Parental Alcohol Exposures Associate with Lasting Mitochondrial Dysfunction and Accelerated Aging in a Mouse Model

Alison Basel, Sanat S. Bhadsavle, Katherine Z. Scaturro, Grace K. Parkey, Matthew N. Gaytan, Jai J. Patel, Kara N. Thomas, Michael C. Golding
Supplementary Figure 1. Experimental flowchart depicting the timeline of parental alcohol exposures, mouse breeding, gestation, and offspring assessments.
Supplementary Figure 2. Maternal, paternal, and dual parental alcohol consumption exert sex- and treatment-specific effects on offspring normalized organ weights. We compared bodyweight-normalized (A) adrenal, (B) heart, (C) kidney, (D) pancreas, (E) spleen, (F) thymus, and (G) testis weights between the treatment groups. We analyzed datasets using a two-way ANOVA followed by Tukey’s post hoc test. Data represent mean ± SEM, (n=9-29) * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
Supplementary Figure 3. Maternal, paternal, and dual parental alcohol consumption induce markers of premature cellular senescence in the postnatal day 300 offspring kidney. We used reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) analysis to compare transcripts encoding (A) p16, (B) p21Ink4a, (C) Cyclin D1 (Ccdn1), and (D) Lamin-B1 (Lmnb1) between treatments. We used a two-way ANOVA followed by Tukey’s post hoc test to compare treatment groups. Data represent mean ± SEM, (n=8) * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
Supplementary Figure 4. Analysis of clinical markers of liver damage in the female offspring of alcohol-exposed parents. Comparison of (A) alanine transaminase (ALT) and (B) aspartate transaminase (AST) between treatments. (C) Comparison of AST:ALT ratios between treatment groups. We used a two-way ANOVA to compare treatment groups. Data represent mean ± SEM, (n=8).
**Supplementary Figure 5.** Maternal, paternal, and dual parental alcohol consumption induce treatment-specific changes in mitochondrial DNA copy number within the postnatal brain and kidney. We used quantitative polymerase chain reaction (qPCR) to measure mitochondrial DNA copy number between the postnatal day 300 (A) brain and (B) kidney between treatment groups and analyzed the data using a two-way ANOVA followed by Tukey’s post hoc test to compare treatment groups. Data represent mean ± SEM, (n=8) * P < 0.05, *** P < 0.001, **** P < 0.0001.

**Supplementary Table 1.** Descriptions of the sample sizes and statistical tests for each figure.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Litter and sex information per treatment group</th>
<th>Number of Litters</th>
<th>Number of Males</th>
<th>Number of Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>11</td>
<td>28</td>
<td>27</td>
</tr>
<tr>
<td>Maternal</td>
<td></td>
<td>10</td>
<td>29</td>
<td>22</td>
</tr>
<tr>
<td>Paternal</td>
<td></td>
<td>12</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td>Dual</td>
<td></td>
<td>9</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td><strong>Graph</strong></td>
<td><strong>Statistical Test</strong></td>
<td><strong>Sample Size</strong></td>
<td></td>
<td><strong>Outliers</strong></td>
</tr>
<tr>
<td>B: Sire body weight</td>
<td>Two-way ANOVA, multiple comparisons using Sidak.</td>
<td>n = 16 control 15 ethanol</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>C-D: Average daily dose of EtOH</td>
<td>One-way ANOVA, multiple comparisons using Tukeys, or Unpaired t test</td>
<td>C: n = 19 paternal 17 maternal preconception 22 maternal gestation 11 paternal 8 dual</td>
<td>J: n = 13 preconception control 17 preconception ethanol 20 gestation control 20 gestation ethanol</td>
<td>2 preconception control 1 gestation control</td>
</tr>
<tr>
<td>E-F: Maternal daily dose and food intake</td>
<td>Two-way ANOVA, multiple comparisons using Sidak.</td>
<td>n = 13 preconception control 17 preconception ethanol 20 gestation control 20 gestation ethanol</td>
<td></td>
<td>2 preconception control 1 gestation control</td>
</tr>
<tr>
<td>G-H: Daily calories and weight gain</td>
<td>Unpaired t test.</td>
<td>G: n = 22 control 20 ethanol</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H: n = 22 control 20 ethanol</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

