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Supplemental Material

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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Supplemental Materials and Methods

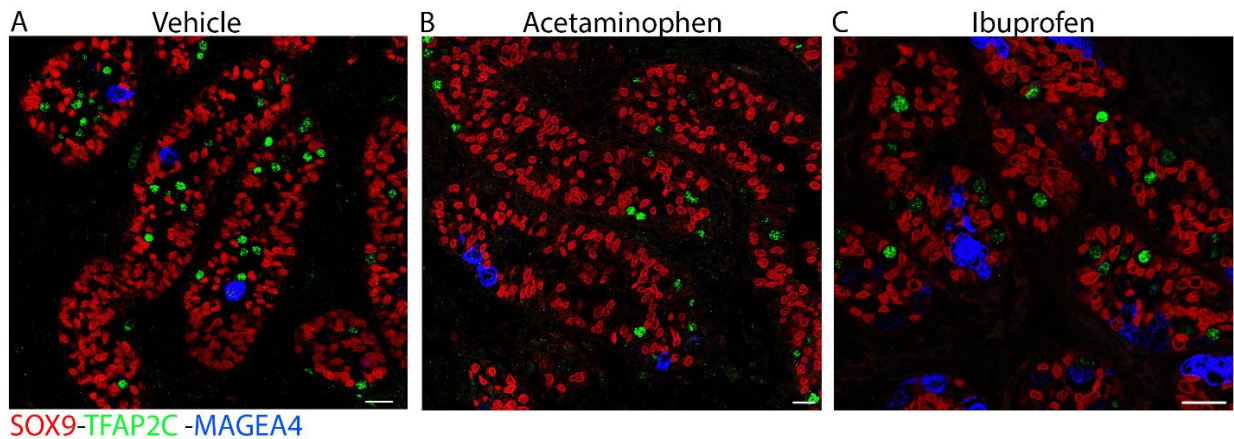
NTERA2

Primer sequences for gene expression analysis

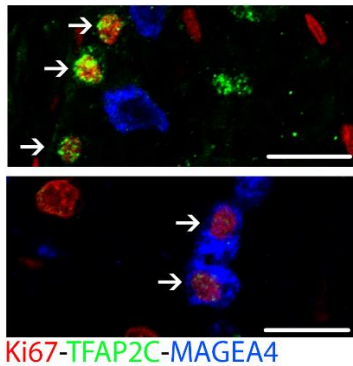
Human primers: *TET1* forward primer: 5'-gatgacagaggttcttgacacat-3', reverse primer: 5'-aggtgacacggctcagtg-3', probe number #86 (cat. no. 04689119001); *EZH2* forward primer: 5'-aagaagagaagaaagatgaaactcg-3', reverse primer: 5'-ttggtgttgacaccgagaat-3', probe number #82 (cat. no. 04689054001); *DNMT3a* forward primer: 5'-cctgaagcctcaagagcagt-3', reverse primer: 5'-tggtctccttctgttcttgc-3', probe number #46 (cat. no. 04688066001); *DNMT3b* forward primer: 5'-ggtgactgagctcgaaag-3', reverse primer: 5'-aagaggtgctggatgacagg-3', probe number #3 (cat. no. 04685008001); *POU5F1* forward primer: 5'-cttcggattcgtctctcg-3', reverse primer: 5'-cttagccaggtccgaggat-3', probe number #22 (cat. no. 04686969001); *TFAP2C* forward primer: 5'-gagccaaatcgaaaaatgga-3', reverse primer: 5'-gccaaatgaacagcttcacc-3', probe number #7 (cat. no. 04685059001); *NANOG* forward primer: 5'-atgcctcacacggagactgt-3', reverse primer: 5'-cagggtgctctgaataagc-3', probe number #69 (cat. no. 04688686001); *TBP* forward primer: 5'-gccatagtgatcttgcagt-3', reverse primer: 5'-cgctggaactgctcacta-3', probe number #67 (cat. no. 04688660001). Rat primers: *Tet1* forward primer: 5'-agagggaaaagaagcccaaa-3', reverse primer: 5'-aacaacccaaccttgc-3', probe number #60 (cat. no. 04688589001); *Ezh2* forward primer: 5'-gactggtgaagagttgttcttga-3', reverse primer: 5'-ctcgttcgatgccacata-3', probe number #122 (cat. no. 04693566001), *Dnmt3a* forward primer: 5'-aacggaagcgggatgagt-3', reverse primer: 5'-gcaatcaccttgcttctt-3', probe number #75 (cat. no. 04688988001); *Dnmt3b* forward primer: 5'-caaatccaggactgcag-3', reverse primer: 5'-accactagcacccttctt-3', probe number #94 (cat. no. 04692110001).

