APPENDIX AVAILABLE ON THE HEI WEB SITE

Research Report 166

Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity

Part 1. Biologic Responses in Rats and Mice to Subchronic Inhalation of Diesel Exhaust from U.S. 2007-Compliant Engines: Report on 1-, 3-, and 12-Month Exposures in the ACES Bioassay

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Appendix I. ACES Three-Way ANOVA Results at 4 and 13 Weeks

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Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award CR-83467701 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

This document was reviewed by HEI's ACES Review Panel
but did not undergo the HEI scientific editing and production process.

ACES – Rats (4 and 13 Weeks)[1]
Traditional Three-Way ANOVA[2] p-values
Exposure Effect Modeled as 4 Experimental Group Means

Endpoint	Gender	Exposure	Gender x Exposure	Time	Gender x Time	Exposure x Time	Gender x Exposure x Time
Cytokine CINC-3 Lung	0.003	0.376	0.751	0.045	0.794	0.895	0.677
Cytokine IL-1β Lung	0.002	0.435	0.084	0.123	0.454	0.596	0.179
Cytokine IL-6 Lung	0.952	0.121	0.234	<0.001	0.321	0.744	0.068
Cytokine KC Lung	0.064	0.609	0.340	0.008	0.276	0.681	0.680
GSH Lung	0.302	0.005	0.570	<0.001	0.507	0.860	0.564
GSSG Lung	0.131	0.342	0.574	<0.001	0.148	0.346	0.602
HO-1	0.368	<0.001	0.709	<0.001	0.355	0.112	0.869
Total Glutathione Lung	0.480	0.012	0.596	0.039	0.385	0.566	0.507
ALP	0.495	0.015	0.963	0.064	0.204	0.398	0.600
LDH	0.006	0.980	0.446	0.161	0.123	0.451	0.444
Macrophages Absolute	<0.001	0.105	0.198	<0.001	0.785	0.618	0.471
PMN Absolute Count	0.890	0.425	0.580	0.146	0.765	0.937	0.536
Total Cells	<0.001	0.099	0.261	<0.001	0.680	0.575	0.491
µTP	0.018	0.018	0.428	0.422	0.268	0.002	0.600
Albumin BALF	<0.001	0.006	0.429	0.050	0.737	0.001	0.926
GSH BALF	0.959	0.187	0.874	<0.001	0.851	0.675	0.582
GSSG BALF	0.040	0.709	0.159	<0.001	0.001	0.617	0.192
Hemoglobin	0.855	0.497	0.806	<0.001	0.793	0.203	0.494
TEAC	0.004	<0.001	0.567	0.080	<0.001	0.367	0.787
Total Glutathion BALF	0.700	0.135	0.868	<0.001	0.557	0.352	0.382

ACES – Rats (4 and 13 Weeks)[1]
Alternative Three-Way ANOVA[3] p-values
Exposure Effect Modeled as Continuous (Trend) Variable[,4]

Endpoint	Gender	Exposure	Gender x Exposure	Time	Gender x Time	Exposure x Time	Gender x Exposure x Time
Cytokine IL-1β Lung	0.002	0.398	0.012	0.125	0.450	0.303	0.920
Cytokine IL-6 Lung	0.947	0.032	0.163	<0.001	0.323	0.452	0.063
GSH Lung	0.309	0.001	0.151	<0.001	0.481	0.746	0.184
HO-1	0.358	<0.001	0.777	<0.001	0.351	0.026	0.515
Total Glutathione Lung	0.478	0.001	0.177	0.038	0.362	0.630	0.190
µTP	0.022	0.030	0.137	0.440	0.286	0.111	0.614
Albumin BALF	<0.001	0.013	0.105	0.056	0.771	0.268	0.855
TEAC	0.007	0.001	0.797	0.099	<0.001	0.099	0.651

