Sex-specific and gender-specific research: Examples

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Epidemiology of Neonatal Encephalopathy

Higher Resource Countries

• NE- 3-4/1000
• HIE- 1-2/1000
• Perinatal stroke- AIS and SVT- 1-4000

International Data

• HIE- 23% of all neonatal deaths
• Top 20 leading causes of burden of disease across the lifespan
• 5th – cause of death in children <5
Most report no effect of gender on incidence or outcome.

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<tr>
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<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Male</td>
<td>33 (80.50%)</td>
<td>57 (81.40%)</td>
<td>54 (77.10%)</td>
</tr>
<tr>
<td>Female</td>
<td>8 (19.50%)</td>
<td>13 (18.60%)</td>
<td>16 (22.90%)</td>
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<tr>
<td>Total</td>
<td>41 (100%)</td>
<td>70 (100%)</td>
<td>70 (100%)</td>
</tr>
</tbody>
</table>

Qureshi et al, J Ayub Med Coll Abbottabad 2010;22(4)

Single center study of 70 cases of placental abruption in 4800 deliveries
17 (27.8%) of the infants died

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
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<tbody>
<tr>
<td>Gender of all cases</td>
<td>(34, 56%)</td>
<td>(27, 44%)</td>
</tr>
<tr>
<td>Gender of infants that died</td>
<td>(4, 24%)</td>
<td>(13, 76%)</td>
</tr>
</tbody>
</table>

Nwosu, JOBGyn, 1999, Vol. 19, No. 6 , Pages 612-614
Epidemiology - Effects of Gender

- Whole body cooling – NEJM: 56% male
- Cool Cap- Lancet: 53% male
- TOBY trial- NEJM: 58% male
- ICE trial-Archives of DC: 57% male

None of the hypothermia studies were designed to determine the gender effect on therapeutic outcomes.
Epidemiology - Effects of Gender

Most report no effect of gender on incidence or outcome of HIE.

- However: Males
  - More vulnerable to perinatal insults
  - Worse cognitive outcome with similar insults
  - Overall increase risk for brain-based developmental disorders
  - Lower IQ than females with same degree of IVH

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### Incidence and Outcome stratified by Gender

<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Male 17</td>
<td>Female 14</td>
</tr>
<tr>
<td>Home</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Stepdown</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Deceased</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Length of Stay</td>
<td>5-31 days</td>
<td>1-55 days</td>
</tr>
<tr>
<td></td>
<td>6-48 days</td>
<td>8-48 days</td>
</tr>
</tbody>
</table>

**Overall : 61% male: 39% female**

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Males are selectively afflicted with the neurodevelopmental and psychiatric disorders of childhood, a broad and virtually ubiquitous phenomenon that has not received proper attention in the biological study of sex differences. The literature has alluded to psychosocial differences, genetic factors and elements pertaining to male “complexity” and relative immaturity, but these are not deemed an adequate explanation for selective male affliction. The structure of sex differences in neurodevelopmental disorders is hypothesized to contain these elements: (1) Males are more frequently afflicted, females more severely; (2) disorders arising in females are largely mediated by the genotype; in males, by a genotype by environment interaction; (3) complications of pregnancy and delivery occur more frequently with male births; such complications are decisive and influence subsequent development. We hypothesize that there is something about the male fetus that evokes an inhospitable uterine environment. This “evocative principle” is hypothesized to relate to the relative antigenicity of the male fetus, which may induce a state of maternal immunoreactivity, leading either directly or indirectly to fetal damage..............

Despite the fact that “most report no effect of gender on incidence or outcome of HIE.”

