Nicotine Exposure Alters Gene Expression of Bone Forming Cells from the Murine Calvaria

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Craniofacial Variation

- Craniofacial Growth and Development/Variation
  - Genetic
    - Heredity
  - Epigenetic
    - Hormones
      - Sex Steroids
      - Thyroid
  - Environment
    - Diet
    - Muscle of mastication

- Most Common Clinical Problems
  - Craniosynostosis
  - Cleft Lip and Palate
Craniosynostosis

- Premature fusion of cranial suture(s) while brain is still growing
- ~150 syndromes
- ~1 in 1800-2500 live births
- Some etiologies identified
- Pathogenesis poorly understood
Clinical Problem

• Major Medical Co-morbidities
  • Elevated intracranial pressure
  • Altered intracranial volume
  • Dilation of the subarachnoid spaces
  • Optic nerve compression
  • Papilledema
  • Optic atrophy
  • Blindness
  • Cognitive disabilities
  • Mental retardation
  • Extensive, costly, and recurrent clinical and surgical management problems

• Dental Issues
  • Asymmetry
  • Cross Bites
  • Impaction
  • Crowding
  • Agenesis
  • Delayed and Ectopic Eruptions
  • Oral Hygiene Problems: caries, periodontal disease

• Craniofacial Issues
  • Maxillary Protrusion
  • Maxillary Hypoplasia
  • Jaw discrepancies
  • Post-Orthognathic Surgery
Gene-Environment Interactions

Positive associations between selected environmental exposures and craniosynostosis based on CDC NBDPS data

<table>
<thead>
<tr>
<th>Exposure</th>
<th>OR (95% CI)</th>
<th>Continuous Model¹</th>
<th>CS Type</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine (&gt;300mg/day)</td>
<td>1.34 (1.01 – 1.77)²</td>
<td>-</td>
<td>All</td>
<td>Browne et al., 2011</td>
</tr>
<tr>
<td>Clomiphene Citrate</td>
<td>1.9 (1.2 – 3.0)²</td>
<td>-</td>
<td>All</td>
<td>Reefhuis et al., 2011</td>
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<tr>
<td>Choline</td>
<td>-</td>
<td>p = 0.03</td>
<td>Metopic</td>
<td>Carmichael et al., 2010</td>
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<tr>
<td>Vitamin B12</td>
<td>-</td>
<td>p ≤ 0.01</td>
<td>Metopic</td>
<td>Carmichael et al., 2010</td>
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<tr>
<td>Smoking (≥15 Cig/day)</td>
<td></td>
<td></td>
<td></td>
<td>Carmichael et al., 2008</td>
</tr>
<tr>
<td>Month Before</td>
<td>4.7 (1.3 - 13.7)</td>
<td>-</td>
<td>Lambdoid</td>
<td></td>
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<tr>
<td>First Month</td>
<td>4.6 (1.1 - 14.7)</td>
<td>-</td>
<td>Lambdoid</td>
<td></td>
</tr>
<tr>
<td>Second Month</td>
<td>7.3 (1.7 - 23.2)</td>
<td>-</td>
<td>Lambdoid</td>
<td></td>
</tr>
<tr>
<td>Third Month</td>
<td>7.4 (1.3 - 26.5)</td>
<td>-</td>
<td>Lambdoid</td>
<td></td>
</tr>
<tr>
<td>Second Trimester</td>
<td>8.7 (1.6 - 31.5)</td>
<td>-</td>
<td>Lambdoid</td>
<td></td>
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<tr>
<td>Thyroid Hormone</td>
<td>2.5 (1.5 – 4.2)²</td>
<td>-</td>
<td>Isolated, Single</td>
<td>Rasmussen et al., 2007</td>
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<tr>
<td></td>
<td>2.6 (1.5 – 4.5)²</td>
<td>-</td>
<td>Sagittal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.1 (1.0 – 4.5)²</td>
<td>-</td>
<td>Sagittal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.3 (1.3 – 8.5)²</td>
<td>-</td>
<td>Coronal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.6 (3.9 – 23.7)²</td>
<td>-</td>
<td>Coronal</td>
<td></td>
</tr>
<tr>
<td>SSRIs</td>
<td>2.5 (1.5 – 4.0)²</td>
<td>-</td>
<td>Mixed</td>
<td>Alwan et al., 2007</td>
</tr>
</tbody>
</table>