[1]Shaded cells reflect endpoints for which there was statistical evidence ($p<0.05$) of exposure effects (either differences between means or exposure-related trends) in one- or two-way time-specific ANOVAs
[2] Degrees of freedom associated with sources of variation: Gender (1); Exposure (3); Gender x Exposure (3); Time (1); Gender x Time (1); Exposure x Time (3); Gender x Exposure x Time (3)
[3] One degree of freedom associated with each source of variation
[4]Coding of Exposure Groups in trend analysis (Control = 0, Low = 1, Mid = 2, High = 3)

ACES – Rats (4 and 13 Weeks)[1]
Interpretation of Three Way ANOVA Results for Endpoints That Were Highlighted in the LRRI Report

Apart from Cytokine 1β, all of the endpoints that exhibited statistically significant ($p<0.05$) evidence of exposure effects in the time-specific one- and two-way ANOVAs also showed statistically significant evidence of exposure effects in three-way ANOVAs based on experimental group means or those based on exposure-related trends. In the interpretations that follow," three-way ANOVA" refers to the traditional approach, unless otherwise specified.

HO-1: There was strong evidence ($p<0.001$) of exposure effects for HO-1 in the three-way ANOVA. As there were no time-related interactions, the three-way ANOVA for HO-1 suggests that the effects were consistent across time points, and the absence of gender-related interactions indicates consistency of effects across genders. This pattern was evident from the one and two-way ANOVAs.

TEAC: The three-way ANOVA for TEAC indicated that the relationship between gender mean values at the two time points differed, and there was marginally significant ($p=0.099$) evidence that exposure effects differed at the two time points. The two-way ANOVAs showed strong evidence of an exposure effect in both genders at 13 weeks, and no substantial evidence of an effect at 4 weeks.

μTP: The three-way ANOVA gave evidence of an exposure effect ($p=0.018$ for differences between experimental group means) that was consistent across genders. There was also evidence that the exposure effect on μTP varied across time (based on the strongly significant $p=0.002$ exposure x time effect). The two-way ANOVAs confirmed this, with statistically significant evidence of exposure-related effects (based on trends) only at 13 weeks.

Albumin BALF: Apart from evidence of systematic temporal variation, the significance pattern for albumin experimental group means in the three-way ANOVA was similar to that of μTP. The two-way ANOVAs indicated consistent effects across genders only at 13 weeks.

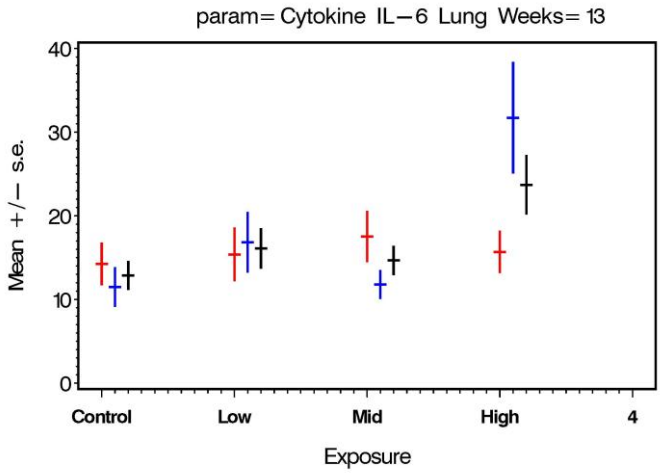
GSH Lung: The three-way ANOVAs based on experimental group means and trends both gave evidence of exposure effects, with little, if any evidence to suggest that effects differed by gender or time. Time specific two-way ANOVAs indicated that gender-based effects could be pooled, resulting in evidence of exposure-related trends at both time points.

Total Glutathione Lung: The three-way ANOVAs based on experimental group means and trends both gave evidence of exposure effects, with little, if any evidence to suggest that effects differed by gender or time. Time specific two-way ANOVAs indicated that gender-based effects could be pooled, resulting in evidence of exposure-related trends at both time points.

