Caffeine Modulates Hyperoxia - Induced Angiogenesis in Newborn Mice

Vikramaditya Dumpa, MD
Lori C Nielsen, MS
Huamei Wang, MD
Vasanth HS Kumar, MD

Supported by AAP Marshall Klaus Perinatal Research Grant
I have no relevant financial relationships with the manufacturer(s) of any commercial product(s) and/or provider(s) of commercial services discussed in this CME activity.
‘Caffeine therapy for Apnea of Prematurity’ (CAP) Trial

• Caffeine reduces BPD when used within the first 10 days in preterm infants < 1250 grams
  \textit{(Schimdt, B et al N Engl J Med, 2006)}

• “The administration of positive airway pressure through an endotracheal tube, the use of any positive airway pressure, and oxygen therapy were each discontinued one week earlier for infants in the caffeine group than for infants in the placebo group” \textit{— (Schimdt, B et al N Engl J Med, 2006)}

• \textbf{Reduced duration of mechanical ventilation vs.}

• \textbf{Direct pulmonary effect of caffeine}
Possible mechanisms of actions of caffeine

• Caffeine decreases the pulmonary tissue expression of chemokines & leukocyte influx following hyperoxia in newborn mice (Weichelt, U et al Eur Respir J 2013)

• Caffeine promotes anti-inflammatory effects in the immature lung of prenatal LPS-exposed rat pups, associated with improvement of respiratory system resistance (Koroglu, O et al Neonatology 2014)

• Caffeine induces alveolar apoptosis in the hyperoxia-exposed developing mouse lung (Dayanim, S et al Pediatr Res 2014)
Dysregulated angiogenesis – Hallmark of BPD

Bhandari, A et al Pediatrics 2009
**Role of HIF in Lung Development**

- **Normoxia / Hyperoxia**
- **Hypoxia**
- **HIF-1α**
- **HIF-1β**
- **PHD Complex**
- **O₂**
- **HIF-1α-OH** → Proteosomal Degradation

Expression of HIF regulated genes:
- VEGF and its receptors / HO-1 / Angiopoietin 1 / Angiopoietin 2

Expression of genes responsible for angiogenesis / alveolar growth:

- Normal lung growth & function
- ?BPD
Does Caffeine Modulate HIF Expression and Regulation?

HIF-1α

O₂

PHD Complex

HIF-1α-OH

Proteosomal Degradation

Expression of genes responsible for angiogenesis / alveolar growth

VEGF and its receptors / HO-1 / Angiopoietin 1 / Angiopoietin 2

Expression of HIF regulated genes

Nucleus

Hypoxia

HIF-1α

HIF-1β

Normoxia / Hyperoxia

↓ Expression of genes responsible for angiogenesis / alveolar growth

?BPD

Normal lung growth & function
Hypothesis

HIF – Angiogenesis / Growth Factors

Microvascular growth drives alveolar / Lung growth

We hypothesize that caffeine modulates HIF / angiogenic gene expression to attenuate BPD
Objectives

• To study the effects of caffeine on gene expression in hyperoxia induced lung injury in newborn mice. The following genes are studied,
  
  • Hypoxia inducible factors (HIF-1α, HIF-2α, HIF-3α & HIF-1β)
  • VEGF & its receptors (VEGFR1)
  • Prolyl hydroxylase-2 (PHD-2)

• To study the effects of caffeine on histopathology and alveolization in adult mice
Study Design

Treatment Groups (C57/BL6 strain mice; n= 6 in each group)

1. Room Air + Normal saline (RA-NS)
2. RA + Caffeine (RA-CAF)
3. O₂ + NS (O₂-NS)
4. O₂ + Caffeine (O₂-CAF)

Caffeine citrate 20mg/kg/day IP injections qd X 5 days

95% O₂ or RA

Postnatal Day

P0

P4

P5

Sacrifice

mRNA expression of
a. HIF-1α, HIF-2α, HIF-3α, HIF-1β
b. VEGF and VEGFR1
c. PHD2
Proteins (to be assayed)
Molecular studies

• mRNA expression in the lung tissue was analyzed by qRT PCR (Quantified by 2-ΔΔC(t) method)

• Data were analyzed on PCR array online web portal (SA Biosciences, MD)

• P values were calculated based on students’ T test from the 2-ΔΔC(t) values for each gene in the control group (room air) and the treatment group (oxygen with and without caffeine)

• Results are expressed as fold change in mRNA expression compared to RA-NS group (p<0.05 considered significant)
Hyperoxia Increases HIF-1α mRNA Expression in the Lung

HIF-1α

Fold change in gene expression Compared to RA-NS

RA-NS  RA-CAF  O2-NS  O2-CAF

*p <0.05 vs. RA-NS
Caffeine or Hyperoxia by Itself Increases mRNA Expression of HIF-2α, HIF-3α, HIF-1β and VEGF
Caffeine or Hyperoxia by Itself Increases VEGFR1 mRNA Expression in the Lung

**VEGFR1**

*\( p < 0.05 \) vs. RA-NS

**Fold change in gene expression compared to RA-NS**

- RA-NS
- RA-CAF
- O2-NS
- O2-CAF
Caffeine by Itself Increases PHD-2 mRNA Expression in the Lung