¹ Exposure modeled as a continuous variable
² Adjusted OR
Significance

• Tobacco product use in pregnancy greatly increases risk for birth defects

• Relative contribution of nicotine, hypoxia, or product additives is unknown

• ~11% of US women smoke through the third trimester of pregnancy (Hall 2014, JAMA)

• E-tobacco products = resurgence in the market

• Need to understand effects on craniofacial development
Hypothesis and Methods

• Hypothesis
  • *Nicotine will alter the osteogenic response of calvarial pre-osteoblasts*

• Methods
  • Murine calvaria cells (MC3T3-E1) exposed to critical doses of caffeine (control) or nicotine in growth or osteogenic media for 3 and 7 days
  • Endpoint assays were designed to determine the effects of the hormone exposure +/- **osteogenic signal**
    • proliferation
    • differentiation (qALP assay)
    • gene expression of *Runx2, Alp, Ocn.*
  • In addition, we performed immunohistochemistry for **nicotinic receptors** (NARA7) in the growth sites of the murine skull, calvarial suture.
Proliferative Response

- Slight increase in proliferation after caffeine administration, $p=0.049$
- Increase in proliferation over time, $p=0.016$
- Greater increase in OM, $p=0.022$
- Nicotine significantly accelerates proliferation with osteogenic environment
Differentiation Response

**Caffeine Effects on Pre-Osteoblast Differentiation**

- OM elicits statistically greater response $p<0.001$
- Overall little observed effect on differentiation

**Nicotine Effects on Pre-Osteoblast Differentiation**

- Greater differentiation by 7 days ($p=0.013$) and OM ($p=0.046$)
- By 7 days there is a statistically significant increase in differentiation with osteogenic environment
Gene Expression

Caffeine Effects on Pre-Osteoblast Gene Expression

- Dose: $10^{-4}$ mol/liter (>average daily use)
- ALP has 2 fold increase at 7 day in GM
- No statistically significant changes

Nicotine Effects on Pre-Osteoblast Gene Expression

- Dose: 25 ng/ml (<plasma nicotine level)
- RUNX2 has two fold increase or greater at 3 and 7 days in GM
- Statistically significant changes in ALP and OCN expression
Nicotinic Receptor in Murine Coronal Suture

A: secondary antibody only; B: positive staining for nicotinic receptor (NARA7) within the suture; C: inset showing density of stain at sutural periosteal junction suggesting rich area of these receptors; D: inset showing similar finding for sutural dural junction
Summary and Conclusions

• Nicotine affects calvarial cells unlike caffeine

• Effects are not simply stimulant driven

• Nicotine has its greatest effect when coupled with an osteogenic signal

• Nicotine increases or decreases gene expression for markers of bone formation depending on condition

• There is a precedent for direct effects of nicotine on craniofacial development via the nicotinic receptors within the calvarial sutures
Future Directions

• Investigate the positive effect of nicotine on osteoblast proliferation
  • TGFβ
  • COL1A1
  • FN1

• Conduct hypoxia directed studies
  • culture mimetics (cobalt chloride)
  • atmospheric conditions (hypoxic chamber)

• Utilize our sensitized murine strain for craniosynostosis (Twist +/-) to model nicotine/hypoxic effects
  • gene expression
  • in-vivo growth and development analysis
Acknowledgments

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  • Dr. Jack Yu, M.D., D.M.D. (Georgia Regents University)