Cytokine IL-1β: As indicated above, there was no substantial evidence from the three-way ANOVA (either for experimental group means or trends) of exposure effects on Cytokine 1β. The two-way ANOVAs gave limited evidence of an exposure effect – only in females at 13 weeks. At this time point, there was also a significant exposure x gender interaction effect ($p=0.012$) that substantiates the absence of a similar effect in males shown with a one-way ANOVA – and the inappropriateness of pooling and reporting effects across genders. Thus, the appropriateness of highlighting the Cytokine 1β pooled gender effect in the report should be reconsidered..

Cytokine IL-6: The three-way ANOVA gives marginal evidence of an exposure effect measured as differences between experimental group means ($p=0.121$), and more substantial evidence from the three-way ANOVA-based assessment of exposure-related trend ($p=0.032$), but also some evidence of a high order interaction between gender, time, and exposure ($p=0.065$),

suggesting the appropriateness of time and gender specific analyses. The two-way and one-way ANOVAs gave limited evidence of an exposure effect, with a significant trend shown only for females at 13 weeks. Although the gender x exposure interaction term in the two-way ANOVA at 13 weeks provides no evidence of a difference in exposure effects across genders (p=0.506), thereby justifying the pooling of effects across genders, the absence of clearer evidence of correspondence of effects across genders (as indicated in the graph below; males red, females blue, both black), makes it difficult to support the existence of an exposure-related trend across genders, even with a significant p-value (p=0.038). Thus, the evidence of an exposure-related effect should be judged as weak.



ACES – Mice (4 and 13 Weeks)[1]
Traditional Three-Way ANOVA[2] p-values
Exposure Effect Modeled as 4 Experimental Group Means

Endpoint	Gender	Exposure	Gender x Exposure	Time	Gender x Time	Exposure x Time	Gender x Exposure x Time
Cytokine IL-1β Lung	<0.001	0.635	0.564	<0.001	<0.001	0.731	0.404
Cytokine IL-6 Lung	<0.001	<0.001	0.750	<0.001	<0.001	0.173	0.099
Cytokine KC Lung	0.494	0.182	0.696	<0.001	0.041	0.334	0.254
Cytokine TNF-a Lung	0.016	0.247	0.910	<0.001	0.143	0.317	.
GSH Lung	<0.001	0.112	0.051	<0.001	<0.001	0.138	0.074
GSSG Lung	0.173	0.735	0.432	<0.001	0.190	0.743	0.419
HO-1	0.290	0.378	0.618	<0.001	0.328	0.422	0.451
Total Glutathione Lung	<0.001	0.158	0.141	<0.001	<0.001	0.189	0.184
Albumin BALF	0.077	0.013	0.170	0.001	<0.001	0.145	<0.001
Alkaline Phosphatase	0.293	0.858	0.709	<0.001	0.338	0.892	0.708
GSH BALF	0.767	0.769	0.476	0.230	0.750	0.727	0.596
GSSG BALF	0.054	0.192	0.327	0.836	<0.001	0.901	0.101
Hemoglobin	0.029	0.859	0.815	<0.001	0.009	0.369	0.464
LDH	0.591	0.002	0.833	0.002	0.929	0.007	0.713
Protein	0.960	0.827	0.540	<0.001	0.901	0.786	0.524
TEAC	0.877	0.374	0.014	<0.001	0.271	0.454	0.015
Total Glutathione BALF	0.513	0.849	0.477	0.030	0.227	0.831	0.520
PMN Absolute	0.354	0.074	0.166	0.923	0.956	0.411	0.906
PMN Differential	0.657	0.042	0.090	0.352	0.967	0.394	0.940

ACES – Mice (4 and 13 Weeks)[1]
Alternative Three-Way ANOVA p-values[3]
Exposure Effect Modeled as Continuous (Trend) Variable[4]

