Abstract # 2414

The National Toxicology Program Research on Synthetic Turf/Recycled Tire Crumb Rubber: 14-Day Exposure Characterization Studies of Crumb Rubber in Female Mice Housed on Mixed-Bedding or Dosed via Feed or Oral Gavage

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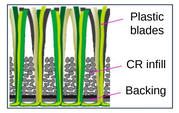
Background

- There is potential for widespread exposure to crumb rubber due to use as infill in synthetic turf fields.
- Crumb rubber manufactured from recycled tires contains potential carcinogenic and toxic substances.



Modified from Cheng et al. 2014

- The National Toxicology Program is conducting research to improve the understanding of potential human exposure and health impacts following crumb rubber exposure, focusing on these questions:
  - What experimental models are useful for characterizing the toxicity of crumb rubber or bioaccessible constituents?
  - What routes of exposure are most likely to result in systemic exposure?
  - What constituents of crumb rubber are bioaccessible, bioavailable, or both?
  - How does the bioaccessibility or bioavailability of constituents vary based on route of exposure?
  - Is biological activity or effect evident following exposure to crumb rubber?
- Presented here are the results from 14-day studies in B6C3F1/N female mice exposed to crumb rubber on mixed-bedding or dosed via feed or oral gavage.
  - Endpoints focus on evidence of systemic exposure through detection of chemical constituents in biological fluids, and by using conventional methods to detect biological effect.

Methods

Material

- Crumb rubber, received from the California Office of Environmental Health Hazard Assessment (OEHHA) (Cristy et al. Abstract # 2415), was used in these studies.
- Benchtop work (Richey et al., Abstract #2417) was performed to determine the feasibility of performing in vivo studies; based on this work the material was size fractionated for use in these studies.
  - The smallest size fraction, 400 mesh (Figure 1), was used in the oral gavage studies and the combined size fraction (Figure 2) was used in the dosed-feed and mixed-bedding studies

Figure 1. Image of 400 mesh Crumb Rubber

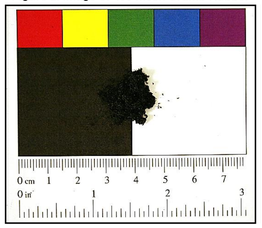


Figure 2. Image of Combined Crumb Rubber

Study Design

- Design Rationale:
  - Female mice were selected to conserve test material in gavage studies, compared with rats, and in the dosed-feed and mixed-bedding studies compared to male mice (individual housing) or rats.
  - Dose selection for the gavage studies was selected based on availability of 400 mesh crumb rubber.
  - For dosed-feed, 50,000 ppm was selected as the exposure concentration as a limit dose.
  - For the bedding studies, a 50/50 wt./wt. bedding mixture was selected to provide a high environmental exposure, while still including absorbent bedding; this mixture extended the length of time between cage changes, conserving crumb rubber material.
- Groups of 15 female B6C3F1/N mice were exposed to crumb rubber for 14 days via oral gavage (0 or 1250 mg/kg/day in corn oil), dosed-feed (0 or 50,000 ppm) or mixed-bedding (0/0 or 50/50 wt./wt.).

Endpoints

- Biological Sample Collection:
  - Following the seventh dose (for gavage), animals (n=5 per group) were transferred to metabolism cages for overnight urine collection. In the morning, animals were returned to their home cages and resumed dosing/exposure.
  - On the final day of dosing (for gavage), animals were euthanized and plasma was collected.
- On the day following the final dose (for gavage), the core animals (n=10 per group) were removed and the following endpoints evaluated:
  - Hematology and bone marrow cytology
  - Histopathology of major organs and glands

Test Article Characterization

Size Characterization

- Characterization data are summarized in Table 1.
- Particle size for the 400 mesh fraction was determined by scanning electron microscopy using an JOEL (Peabody, MA) JSM-7600F.
- Particle size of the combined fraction was determined by optical microscopy using an Olympus (Tokyo, Japan) SZK12 stereomicroscope.

Table 1. Crumb Rubber Particle Size Summary[a]

Study	Fraction Used	Size Range (µm)	Avg. Length (µm)	Length Range (µm)	% Total by Weight
Oral gavage	400	37 - 170[b]	46.0	8.3 - 167	0.34
Dosed-feed Mixed-bedding	Combined	Greater than 170[c]	2135	500 - 4400	99.66

[a] Size characterization was performed on 65 and 23 particles for the 400 mesh and combined fractions, respectively.

Elemental Composition

- Energy dispersive spectroscopy analysis for both size fractions of material were obtained using an EDAX (Mahwah, NJ) ApolloX Silicon Drift Detector.
- Spectra of ten particles from each size fraction were analyzed and the weight percent of the detected elements was determined (Table 2).
- These results are consistent with preliminary results of the characterization of the constituents of the original crumb rubber test article received from OEHHA (Cristy et al. Abstract #2415).