**Prolyl Hydroxylase-2**

*\( p < 0.05 \) vs. RA-NS
Angiogenic mRNA Expression

CAFFEINE

HIF-1α
HIF-2α
HIF-3α
HIF-1β
VEGF
VEGFR1
PHD-2

HYPEROXIA

Expression Similar to RA

CAFFEINE + HYPEROXIA
Conclusions

• Caffeine in room air, produces transcriptional changes in HIF gene expression resulting in increased angiogenic mRNA expression

• Exposure to hyperoxia alone causes increased angiogenic mRNA expression, possibly leading to dysregulated angiogenesis

• Caffeine reverses the hyperoxia induced changes in HIF expression and other angiogenic genes in the lung, to that comparable to the room air group.
Histopathology

Treatment Groups (C57/BL6 strain mice; n= 6 in each group)

Room Air + Normal saline (RA-NS)
RA + Caffeine (RA-CAF)
O₂ + NS (O₂-NS)
O₂ + Caffeine (O₂-CAF)

Molecular Studies (HIF / Angiogenesis)
Protein Expression - Pending

Histopathology Quantification - RAC / MLI
Lung Histopathology in 12 Week Mice

• Trachea was cannulated and the lungs were inflation fixed with 10% formalin at 25 cm H₂O pressure

• Lung sections stained with H&E for assessment of alveolization and lung morphometry

• Morphometric analysis was performed by Radial alveolar count (RAC) and Mean linear intercept (Lm)
Hyperoxia decreases alveolization, however caffeine may be protective to the lung.
Hyperoxia decreases alveolization, however caffeine may be protective to the lung.

RA-NS

40X MAGNIFICATION

RA-CAF

O2-NS

O2-CAF
Radial Alveolar Count

- H & E Staining of formalin fixed lung sections –
  - Alveolization
  - Radial alveolar count

- Images captured on Aperio Imaging software (Leica Biosystems, Buffalo Grove, IL)

- Postnatal lung growth was assessed by Radial Alveolar Count of Emery & Mithal, which assesses the complexity of the terminal respiratory unit (Arch Dis Child 1960;35:544-7)
Caffeine Enhances Alveolization In The Presence Of Hyperoxia As Measured By Radial Alveolar Count

*p <0.05 vs. other 3 groups
Mean Linear Intercept

- Images from H & E staining of lung sections were captured on Aperio Imaging software (Leica Biosystems, Buffalo Grove, IL)

- Mean linear intercept (Lm), an indicator of mean alveolar distension, was assessed in 20 non-overlapping fields of lung parenchyma per animal at × 40 magnification in digitalized images
Caffeine Decreases Hyperoxia Induced Air Space Enlargement

*p <0.05 vs. other 3 groups
Conclusions

• Exposure to neonatal hyperoxia leads to impaired alveolization as suggested by lower RAC and airspace enlargement demonstrated by higher Lm.

• Addition of Caffeine in the newborn period during hyperoxia exposure significantly improves alveolization with an absence of air space enlargement.

• This may suggest that caffeine may have a lung protective effect in the neonatal period in the presence of hyperoxia.
Speculation

• The lung protective effect of caffeine in improving alveolization may be related to microvascular growth in the newborn period leading to improved alveolar growth.

• Similar mRNA expression with room air caffeine and hyperoxia groups resulting in different alveolization patterns needs to be explored further.

• Caffeine may modulate HIF-1 signaling pathway & hence regulate the angiogenic gene expression in the presence of oxygen, to promote normal vascular and alveolar growth in the lung, thereby decreasing BPD.
THANK YOU
Caffeine Dosing

- 20 mg/kg caffeine in mice translates to a dose of 2.4 mg/kg in humans.
- Nonetheless, gene expression changes are noted at relatively low doses of caffeine in mice.

*Reagan-Shaw S, The FASEB Journal 2007*
Caffeine with Hyperoxia group demonstrate weight gain as Adult Mice.

Weight at Birth

Weight at 12 weeks
Molecular Studies

- Gene expression in the lung tissue was analyzed by qRT-PCR (Quantified by 2-ΔΔC(t) method; reference genes – phosphoglycerate kinase 1 & peptidylprolyl isomerase A)

- HIF-1α, HIF-2α, HIF-3α and HIF-1β subunits
- VEGF
- VEGFR1
- Prolyl Hydroxylase-2

Pfaffl, MW et al Nucleic acid research, 2001
Alveolization is similar with or without caffeine in adult mice exposed to room air

RA-Saline Group

RA-Caffeine Group
Hyperoxia decreases alveolization, however caffeine may be protective to the lung

O2-Saline Group

100x

O2-Caffeine Group

400x
Cytokine Measurements

The following cytokines were measured in the lung by enzyme immune assay in all the groups:

- TNF – α
- Interferon-γ
- Interleukin-6
No significant Difference in TNF-α Among the Groups
No significant Difference in Interferon-γ in the Lung Among the Groups
No significant Difference in IL-6 in the Lung Among the Groups