Endpoint	Gender	Exposure	Gender x Exposure	Time	Gender x Time	Exposure x Time	Gender x Exposure x Time
Cytokine IL-6 Lung	<0.001	0.005	0.922	<0.001	<0.001	0.106	0.265
Albumin BALF	0.090	0.021	0.100	0.002	<0.001	0.062	0.059
LDH	0.605	0.024	0.964	0.003	0.929	0.124	0.825
PMN Absolute	0.354	0.003	0.166	0.923	0.956	0.411	0.906
PMN Differential	0.657	0.271	0.090	0.352	0.967	0.394	0.940

[1]Shaded cells reflect endpoints for which there was statistical evidence ($p<0.05$) of exposure effects (either differences between means or exposure-related trends) in one- or two-way time-specific ANOVAs
[2] Degrees of freedom associated with sources of variation: Gender (1); Exposure (3); Gender x Exposure (3); Time (1); Gender x Time (1); Exposure x Time (3); Gender x Exposure x Time (3)
[3] One degree of freedom associated with each source of variation
[4]Coding of Exposure Groups in trend analysis (Control = 0, Low = 1, Mid = 2, High = 3)

ACES – Mice (4 and 13 Weeks)[1]
Interpretation of Three Way ANOVA Results for Endpoints That Were Highlighted in the LRRI Report

All of the endpoints that exhibited statistically significant ($p<0.05$) evidence of exposure effects in the time-specific one- and two-way ANOVAs also showed statistically significant evidence of exposure effects either in three-way ANOVAs based on experimental group means or those based on exposure-related trends. In the interpretations that follow," three-way ANOVA" refers to the traditional approach, unless otherwise specified.

Cytokine IL-6: The three-way ANOVA indicates a strong difference between experimental group means for genders at the two time points, and marginal evidence of a high order interaction between gender, exposure, and time ($p\approx0.1$) that suggests that time specific analyses may be more appropriate. The two-way ANOVA at 4 weeks provides evidence of an exposure-related effect across genders, but there is little, if any evidence of the same cross-gender effect at 13 weeks.

Albumin BALF: The three-way ANOVA gives strong evidence ($p<0.001$) of a gender x time x exposure interaction effect that suggests that individual analyses at the gender/time combinations would be most appropriate. The two-way and one-way time-specific ANOVAs gave no evidence of an exposure effect at 4 weeks. There was evidence of an effect for females only at 13 weeks from the one-way ANOVA, and the significant gender x time interaction at 13 weeks ($p=0.038$) indicates that the albumin effects were not consistent across genders, and should not be pooled. Given the absence of evidence in males at 13 weeks, the overall evidence of an exposure effect is weak.

LDH: The three-way ANOVA indicates that exposure effects varied across time point (exposure x time $p=0.007$). Time-specific two-way ANOVAs showed no evidence of exposure effects at 4 weeks and evidence of an increasing trend across both genders at 13 weeks.

PMN absolute: The three-way ANOVA gives marginal ($p=0.074$) evidence of an exposure effect, with little, if any evidence to suggest differences in effects relating to gender or time. The alternative ANOVA based on exposure-related trends gives substantial ($p=0.003$) evidence of an exposure-related trend across genders and time points. The time specific two-way ANOVAs suggest that exposure effects can be pooled, but the one-way ANOVAs gives marginal evidence of a positive trend in females at 13 weeks ($p=0.051$) and 4 weeks ($p=0.110$), but no evidence of exposure trends in males at either time point. Given the relatively weak evidence in females, and the absence of systematic evidence across genders, the significance of the effect is questionable.

PMN differential: The three-way ANOVA gives statistically significant ($p=0.047$) evidence of an exposure effect, with little, if any evidence to suggest differences in effects relating to gender or time. There is no evidence of an exposure effect in the alternative three-way ANOVA based on exposure-related trends ($p=0.271$). The time specific two-way ANOVAs suggest that exposure effects can be pooled, but the one-way ANOVAs showed evidence a positive trends in females only at 13 weeks ($p=0.034$), and 4 weeks ($p=0.098$); there was no evidence of exposure trends in males at either time point. Given the relatively weak evidence in females, and the absence of systematic evidence across genders, the significance of the effect is questionable.