6$, $p < 0.05$, Figure 2C and 2D). In contrast, Ang-2 (70 kDa) protein level was significantly lower in PPHN lambs ($n=6$, $p < 0.05$, Figure 2E), while COX-2 (72 kDa) protein level was not different ($n=6$, $p < 0.05$, Figure 2F).

3.3 Effect of PPHN on the mRNA levels of key angiogenic pathway genes

The mRNA levels for HIF-1 α , VEGF, COX-2, Tie-2, Ang-1 or Ang-2 genes measured in the fetal cerebral cortex were not significantly different between control and PPHN lambs ($n=6$, figure 3)

4. Discussion

The novel findings of this study are that 1) in utero ductus arteriosus constriction is associated with decreased brain capillary density and 2) this finding is accompanied by decreases in key angiogenesis pathway proteins that mediate cerebrovascular responses to hypoxia in fetal lamb brains. The brain normally adapts to decreased oxygen supply by increasing the capillary density in an effort to maintain adequate cortical tissue oxygenation. Since PPHN is associated with episodes of postnatal hypoxia, impaired mechanisms of angiogenesis in these infants may render them more vulnerable to brain injury.

Cerebrovascular disturbances are the major causes of perinatal brain damage and contribute to the pathogenesis of cerebral palsy and developmental impairments²². An autopsy study of the frontal cortical development from fetal to adult age in humans showed that the density and diameter of vessels increase rapidly after 26 weeks gestation and peaks at 35 weeks²². After 35 weeks of gestation, the density and diameter of vessels are the same as those seen in adults. Their observations suggest that angiogenesis is more rapid than cortical expansion but is equal to the growth of the white matter during third trimester. Therefore, neovascularization may be closely related to normal brain development and its impairment may also lead to impaired white matter development.²⁶ This period of gestation coincides with increasing vulnerability to PPHN from ductus arteriosus constriction in the fetus as well.^{17, 24} In this context, our observations of decreased cortical capillary density in the PPHN exposed animals suggests that this period of angiogenesis is vulnerable to changes in fetal circulation and oxygenation.

At the time of prolific angiogenesis in the developing brain, endogenous HIF-1 α regulated mechanisms are crucially involved in the survival and proliferation of neurons^{23,24}. Several studies have shown that the immature brain is more resistant to hypoxic/ischemic insults than the mature brain, probably related to HIF-1 α signaling^{4,25}. Chronic postnatal hypoxia was associated with increased HIF-1 α expression and increased oxidative metabolism and glucose transporter activity, suggesting a neuroadaptive role for HIF-1 α ²⁶. There is also differential expression of HIF-1 α and its target genes at different stages of brain development during exposure to hypoxia, which implies cellular susceptibility to hypoxic distress^{4,25}. Our study showed decreased expression of HIF-1 α in the fetal brain exposed to PPHN; a result that supports the concept that HIF-1 α is important for normal cerebral angiogenesis. HIF-1, a heterodimer of α and β subunits²⁷, is targeted for proteasomal degradation by prolylhydroxylation of specific domains on the subunit^{28,29}. This process is dependent on the von-Hippel-Lindau tumor suppressor³⁰ which serves as the recognition component of an ubiquitin ligase that promotes degradation of HIF-1 α . PPHN may increase degradation of HIF-1 α through this mechanism, thereby explaining the decreased levels of HIF-1 α seen in our study. This possibility is supported by a lack of difference in the HIF-1 α mRNA levels between control and PPHN samples. The lack of HIF-1 response in PPHN may result in impaired ability to match neuronal activity and capillary density, and explain in part the vulnerability for neurodevelopmental impairment. A recent study in which adult mice that received a HIF prolylhydroxylase inhibitor had both increased HIF-1 α expression in the hippocampus and improved hippocampal memory in fear conditioning suggests that this could be a future target for pharmacologic manipulation³¹.