Figure S1

1st trimester fetal testis culture



D Proliferation



1st trimester fetal ovary culture

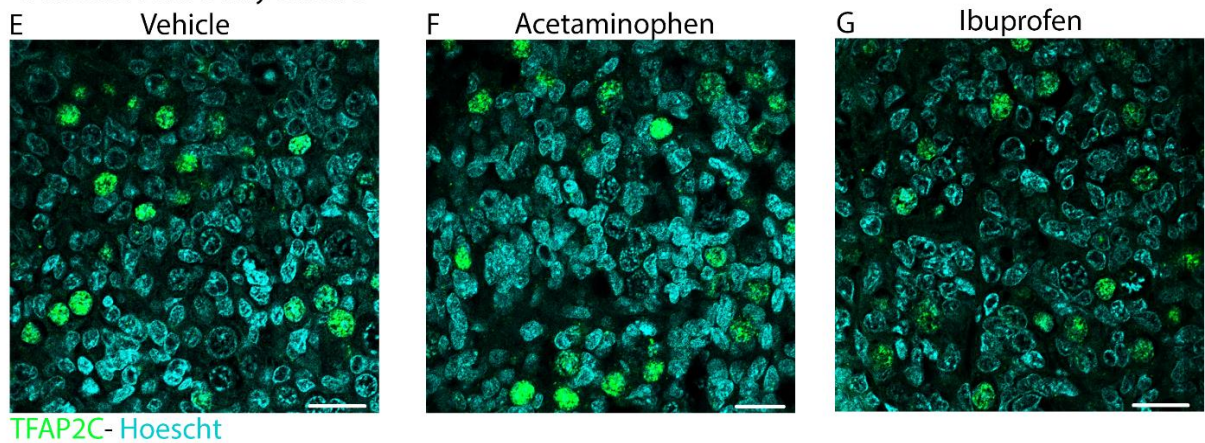
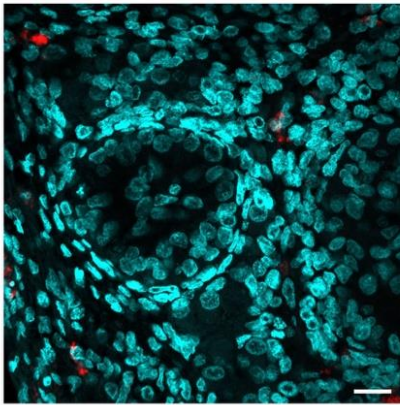


Figure S1 – Immunofluorescence in first trimester human fetal testis and ovary culture. A-C) SOX9 (Sertoli cells), TFAP2C (GC) and MAGEA4 (differentiated GC) immunostaining in first trimester fetal testis exposed to vehicle (A), acetaminophen (B) and Ibuprofen (C). D) Proliferation (Ki67, white arrows) in germ cells (TFAP2C or MAGEA4). E-G) TFAP2C (GC) and Hoechst (counterstaining) immunostaining in first trimester fetal ovaries exposed to vehicle (E), acetaminophen (F) and Ibuprofen (G). Scale bars, 20 μ m.

