Effects of Exposure to Acetaminophen and Ibuprofen on Fetal Germ Cell Development in both sexes in Rodent and Human Using Multiple Experimental Systems

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September 17, 2018
Over the Counter Analgesics in America

- More than 300,000 over the counter products on the market (on the rise).

- Acetaminophen is the most common drug ingredient in the US. It’s in more than 600 medications (Rx + OTC). Estimated 50 million users weekly.

- Ibuprofen is the most popular OTC NSAID. More than 30 billion doses of NSAID (Rx + OTC) consumed annually in the US.

- Both acetaminophen and ibuprofen are known as analgesics (pain relievers) and fever reducers. Only ibuprofen is classified as a non-steroid anti-inflammatory drug.

References:
2) CPHA.org
3) Medscape.com
**OTC Analgesics: Potential for Abuse**

- Misuse of OTC Analgesics may cause:
  - Liver damage
  - Stomach ulcers
  - Kidney failure
  - Increased CVD risk

- In a study of 500 people, 24% take over the maximum daily dose of acetaminophen.

- Overdoses of acetaminophen hospitalized 30,000 people annually and more than 1,500 people died as a result of accidental overdose between 2001-2010.

- In a study with 1,300 participants, 15% commonly exceed the maximum daily dose.
Pregnancy and Analgesic Use

- In the US, 70-76% of women reported using an analgesic at least once during pregnancy.
- Both Acetaminophen and Ibuprofen can cross the placenta and affect fetal development.
- Epidemiological Studies have associated analgesic use during pregnancy with:
  - Preterm and low birth weight
  - Altered male reproductive development
  - Attention deficit disorder
  - Bronchial asthma
Pregnancy and Analgesic Use

- Studies using in utero exposure to acetaminophen or NSAID have focus on development of fetal reproductive systems.
- Analgesic exposure decreased fetal germ cell number by 40-50% affecting reproductive development.
- Intergenerational effects have been observed for both males and females exposed in utero and could be mediated by epigenetic mechanisms.
Analgesic Inhibition of Cyclooxygenase Pathway

- Tissue injury stimulates production of prostaglandin metabolites.
- Prostaglandins stimulate inflammatory mediators and nociceptors on sensory neurons.
- Both NSAIDs and acetaminophen inhibit prostaglandin production.
Proposed Mechanism for Analgesic Alteration of Epigenetic Patterns in Fetal Germ Cells

- NSAID (Ibuprofen), Acetaminophen
- Inhibition of COX2 enzyme
- Fetus has increased COX2 expression during development
- PGE2
- Altered pluripotency
- Altered expression of DNMTs
- Altered chromatin marks
- Altered expression of epigenetic regulators
Objective

This study aims to confirm their proposed mechanism for in utero analgesic exposure effect on germ cell development using a combination of in vitro and in vivo approaches.

Significance

The study uses therapeutically relevant doses for analgesic treatment. The study will investigate a possible epigenetic component for the previously observed reproductive effects and decrease in germ cell numbers.
First trimester human fetal gonads (testes and ovaries) were cultured using the hanging drop method and treated with 10uM Acetaminophen and Ibuprofen for 7 days. The number of germ cells were recorded.
Second trimester human testes were xenografted onto CD1 nude mice and treated with either acetaminophen or ibuprofen. The number of germ cells were recorded.
Studies to determine the mechanism for analgesic action on fetal germ cells were done in vitro using both a human tumor derived germ cell line and fetal rat gonads cultured using hanging drop method. Used prostaglandin antagonists/agonists to observe PGE2 role in reduction of GC number.
Figure 1: Study Design

Pregnant dams were exposed to analgesics during fetal germ cell development and epigenetic reprogramming. Fetal gonads were harvested and used to study the expression of epigenetic regulators.
They use 1mm³ sections of first trimester fetal testes and ovary tissue and grow each section in a different well of the hanging drop plate.

Gravity and tension allow 3D growth rather than growing flat on a dish.

Tissues grown in hanging drop culture are fixed in formaldehyde and stained with antibodies marking different germ cell populations within the gonads.
Figure 2. Effect of Acetaminophen or Ibuprofen on GC number in 1st trimester fetal testes

Germ cell number was determined with fluorescence immunostaining.