HIF-1 α induction of VEGF gene expression often represents a critical rate-limiting step in angiogenesis^{15,32,33}. HIF-1 α accumulates in hypoxic states and binds to the hypoxia response element sequence, which upregulates several genes including VEGF³⁴. VEGF functions as a neuronal survival factor and mediates both auto- and paracrine signaling in neurons in vitro³⁵. Astrocyte-derived VEGF mediates survival and tube stabilization of hypoxic brain microvascular endothelial cells in vitro³⁶. VEGF has been shown to have neuroprotective effects in response to ischemia and hypoxia in postnatal rodent brain^{35,37}. Immediate induction of VEGF in developing hypoxic brain contributes to hypoxia tolerance of neurons and astrocytes and the loss of VEGF induction may therefore, increase vulnerability to injury. We observed in this study that VEGF protein expression in the brain was decreased in PPHN, correlating with decreased capillary density in the cortical tissue. These observations may provide a mechanistic basis for increased vulnerability to neuronal injury in PPHN. VEGF mRNA levels remaining unchanged from control despite decreased protein expression may be explained by post-transcriptional gene regulation in this model of PPHN and requires further investigation³⁸.

In the presence of VEGF, capillary destabilization occurs in hypoxic states as a result of Ang-2 binding to its receptor, Tie-2. Under normal oxygen states, Ang-1 is bound to the Tie-2 receptor, resulting in stabilization of the capillary structure and plays a crucial role in mediating reciprocal interactions between the endothelium and surrounding matrix and mesenchyme. Increased levels of Ang-2 can bind Tie-2 and decrease its availability for interaction with Ang-1 during reduced oxygen availability. This can lead to release of pericytes and destabilization of the capillary, a required step in sprouting angiogenesis³⁹. COX-2 signaling is the main pathway for Ang-2 induction in hypoxia⁴⁰. We showed that Ang-1 protein was increased in our model and Ang-2 protein level was decreased in PPHN, suggesting that the vessel remains in a stable state with halted capillary development in PPHN. Astrocytes appear to be a major source of VEGF and Ang production in the brain. Interestingly, dexamethasone treatment of astrocytic cells in vitro showed similar increased expression of Ang-1 and decreased expression of VEGF suggesting a novel role of astrocytes in blood-brain barrier (BBB) stabilization⁴¹. Our findings may reflect an adaptive mechanism for BBB stabilization in the fetal brain that subsequently could impair adaptive responses to postnatal hypoxia. These speculations require further investigation

Our data taken together shows significant brain vascular remodeling in response to in utero exposure to PPHN. This suggests that late gestation is a vulnerable time for vascular development in the brain. These findings are supported by other models of in utero oxidative stress such as intrauterine growth restriction where altered expression of key angiogenic factors occurs⁴². One limitation of our study is that we were unable to measure cerebral blood flow during our studies due to the fragile nature of the ductal ligation model. Further manipulation of the fetus before and after ductal ligation leads to compromised survival of the fetus in our experience. However, our findings of impaired cerebral angiogenesis and its association with our previous report of impaired angiogenesis in the lung in PPHN are novel^{9,10}. These observations may suggest a mechanism for vulnerability to impaired neurodevelopmental outcomes observed in PPHN patients. We were also unable to find significant differences in mRNA levels to correlate transcriptional differences with the significant translational difference of key angiogenic proteins. This result is not so surprising

since typically ~30 to even ~85% of the variation in protein levels in prokaryotic and eukaryotic organisms can be attributed to variation in mRNA expression. The other 15 to 70% of the variation is explained by post-transcriptional and post-translation regulation and by measurement errors⁴³. In fact, the most significant measurements of protein vs. mRNA concentrations collected in tissue and cell culture may center on $R^2 = 0.4^{43}$. That is, agreement between the two measurements occurs less than 40% of the time. For these reasons, our protein expression differences require further investigation of organism-specific regulation of translation and protein degradation to better understand the microvascular changes we see with this model.