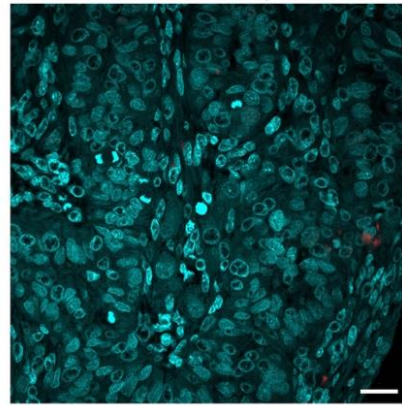
Figure S2

1st trimester fetal testis culture

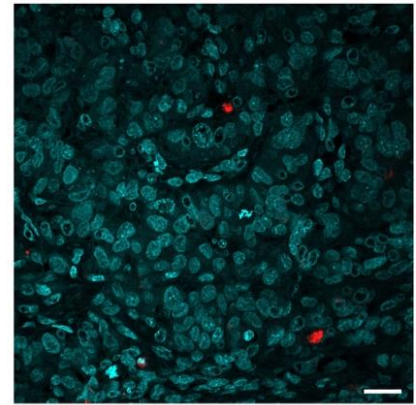
A Vehicle



B Acetaminophen

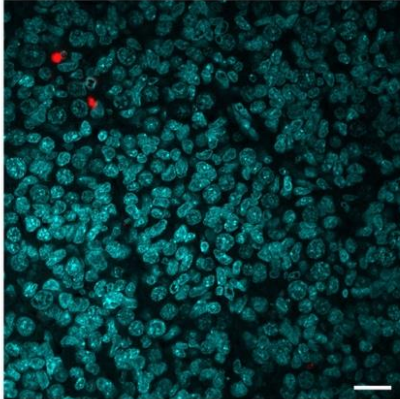


C Ibuprofen

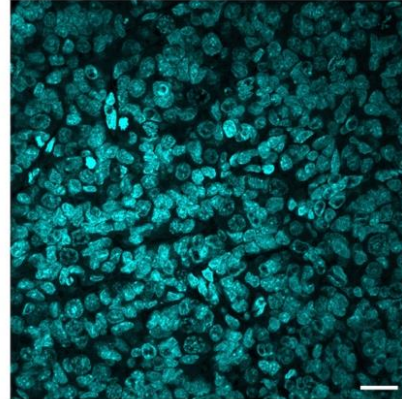


1st trimester fetal ovary culture

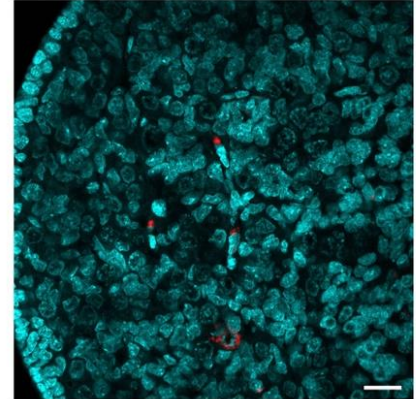
D



E

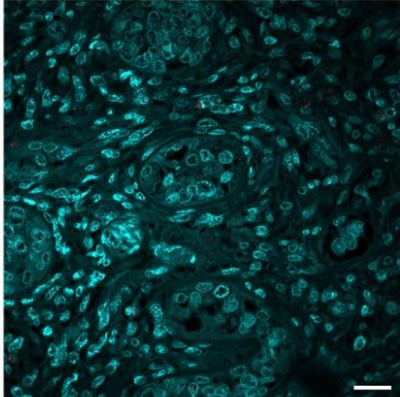


F

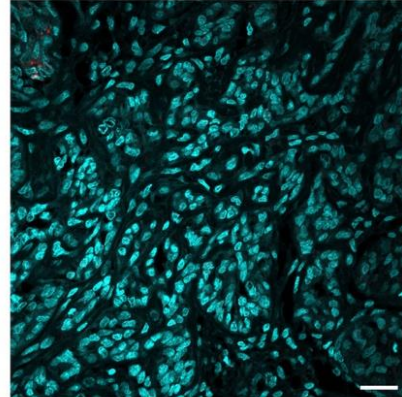


2nd trimester fetal testis xenograft

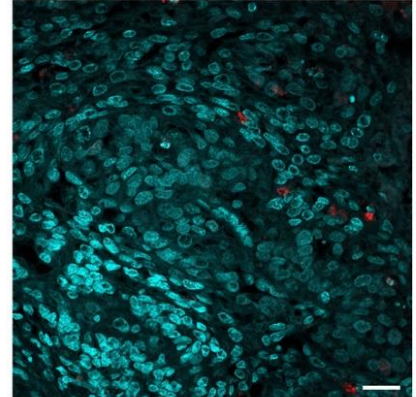
G



H



I



Cleaved caspase 3- Hoescht

Figure S2. Effect of analgesic exposure on apoptosis. Representative images of immunofluorescence for apoptosis (Cleaved caspase 3) as a consequence of analgesic exposure was studied in 3 different systems: 1st trimester fetal testis (A-C) and ovary (D-F) culture, and 2nd trimester fetal testis xenograft (G-I) exposed to human relevant doses of acetaminophen (10 μ M, A-F; 20mg/kg three times daily, G-I) and ibuprofen (10 μ M, A-F; 10mg/kg three times daily, G-I). Quantification of apoptotic cells was not performed due to the rarity of these cells. Hoescht (counterstain). Scale bar, 20 μ m.