<table>
<thead>
<tr>
<th>Experimental evidence to the contrary</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rodent males have significantly higher testosterone levels from E18-P5 Weisz and Ward, Endocrinology January 1, 1980 vol. 106 no. 1 306-316</td>
</tr>
<tr>
<td>3. p7 Males and Testosterone-treated HI females performed worse on auditory, water maze, and histopathology compared to vehicle treated HI females. Hill et al, Int. J. Devl Neuroscience 29 (2011) 621–628</td>
</tr>
</tbody>
</table>

**Hippocampus**

- Relative volume

**Cerebellum**

- Relative volume

**Brd U IR**

- Images showing differences in cell proliferation.
Putative Mechanisms of Gender-based differences response to HI

Testosterone exacerbates and 17β-Estradiol protects against Glutamate Neurotoxicity

Testosterone withdrawal protects Against MCAO ischemia

Innate Gender specific responses in vitro

Different sensitivity to forms of Cytotoxicity To Glutamate receptor agonists

Innate Gender specific responses *in vitro*

Different Mechanistic responses

**OONO**⁻  **STS**

Different GSH responses

*In vitro and in vivo*

PARP 1 gene effect on response to HI injury and stroke

Effect of PARP 1 gene deletion is exacerbated in adult females

Fundamental differences in injury mechanisms in neonatal HI

**AIF + cells**

**Caspase 3 activity**

**PAR and AIF coexpression**

**ONOO⁻⁺ cells**

No differences in injury, cytochrome c release, autophagy markers,
Gender differences in response to EPO

Gender responses to Experimental HI and Hypothermia

Females

Males

Males p18

J Burnsed, MD, J Zhang, PhD, R Chavez-Valdez, MD, D Flock, BA, K Kesavan, MD, I Burd, MD, and FJ Northington, MD.
PAS meeting 2013
Gender has an effect on response to injury and treatment with Necrostatin after neonatal HI

Northington, Chavez-Valdez et al; JCBFM (2011); 31(1):178-89
Regional and gender specific Neurotrophin responses to HI and Nec treatment

Forebrain

Equivalent activation of TrkB signaling
Predominance of alternative BDNF receptors in females

Diencephalon

Predominance in activation of TrkB signaling in males
Predominance of alternative BDNF receptors in females

Chavez-Valdez, et al Manuscript in preparation
No differences in measures of NO/mitochondrial function after HI or Nec Tx

Chavez-Valdez, Martin et al; Neuroscience (2012); In-press
HI ER dilation is attenuated by Nec tx after HI - are there gender differences?

Vehicle

Necrostatin-1

Chavez-Valdez et al. Unpublished observations.
Endoplasmic Reticulum

nNOS

NO

ROS

Misfolded protein

Endoplasmic Reticulum Response (UPR)

Cytosol

Ca^{2+}

Global translation

ATF4

Xbp1 mRNA

GADD34

CHOP

41kDa XBP1

Golgi

GRP78

PERK

eIF2α

Splicing

Nucleus

ATF6

GRP78

GRP78

ATF6

XBP1

Transcription

Cleavage

Cleaved ATF6

Apoptosis Continuum

Necrosis
Transient increase in GRP78 expression in female mice at 3h

* p< 0.05 (n= 6/group), Wilcoxon test
Relative expression vs. naïve (Pfaffl method)

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Increase in PERK phosphorylation in males at 3h delayed to 24h by NEC tx. EIF2α phosphorylation in male mice at 3h does not occur with Nec-1 treatment.

ANOVA (males) 3h, p=0.04; 24h, p=0.01/ (females) NS
* p<0.05 (n= 3/ group), Tukey Post-hoc.