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### SUPPLEMENTARY DATA

#### I-J: Gestation length and litter size

<table>
<thead>
<tr>
<th></th>
<th>Analysis</th>
<th>Comparison Method</th>
<th>Sample Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Kruskal-Wallis</td>
<td>Multiple comparisons using Dunn’s.</td>
<td>11 control 12 maternal 11 paternal 8 dual</td>
</tr>
<tr>
<td>J</td>
<td></td>
<td></td>
<td>10 control 12 maternal 11 paternal 8 dual</td>
</tr>
</tbody>
</table>

#### K: Sex ratio

<table>
<thead>
<tr>
<th></th>
<th>Analysis</th>
<th>Comparison Method</th>
<th>Sample Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chi-Square analysis followed by Fisher’s Exact test for individual comparisons.</td>
<td></td>
<td>55 control 51 maternal 46 paternal 27 dual</td>
</tr>
</tbody>
</table>

#### Figure 2: Parental alcohol exposures induce sex- and treatment-specific effects on offspring lean weight and normalized organ weights

##### A-B: Body weight analysis

<table>
<thead>
<tr>
<th></th>
<th>Analysis</th>
<th>Comparison Method</th>
<th>Sample Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Two-way ANOVA</td>
<td>Multiple comparisons using Uncorrected Fisher’s LSD.</td>
<td>28 control 21 maternal 29 paternal 19 dual</td>
</tr>
<tr>
<td>b</td>
<td></td>
<td></td>
<td>27 control 25 maternal 23 paternal 9 dual</td>
</tr>
</tbody>
</table>

##### C-G: DEXA scan analysis

<table>
<thead>
<tr>
<th></th>
<th>Analysis</th>
<th>Comparison Method</th>
<th>Sample Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>Two-way ANOVA</td>
<td>Multiple comparisons using Sidak.</td>
<td>10 control 6 maternal 11 paternal 11 dual</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td>9 control 8 maternal 8 paternal 9 dual</td>
</tr>
</tbody>
</table>

##### H-I: Organ to body weight

<table>
<thead>
<tr>
<th></th>
<th>Analysis</th>
<th>Comparison Method</th>
<th>Sample Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>We inserted organ weights into Excel, then divided by total body weight. Two-way ANOVA, multiple comparisons using Tukey.</td>
<td></td>
<td>28 control 21 maternal 29 paternal 19 dual</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td>27 control 25 maternal 23 paternal 9 dual</td>
</tr>
</tbody>
</table>

#### Figure 3: Increased markers of cellular senescence in the brains of offspring derived from alcohol-exposed parents

##### B: B-gal quantification

<table>
<thead>
<tr>
<th></th>
<th>Analysis</th>
<th>Comparison Method</th>
<th>Sample Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>Two-way ANOVA</td>
<td>Multiple comparisons using Tukey.</td>
<td>6 control 6 maternal 6 paternal 6 dual</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td>6 control 6 maternal 6 paternal 6 dual</td>
</tr>
</tbody>
</table>

##### C: Senescent genes

<table>
<thead>
<tr>
<th></th>
<th>Analysis</th>
<th>Comparison Method</th>
<th>Sample Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>Two-way ANOVA</td>
<td>Multiple comparisons were done to the control group using Dunnett.</td>
<td>8 control 8 maternal 8 paternal 7 dual</td>
</tr>
</tbody>
</table>

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### Figure 4: parental alcohol exposures program cumulative effects on the male offspring’s predisposition to develop senescence-associated liver disease