Figure S3

2nd trimester fetal testis xenografts

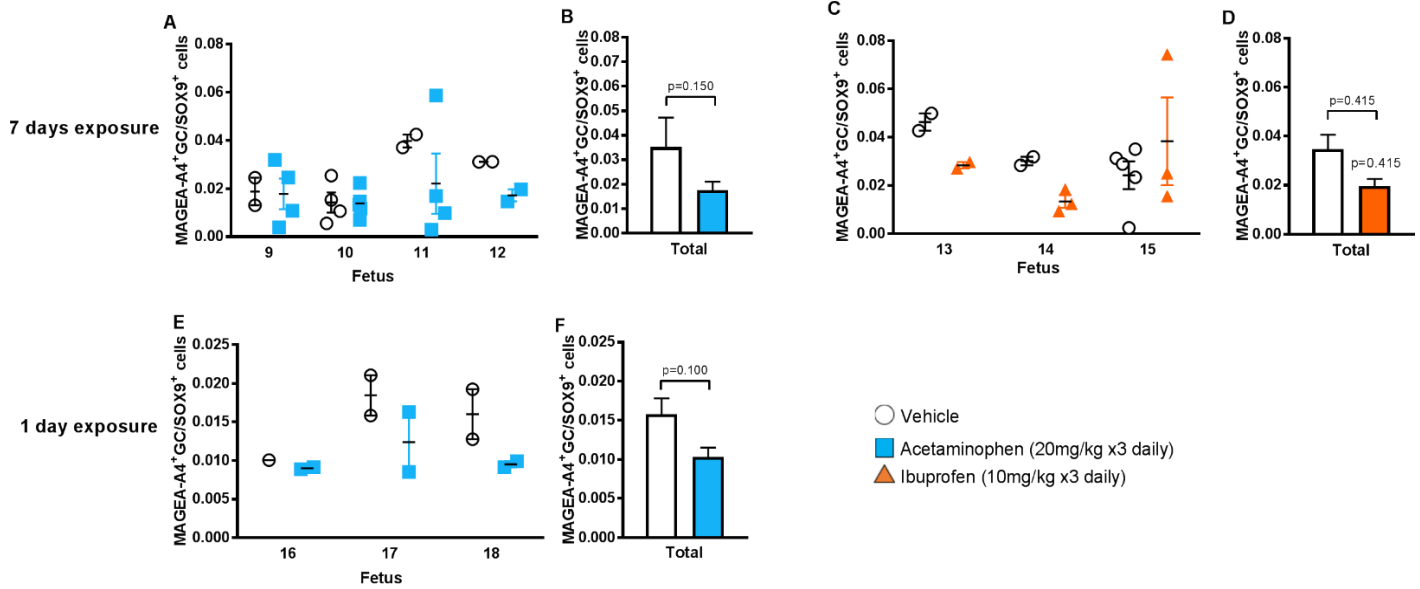


Figure S3. Effect of acetaminophen or ibuprofen exposure of 2nd trimester fetal human testis tissue xenografts on MAGE-A4⁺ germ cell (GC) number. Testis tissue pieces (~1mm³) from 4 fetuses (acetaminophen) or 3 fetuses (ibuprofen) were xenografted into nude mice. Host mice were administered vehicle, acetaminophen (20mg/kg, three times daily) for 1 or 7 days, or ibuprofen (10mg/kg three times daily) for 7 days before xenograft recovery at day 7. Tissues were fixed and immunostained to identify Sertoli cells (SOX9⁺), undifferentiated GC cells (TFAP2C⁺), and differentiated GC cells (MAGEA4⁺). MAGEA4⁺ GC counts were determined as a proportion of MAGEA4⁺ cells per SOX9⁺ Sertoli cells. **A, C** and **E** show individual data points representing total MAGEA4⁺ cells for each tissue piece per treatment and fetus, with horizontal lines indicating the mean for each treatment and fetus.. **B, D** and **F** show means (\pm SEM) over all tissue grafts for each