TFAP2C+ = gonocytes

MAGEA4+= spermatogonia

KI67+= Proliferating
Figure 3. Effect of Acetaminophen or Ibuprofen on GC number in first trimester fetal ovaries

Germ cell number was determined with fluorescence immunostaining. TFAP2C= gonocyte
Human Fetal Testis Xenograft Method

- Second trimester human fetal testis xenografted into castrated male CD1 mouse (immunodeficient)
- hCG injections to mimic *in utero* environment
Figure 4. Effect of 7 d acetaminophen or ibuprofen exposure of second-trimester fetal human testis tissue xenografts on GC number.

Total Germ Cells

Gonocytes
Figure 5. Effect of 7 d acetaminophen or ibuprofen exposure of second-trimester fetal human testis tissue xenografts on GC proliferation.

**Acetaminophen (7 days)**

- **A**: KI67⁺ GC %
- **B**: KI67⁺ GC %

**Ibuprofen (7 days)**

- **C**: KI67⁺ GC %
- **D**: KI67⁺ GC %

- **Total**: Fetus

**Proliferating total GC**

**Proliferating gonocytes**
Figure 6. Effect of 1 d acetaminophen exposure of second-trimester fetal human testis tissue xenografts on GC number and GC proliferation.
Figure 7. Effect of exposure of NTera2 cells to analgesics or prostaglandin E2-receptor modulators on cell number

NTera2 cells

- Pluripotent human embryonal carcinoma cell line
- Express markers of fetal GC
- Passage number: 25 - 35
- Good practices:
  - Tested for mycoplasma infection by ELISA
  - Genotyping to confirm cell lineage?
Figure 8. Effects of Acetaminophen, Ibuprofen, or EP2 + EP4 Antagonists on mRNA Expression of GC differentiation Markers in NTera2 Cells
Figure 8. Effects of Acetaminophen, Ibuprofen, or EP2 + EP4 Antagonists on Expression of Epigenetic Regulatory Genes in NTera2 Cells

- **D:** Acetaminophen
  - TET1
  - EZH2
  - DNMT3A
  - DNMT3B

- **E:** Ibuprofen
  - TET1
  - EZH2
  - DNMT3A
  - DNMT3B

- **F:** EP antagonists
  - TET1
  - EZH2
  - DNMT3A
  - DNMT3B

- **G:** H3k27me3
  - Veh
  - Acet
  - Ibu
  - EP-Antags
Figure 9. In vitro Effects of Acetaminophen, Ibuprofen, or EP2 + EP4 Antagonists on Fetal Rat Gonads
Figure 10: Effect of Acetaminophen on rat fetal gonads exposed in utero

***Exposed once daily by gavage

In-vivo exposure of rat fetal gonads

A B

e15.5 e17.5

Testis

Fold difference

Tet1 Ezh2 Tet1 Ezh2

* p=0.011 * p=0.01

p=0.039 p=0.17

C D

e15.5 e17.5

Ovary

Fold difference

Tet1 Ezh2 Dnmt3a Dnmt3b Tet1 Ezh2

*** p=0.0001 ** p=0.0016

p=0.371 p=0.185 p=0.764

Vehicle
Acetaminophen (350mg/kg/day)
Conclusions

- Acetaminophen and Ibuprofen treatment for seven days reduce germ cell number in both first trimester male and female human gonads treated in vitro.
- Acetaminophen and Ibuprofen treatment for seven days reduce germ cell number in second trimester human testes xenografts. Acetaminophen reduces germ cell number after only 24h.
- Prostaglandins are associated with reduction in germ cell number in vitro after treatment with Acetaminophen and Ibuprofen for 48h.
- Acetaminophen alters pluripotency markers in NT2 germ cells.
- Acetaminophen and Ibuprofen alter epigenetic regulatory genes EZH2, TET, DNMTs in NT2 germ cells and in rat fetal gonads treated in vitro.
- Acetaminophen alters EZH2, TET, and DNMT expression in rat fetal gonads exposed in utero during epigenetic reprogramming.
Discussion Questions

**Team Pulczinski**
Did the authors achieve their stated objective to determine if analgesic reduction in GC number in male and female gonads is mediated by the PGE2 pathway? Are there any other pathways that could be involved?

**Team Casin**
The authors show epigenetic regulatory proteins TET1, EZH2, and DNMT3a/3b have altered expression following exposure to analgesics. What are some follow-up experiments that could be used to further elucidate the epigenetic component of their proposed mechanism?

**Team Illingworth**
Assess the strength of their methods. Were any controls missing from these experiments? Were the statistical methods appropriate? Were there other major limitations worth discussing?

**Team Yeung**
Assuming their mechanism for PGE2 mediated effects on germ cell development is correct, what effect could this study have on public health (research and in general)?