<table>
<thead>
<tr>
<th>A-D: Senescent genes</th>
<th>Two-way ANOVA, multiple comparisons were done to the control group using Dunnett.</th>
<th>Males: n = 8 control 8 maternal 8 paternal 8 dual</th>
<th>B: 1 dual male 1 paternal female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Females: n = 8 control 8 maternal 8 paternal 8 dual</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-H: Histology</td>
<td>Ordinary One-way ANOVA, multiple comparisons using Tukeys.</td>
<td>n = 8 control 8 maternal 8 paternal 8 dual</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Females: n = 8 control 8 maternal 8 paternal 8 dual</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-K: Liver function</td>
<td>I&amp;K: Ordinary One-way ANOVA, multiple comparisons using Tukeys.</td>
<td>n = 8 control 8 maternal 8 paternal 8 dual</td>
<td>1: 1 maternal</td>
</tr>
<tr>
<td></td>
<td>J: Kruskal-Wallis, multiple comparisons using Dunn’s.</td>
<td></td>
<td>K: 1 maternal</td>
</tr>
</tbody>
</table>

### Figure 5: Stress-induced senescence induced by chronic parental alcohol use correlates with evidence of hepatic mitochondrial dysfunction

<table>
<thead>
<tr>
<th>C-D: S/OPA1-L/OPA1</th>
<th>Ordinary One-Way ANOVA, multiple comparisons using Fisher’s LSD.</th>
<th>Males: n = 9 control 9 maternal 9 paternal 9 dual</th>
<th>C: 1 control 1 dual</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Females: n = 6 control 6 maternal 6 paternal 6 dual</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E: Total OPA1</td>
<td>Two-way ANOVA, multiple comparisons were done to the control group using Dunnett.</td>
<td>Males: n = 9 control 9 maternal 9 paternal 9 dual</td>
<td>1 control male 1 maternal male</td>
</tr>
<tr>
<td></td>
<td>Females: n = 6 control 6 maternal 6 paternal 6 dual</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F: Total OMA1</td>
<td>Two-way ANOVA, multiple comparisons using Tukey.</td>
<td>Males: n = 6 control 6 maternal 6 paternal 6 dual</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Females: n = 5 control 6 maternal 6 paternal 6 dual</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G: Mt copy number</td>
<td>Two-way ANOVA, multiple comparisons using Tukey.</td>
<td>Males: n = 8 control 8 maternal 8 paternal 8 dual</td>
<td>1 control female</td>
</tr>
<tr>
<td></td>
<td>Females: n = 8 control 8 maternal 8 paternal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### SUPPLEMENTARY DATA

| H: ELISA IL-6 | Two-way ANOVA, multiple comparisons using Tukey. | Males: n = 5 control, 5 maternal, 5 paternal, 5 dual | 8 dual | 0 |
| None | None | None | None | None |
| None | None | None | None | None |

| I: NAD/NADH ratio | Two-way ANOVA, multiple comparisons were done to the control group using Dunnett. | Males: n = 4 control, 4 maternal, 4 paternal, 4 dual | 0 |
| None | None | None | None | None |
| None | None | None | None | None |

### Figure 6: Offspring of alcohol-exposed parents exhibit decreased Sirtuin protein abundance and increased measures of oxidative damage

#### A: ELISA Sirt1
Ordinary One-way ANOVA, multiple comparisons were done to the control group using Dunnett.

| n = 8 control, 8 maternal, 8 paternal, 8 dual | 0 |
| None | None |

#### B: SIRT3 quantification
Ordinary One-Way ANOVA, multiple comparisons using Fisher’s LSD.

| n = 6 control, 6 maternal, 6 paternal, 6 dual | 0 |
| None | None |

#### C: MDA assay
Ordinary One-way ANOVA, multiple comparisons using Tukeys

| n = 8 control, 8 maternal, 8 paternal, 8 dual | 0 |
| None | None |

#### D: H3K9Ac quantification
Ordinary One-way ANOVA, multiple comparisons using Tukeys

| n = 6 control, 6 maternal, 6 paternal, 6 dual | 0 |
| None | None |

#### M-N: H3K27me3 and H3K9me3 quantification
Ordinary One-Way ANOVA, multiple comparisons using Fisher’s LSD.

| n = 6 control, 6 maternal, 6 paternal, 6 dual | 1 paternal |
| None | None |

### Supplemental Figure 2: Normalized organ weights

#### A: Adrenal gland
Two-way ANOVA, multiple comparisons using Uncorrected Fisher’s LSD.

| Males: n = 28 control, 21 maternal, 29 paternal, 19 dual | Males: 1 paternal, 2 dual |
| Females: n = 27 control, 25 maternal, 23 paternal, 9 dual | Females: 1 control, 1 maternal |