5. Conclusions

In conclusion, we observed reduced cortical capillary density in fetal lambs exposed to PPHN induced by in utero ductal constriction. This study is among the first to examine blood vessel formation in the brain associated with PPHN. These findings in the brain are associated with decreased expression of proteins important for angiogenesis, including HIF-1 α , VEGF, and Ang-2. Our results are consistent with findings seen in the fetal lung, and suggest that impaired angiogenesis extends beyond the lung in PPHN. Impaired angiogenesis and reduced HIF-1 α and VEGF expression are typically associated with impaired white matter development and decreased neuroprotective mechanisms in the presence of hypoxia. Our observations suggest that evolution of PPHN is associated with inhibition of normal microvascular development in the brain by down-regulation of angiogenic proteins. The mechanism of decreased cerebral angiogenesis protein expression in PPHN requires further investigation.

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Abbreviations

CNS	Central nervous system
PPHN	Persistent pulmonary hypertension in the newborn
PDA	Patent ductus arteriosus
Glut-1	Glucose transporter-1
HIF-1	Hypoxic inducible factor-1
VEGF	Vascular endothelial growth factor
COX-2	Cyclooxygenase-2
Ang-1	Angiopoetin-1
Ang-2	Angiopoetin-2
Tie-2	Tyrosine-protein kinase receptor

Highlights

- PPHN predisposes infants to long-term neurologic impairments
- PPHN is associated with impaired angiogenesis in the brain
- Impaired cerebral angiogenesis may lead to maladaptation to hypoxic injury

Figure 1. Microvascular density in fetal lamb cerebral cortex in PPHN and gestation matched, twin controls

Images shown at 20× magnification for Glut-1 and isolectin B4 and 10× magnification for factor VII. Capillary density (number per square millimeters) of Glut-1, isolectin B4, and factor VIII-stained parietal lobe sections of control lambs compared to gestation matched PPHN lambs. * $p < 0.05$ from control. Values are means $\pm$ SD; Open bars are control; Closed bars are PPHN, $n=6$.

Figure 2. Angiogenesis pathway protein levels in fetal lamb cerebral cortex in PPHN and gestation matched, twin controls

A, HIF-1 α protein optical density (OD) ratio to internal control, β -actin in frontal lobe cell lysate of control lambs compared to gestation matched PPHN lambs. Similarly, optical density ratios of angiogenesis protein to β -actin are shown for B, VEGF protein levels. C, Ang-1 protein levels. D, Tie-2 protein levels. E, Ang-2 protein levels, and F, Cox-2 protein level. *p<0.05 from control. Values are means $\pm$ SD; Open bars are control; Closed bars are PPHN, n=6.

Figure 3. Comparison of angiogenesis pathway mRNA levels from PPHN fetal lamb cerebral cortex and gestation matched, twin controls

Relative mRNA level was calculated per equation for each gene of interest from frontal lobe as fold difference (2^{-Ct}) with control condition as 1 and the fold increase/decrease from this value for PPHN shown as mean $\pm$ SD from six separate experiments. Each target mRNA was normalized to β -actin mRNA. Open bar indicate control condition and closed bars indicate PPHN condition.

Table 1

The sequence for primers used in RT-PCR

Primer Name	Sequence
HIF-1 α Forward	CAGAAGAACTTTTGGGCCGC
HIF-1 α Reverse	TACAATGCACTGGGGCTGAG
VEGF Forward	TGCTCTCTTGGGTGCATTGG
VEGF Reverse	GAAGCTGCGCTGGTAGACAT
COX-2 Forward	TGTATCCCGCCCTTCTGGTA
COX-2 Reverse	CCGGCTTCTACCATGGTCTC
Ang-1 Forward	CATGCTCGAGATAGGCACCA
Ang-1 Reverse	TCCAGCAGTTGTATTTCAAGTCG
Ang-2 Forward	AGAAGCAACTGGTGACTGCC
Ang-2 Reverse	GTCCTTGGCAAGCAAGGAGTA
B-actin Forward	CAAAGACCTCTACGCCAACAC
B-actin Reverse	GACTCGTCGTA CTCTGCTTG