Table 2. Elemental Composition (weight %) of 400 mesh and Combined Crumb Rubber

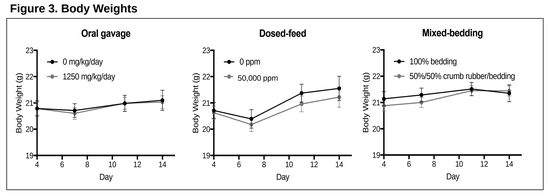
Element	400 Mesh	Combined
C	81.9 ± 1.90	80.0 ± 5.20
O	9.67 ± 1.86	10.5 ± 1.10
Si	0.17 ± 0.15	3.60 ± 5.22
S	3.90 ± 1.21	2.53 ± 0.67
Zn	3.64 ± 1.71	2.23 ± 1.21
Al	0.11 ± 0.15	0.39 ± 0.65
Ca	0.07 ± 0.08	0.35 ± 0.64
Cl	0.33 ± 0.13	0.26 ± 0.30
Mg	0.16 ± 0.07	0.14 ± 0.06
Ti	0.00 ± 0.01	0.01 ± 0.02

In-Life Data and Toxicological Endpoints

Survival and Body Weights

- There were no effects on survival or body weights (Figure 3) following exposure to crumb rubber by oral gavage (1250 mg/kg/day), dosed-feed (50,000 ppm) or mixed-bedding exposure (50/50 wt./wt.) for 14 days.

Figure 3. Body Weights



Crumb Rubber Consumption

- Qualitatively, the feces from animals exposed to crumb rubber by oral gavage and in feed appeared darker in color (dark brown to black) compared to feces from control animals.
  - When viewed under microscopic magnification, feces contained black particulate not typical of rodent feces. The dark color of a subset of feces and the presence of black particulate suggest crumb rubber passed through the gastrointestinal tract.
- By observation, fecal output from 1250 mg/kg animals was similar to controls, indicating crumb rubber did not obstruct movement of ingested material.
- Feces from 50,000 ppm animals appeared black in color on study day 4, but much lighter than day 4 on subsequent observations, indicating avoidance of crumb rubber in feed compared to early in the study.

Toxicological Endpoints

- Hematology, bone marrow cytology and histopathology of all major organs and glands were performed as tools to assess whether systemic exposure to crumb rubber constituents occurred and if there was evidence of biological effect following crumb rubber exposure.
- Preliminary review of hematology and bone marrow cytology data indicate that no biologically relevant changes were observed following exposure to crumb rubber.
- Higher incidences of esophageal inflammation were observed in crumb rubber gavage animals compared to respective controls, however the severity was similar between treated and control animals.

Evaluation of Systemic Exposure

Plasma and Urine Samples

- Individual animal samples: (5 animals per route per group; n=30 urine and n=29 plasma)
- Pooled samples (1 per route per group; n=6 urine, n=6 plasma): Based on availability of individual animals samples, equal volumes of samples within a group were pooled.

Chemical Analysis

- Samples were prepared for a reversed phased analysis using a metabolomics approach on a high resolution UPLC-MS system (positive and negative ion mode).
  - Waters Acquity UPLC and a Synapt G2-Si ESI-Q-TOF mass spectrometer; chromatographic separation was accomplished on an Acquity HSST3 C18 column (2.1 X 100 mm, 1.8 µm)
- Spectra of samples were imported into Progenesis QI software (Nonlinear Dynamics, UK) to determine differentiating features between treated and control samples by route.

Data Analysis

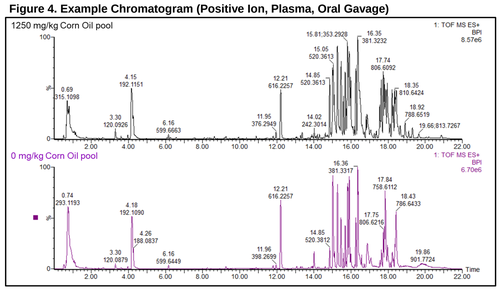
- Principal component analysis (PCA) was conducted using SIMCA 14.1 (Umetrics, Umeå, Sweden)
- Fold-changes between treated and untreated groups were obtained with SAS (*p*-values using an Exact Wilcoxon Rank Sum Test).
  - Fold changes were calculated for each compound by comparing the median values of the treated and untreated group.
  - In the presence of a near-zero median value, the median value of the non-zero group served as the “pseudo” fold change.
- Compound Identification:
  - Untargeted: In-house Retention Time library, In-house Exogenous database, HMDB, T3DB, EPA Toxcast, and EPA DSSTox. IDs were accepted based upon their exact mass, isotope ratio, fragmentation, and retention time (if available).
  - Targeted: match exact mass isotope distribution and fragmentation in public databases for components identified in crumb rubber

Untargeted Analysis

Chromatogram

- An example chromatogram from the LC-MS analysis for oral gavage 1250 mg/kg pool and 0 mg/kg control pool is shown in Figure 4.

Figure 4. Example Chromatogram (Positive Ion, Plasma, Oral Gavage)



Principal Component Analysis

- Representative PCA score plots for plasma and urine, using positive ion analysis, are shown in Figures 5 and 6, respectively, for oral gavage, dosed-feed and mixed-bedding individual animal samples, as well as pooled treatment and pooled control samples.
- Using PCA, data did not reveal a clear distinction between control and treated groups, suggesting animal to animal variability was higher than variability between treated and control groups.