ANOVA (males) 3h, p=0.01; 24h, NS/ (females) 3h/24h, NS
* p<0.05 (n= 6/ group), Tukey Post-hoc.
Increase in GADD 34 expression in vehicle male mice at 24h blocked with Nec-1 treatment

* p< 0.05 (n= 6/ group), Wilcoxon test
Relative expression vs. naïve (Pfaffl method)

ANOVA (males) 3h, NS; 24h p=0.04/ (females) 3h/24h, NS.
* p< 0.05 (n= 6/ group), Tukey Post-hoc.
Upregulation of Xbp-1 transcription and alternative splicing in veh tx male mice at 24h blocked by Nec tx

* p< 0.05 (n= 6/group), Wilcoxon test
Relative expression vs. naïve (Pffafl method)

Xbp-1 mRNA relative expression

(Males)
Kruskal-Wallis
p = 0.001

(Females)
Kruskal-Wallis
(24H) p=0.37

* p< 0.05 (n= 6/group), Wilcoxon test
Relative expression vs. naïve (Pffafl method)
Increase in CHOP expression in male mice at 24h persist despite Nec-1 treatment

CHOP - mRNA relative expression

ANOVA (males) 3h, NS; 24h, p=0.05/ (females) 3h/24h, NS.

* p< 0.05 (n= 6/ group), Tukey Post-hoc.

CHOP-Protein expression

* p< 0.05 (n= 6/ group), Wilcoxon test

Relative expression vs. naïve (Pfafl method)

* p< 0.05 (n= 6/ group), Tukey Post-hoc.
Hypotheses:
- Neonatal HI induces early ER stress, activating the UPR differentially in males and females.
- Nec-1 protects against ER stress in a gender-biased manner.
Current observations

Gender differences exist in incidence and outcome of neonatal brain injury.

Sex steroid levels likely play a small role in male susceptibility to neonatal brain injury.

Neurons have innately different responses to injury, therapeutics and different mechanisms.

AIF and caspase 3 mechanisms consistently exhibit significant gender dependence.

Gender differences may exist in

– responses to therapeutic hypothermia
– neurotrophin responses after HI
– organelle response to HI.
Neonatal Hypoxia Ischemia

**GRP78**

FEMALES

P<sub>eIF2α</sub>

Xbp1 mRNA

GADD34

PERK

CHOP

ATF4

MALES

NO•

ROS

In forebrain of male mice, neonatal HI induces ER stress with early UPR. Nec-1 prevents UPR without modulating CHOP.

In female mice, neonatal HI induces early GRP78 upregulation.

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**Northington, Chavez-Valdez et al.** JCBFM (2011); 31(1):178-89

Conclusion

• To provide maximal benefit, adjuvant therapies need to address gender specific mechanisms in injury mechanisms, therapeutic and organelle response and be tested with sample sizes adequate to detect gender differences.
Attenuated ER stress in female mice following HI is linked to:
- upregulation of GRP78 \(^{(1)}\), and perhaps to
- differential calcium regulation and/ or
- calpain activation.

Nec-1, by preventing NO• \(^{(2)}\), may attenuate ER stress and need for UPR in male mice.

\(^{(2)}\) Chavez-Valdez et al. (2012) Neuroscience. In-Press
ER STRESS MODEL IN NEONATAL HI

Hypotheses:
- Neonatal HI induces early ER stress, activating the UPR differentially in males and females.
- Nec-1 protects against ER stress in a gender-biased manner.
### Methods:

<table>
<thead>
<tr>
<th>MODEL</th>
<th>TREATMENTS</th>
<th>REGIONS</th>
<th>ANALYSIS</th>
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<tbody>
<tr>
<td>Mice C57B6</td>
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<tr>
<td>PND 7</td>
<td>➔ 15 min</td>
<td>3 h</td>
<td>24h</td>
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<tr>
<td></td>
<td>Naive control</td>
<td></td>
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<tr>
<td>Vannucci HI</td>
<td>Vehicle 0.1 µl</td>
<td></td>
<td></td>
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<tr>
<td>Model</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Unilateral</td>
<td>Necrostatin 0.1 µl (80µM)</td>
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<tr>
<td>carotid ligation</td>
<td>ICV injection 15 min after hypoxia</td>
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- **GRP78**
- **PERK**
- **EIF2α**
- **GADD34**
- **CHOP**
- **XBP-1**

### Gender stratification

- **FOREBRAIN**

### Western blot

- Real time qRT-PCR

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