#### B: Heart
Two-way ANOVA, multiple comparisons using Tukeys.

| Males: n = 28 control, 21 maternal, 29 paternal, 19 dual | Males: 1 control, 2 maternal |
| Females: n = 27 control, 25 maternal, 23 paternal, 9 dual | Females: 0 |

#### C: Kidney
Two-way ANOVA, multiple comparisons using Uncorrected Fisher’s LSD.

| Males: n = 28 control, 21 maternal, 29 paternal | Males: 0 |
| Females: n = 27 control, 25 maternal, 23 paternal, 9 dual | Females: 0 |

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<table>
<thead>
<tr>
<th>A-D</th>
<th>Senescent genes qPCR</th>
<th>Two-way ANOVA, multiple comparisons were done to the control group using Dunnett.</th>
<th>Males: n = 8 control 8 maternal 8 paternal 7 dual</th>
<th>Females: n = 8 control 8 maternal 8 paternal 8 dual</th>
<th>B: 1 maternal female C: 1 control male D: 2 maternal males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplemental Figure 3: RT-qPCR to compare senescent transcripts in the kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-D</td>
<td>ALT and AST analysis</td>
<td>Ordinary One-Way ANOVA, multiple comparisons Tukeys</td>
<td>n = 8 control 8 maternal 8 paternal 8 dual</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Supplemental Figure 4: Female ALT and AST liver analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-D</td>
<td>mtDNA/ncDNA analysis in brain</td>
<td>Two-way ANOVA, multiple comparisons using Tukeys.</td>
<td>Males: n = 8 control 8 maternal 8 paternal 7 dual</td>
<td>Females: n = 8 control 8 maternal 8 paternal 8 dual</td>
<td>Males: 1 maternal female Females: 1 control</td>
</tr>
<tr>
<td>Supplemental Figure 5: Mitochondrial copy number in the brain and kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-D</td>
<td>mtDNA/ncDNA analysis in kidney</td>
<td>Two-way ANOVA, multiple comparisons using Tukeys.</td>
<td>Males: n = 8 control 8 maternal 8 paternal 7 dual</td>
<td>Females: n = 8 control 8 maternal 8 paternal 8 dual</td>
<td>Males: 1 maternal female Females: 1 dual</td>
</tr>
</tbody>
</table>

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Supplementary Table 2: Sequence information for the PCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>CCACCATGTACCCAGGCATT</td>
<td>CGGACTCATCGTACTCCTGC</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>CTGATGTATGCCAAGCGTGC</td>
<td>TCGCCTTCCACAGAATCCAC</td>
</tr>
<tr>
<td>P21(WAF/Cip1)</td>
<td>GTTCCTTGGCCACTTCTTACCT</td>
<td>GGTGAGTCTCTAATGCCATCC</td>
</tr>
<tr>
<td>P16Ink4a</td>
<td>CGCTGAGGTTGGTCTTGTGTA</td>
<td>GCTCTGCTCTTGAGATTGGC</td>
</tr>
<tr>
<td>CCND1</td>
<td>TGGCGCAAGAGATTGTG</td>
<td>CTTCCTCAAGGGCTCAAGG</td>
</tr>
<tr>
<td>LMNB1</td>
<td>ATCAACCAATGGTGCTT</td>
<td>TCCTCGGCTATGGTGCTT</td>
</tr>
<tr>
<td>D-Loop3</td>
<td>TCCTCGTGAACCAACAAA</td>
<td>AGCGAGAAGAGGGGCAATT</td>
</tr>
<tr>
<td>Tert</td>
<td>CTAGCTCATGTGCAAGACCCTTT</td>
<td>GCCAGGAGGTCTCTCAGTT</td>
</tr>
</tbody>
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