Figure 5. PCA Plots, Positive Ion, Plasma

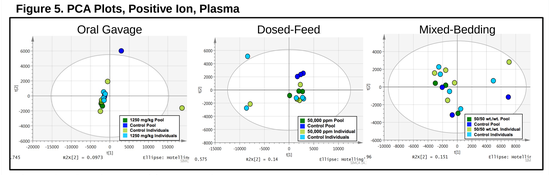
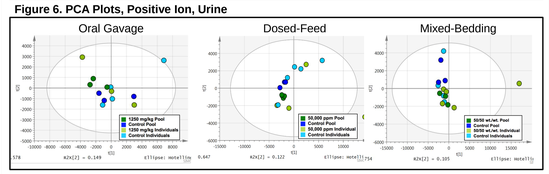


Figure 6. PCA Plots, Positive Ion, Urine



Compounds Found in Plasma and Urine

- A select summary of compounds, tentatively identified in plasma and urine of crumb rubber exposed animals, is shown below in Table 3.
- Identifications were made using database matches to mass and fragmentation, not standard references.
  - Many of the identified compounds had multiple potential matches.
- Compounds in Table 3 are significantly ($p < 0.1$) elevated in treated groups compared to corresponding controls (fold-change > 2).
  - Some compounds were identified from the targeted analysis of constituents known to be found in crumb rubber (in red).
  - Other chemicals were identified in the untargeted analysis; those listed are either anticipated to be related to crumb rubber exposure or had very large fold changes between control and treated groups.
    - It is important to note that fold-change does not equate to abundance; compounds with very large fold-changes may be present at low levels in plasma/urine of exposed animals.

Table 3. Compounds Found in Plasma and Urine

	Tentative Compound Identification	Fold Change
Plasma		
Oral Gavage	5-[4-(Heptyloxy)phenyl]-2-(methylsulfonyl)pyrimidine	1080.5
	6-Acetamido-2-naphthalenesulfonic acid	1077.1
	N-Acetyl-s-1,3-benzothiazol-2-yl-l-cysteine	151.5
	1,2-Dihydro-2,2,4-trimethylquinoline	12.6
	2,6-Di-tert-butylbenzoquinone	4.6
	2,6-Di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	2.7
	3,5-Di-tert-butyl-4-hydroxybenzaldehyde	2.1
Dosed-Feed	6-Acetamido-2-naphthalenesulfonic acid	635.1
Mixed-Bedding	(2R)-2-(Benzyloxy)-3-heptanone	5491.8
	6-Acetamido-2-naphthalenesulfonic acid	609.2
	1-Hydroxy-4-[2-hydroxy-5-(1-hydroxy-2-{[6-(4-phenylbutoxy)hexyl]amino}ethyl)benzyl]-2-naphthoic acid	65.3
Urine		
Oral Gavage	4-{[2-(1-Cyclohexen-1-yl)ethyl]amino}-1-butanol	15,380.2
	2(3h)-Benzothiazolethione	4631.2[a]
	2-Isobutyl-4-methyltetrahydro-2H-pyran-2-ol[b]	3259.4
	Neodecanoic acid[b]	3259.4
	3-Phenyl-2-thioxo-1,3-thiazolan-4-one	532.8
	2-(Carboxymethylthio) benzothiazole	326.5
	o-(2-Benzothiazolyl)phenol	97.4
	N-[3-(1,3-Benzothiazol-2-yl)phenyl]-3H-purin-6-amine	3.2
	2-Ethylhexyanoic acid	2.2
Dosed-Feed	Hexanal	2.4
Mixed-Bedding	3-Aminopyrazine-2,6-dicarbonitrile	112.6
	1-Methyl-pyrrole-2-acetic acid	6.1
	4-Oxocyclohexanecarbaldehyde	5.1

Compounds in red were identified using targeted analysis, matching the exact mass isotope distribution and fragmentation for compounds known to be found in crumb rubber.
[a] Highest fold change of three features identified as 2(3h)-Benzothiazolethione.
[b] The same feature was identified in the targeted and untargeted analyses and given different identifications.

Conclusions

Feasibility

- In these studies, exposure to crumb rubber was well tolerated in female mice by oral gavage, dosed-feed and mixed-bedding.
- Due to apparent avoidance of crumb rubber in the dosed-feed study, it is not feasible to accurately estimate crumb rubber dose from consumed feed.

Evidence of Biological Effect or Systemic Exposure

- Based on our preliminary review of data, there were no treatment-related effects on in-life data or toxicological endpoints.
- Based on tentative identifications, the metabolomics analysis of urine and plasma from animals exposed to crumb rubber revealed evidence of systemic exposure to compounds likely originating from the test materials (e.g. benzothiazole compounds).
  - Due to the low levels of these compounds present in the native test material, it is likely that systemic exposure levels were very low for all routes of exposure.
  - While compounds potentially from crumb rubber were identified, PCA plots were unable to differentiate treatment from control groups.

Acknowledgements

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