fetus, and treatment. *P*-values shown in panels **B, D**, and **F** compare values in treated samples to vehicle controls using two-factor ANOVA to account replicate tissue pieces for each fetus and multiple fetuses per treatment. Gestational age of fetuses 9-18 were

Figure S4

NTERA2 cell culture

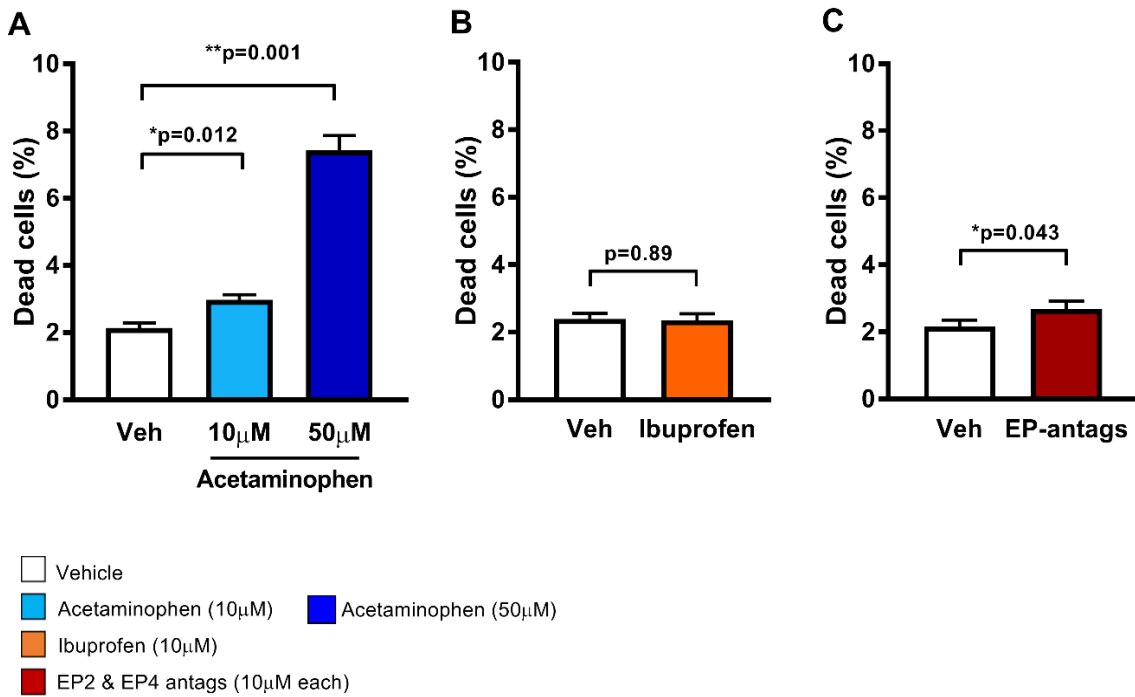


Figure S4. Effect of exposure of NTERA2 cells to analgesics or prostaglandin E2-receptor modulators on cell death. NTERA2 cells (10^5 ; $n=3$) were cultured for 48h in medium supplemented with either vehicle (Veh), acetaminophen (10-50 μ M), ibuprofen (10 μ M) or EP2+EP4 antagonists [EP-antags: 10 μ M L-161,982 (EP2 antagonist) + 10 μ M PF04418948 (EP4 antagonist)]. The percentage of NTERA2 dead cells at the end of the experiment for each treatment is shown in panels A – C. NTERA2 dead cells were determined by flow cytometry from the same cultures as in Figure 7. Graphs show means \pm SEM from three independent experiments with three replicates each and statistical analysis was by 2-factor ANOVA;