The National Toxicology Program Research on Synthetic Turf/Recycled Tire Crumb Rubber: Characterization of the Leachability and Cytotoxicity of Crumb Rubber In Vitro

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Abstract
Public health concerns for playing on synthetic turf fields with crumb rubber (CR) infill have increased in recent years. CR manufactured from recycled tires contains potent carcinogenic and toxic substances and there is potential for widespread exposure with over 12,000 synthetic turf fields in the US. The NTP recently conducted in vivo and in vitro research studies to understand the leachability and biological effects of CR using multiple human cell types to reflect different potential routes of CR exposure (cytotoxicity of CR using human keratinocytes (HaCaT) and peripheral lung (HPL)-1D cells to reflect dermal and inhalation routes of exposure). CR (100 mg/g) was incubated in cell culture medium for 1, 4 or 7 days at 60ºC. CR conditioned medium (CRCM) was then removed and conditioned medium (CR) was recovered and isolated for analysis. CR was cytotoxic to human epithelial (HaCaT, HPL)-1D cells, which were used to reflect oral, nasal and ocular exposure, but not to human haematopoietic (HepaRG) cells. To determine if the cytotoxicity of CRCM was an artifact of CR leaching in serum-free culture medium, CR was incubated in PBS or artificial lung fluid (ALF). In contrast to CRCM, CR-conditioned PBS or ALF was not cytotoxic to HPL-1D cells. Untargeted analysis by untargeted UPLC-MS was used to characterize the chemical composition of CRCM and CR-conditioned medium (CRM). Chromatograms were recorded using XIC (7.5 ms) for MS and 7.2 ms for MS/MS. CRCM from non-cytotoxic CR-conditioned PBS and ALF. Chemical compounds were identified using the National Institute of Standards and Technology database (NIST). CRCM and CRM were also tentatively identified by untargeted UPLC-MS. CRCM and CRM were subjected to in vitro cytotoxicity testing with HaCaT and HepaRG cells.

Objective
- Characterize the leachability and biological effects (cytotoxicity) of crumb rubber constituents in vitro using multiple human cell types to reflect different potential routes of CR exposure.

Experimental Design
- CR was extracted from CR samples with methanol. The extract was evaporated to dryness to prepare for analysis. CRCM and CRM were subjected to in vitro cytotoxicity testing with HaCaT and HepaRG cells. CRCM and CRM were subjected to in vitro cytotoxicity testing with HaCaT and HepaRG cells.

Conclusions
- CRM was cytotoxic to multiple human cell lines (K562 and MCF-7) and was not cytotoxic to HepaRG cells.
- Principal component analysis showed segregation of cytotoxic CRCM from non-cytotoxic CR-conditioned PBS and ALF.
- A number of chemicals were tentatively identified using untargeted analysis to be cytotoxic to CRCM-conditioned cells.
- Chemical compounds used in the vulcanization of rubber (such as 2-Mercaptobenzothiazole, N,N'-Dicyclohexylurea, and 1,3-di-o-Tolylguanidine) were found to be cytotoxic to CRCM-conditioned cells.

Table 1

<table>
<thead>
<tr>
<th>Chemical ID</th>
<th>% Matcha F12</th>
<th>PBS</th>
<th>ALF</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG n12</td>
<td>89-94</td>
<td>5.8</td>
<td>6.1</td>
</tr>
<tr>
<td>PEG n8</td>
<td>89-91</td>
<td>7.0</td>
<td>7.4</td>
</tr>
<tr>
<td>N,N'-Dicyclohexylurea</td>
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<tr>
<td>Decanamide</td>
<td>64</td>
<td>1.6</td>
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</tr>
<tr>
<td>Metolachlor morpholinone</td>
<td>59</td>
<td>4.8</td>
<td>6.9</td>
</tr>
<tr>
<td>4'-(Imidazol-1-yl)acetophenone</td>
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Figure 1
CRCM is cytotoxic to HaCaT cells

Figure 2
CRCM is cytotoxic to HPL-1D cells

Figure 3
CRCM is cytotoxic to FcγT-74-int, but not HepaRG, cells

Figure 4
CR-conditioned PBS is not cytotoxic

Figure 5
Principal component analysis shows segregation of cytotoxic CRCM from non-cytotoxic CR-conditioned PBS and ALF.

Introduction
In recent years, there has been a risk in public health concern for playing on synthetic turf fields due to reported health effects in young adult soccer players in Washington State. Based on these initial reports, the Washington State Department of Health conducted an epidemiological investigation and found that the number of soccer players with cancer in the initial reports was less than the expected rate for Washington state residents. Despite this, the level of public concern increased which led to the development of the National Toxicology Program Research on Synthetic Turf/Recycled Tire Crumb Rubber: Characterization of the Leachability and Cytotoxicity of Crumb Rubber In Vitro (NTP). This study aimed to investigate potential health effects of playing on synthetic turf fields made from recycled tires. Synthetic turf fields have been used for decades, and throughout this time the technology and composition of the fields have evolved. In an attempt to ban heavy metals and plastics from grass fields, infill materials were added to enhance shock and traction. Most recently, crumb rubber (CR) has been employed as the infill material. CR is manufactured by shredding recycled automobile tires into rubber particles. Due to the fast that recycled tires are known to contain numerous potential carcinogenic and toxic substances, investigating potential health effects of playing on synthetic turf fields has focused on CR. Several National Toxicology Program (NTP) and research efforts are ongoing to determine the potential risks associated with playing on synthetic turf fields made from recycled tires. The NTP research program is focused on understanding uncertainties regarding potential human exposure and health risks associated with exposure to CR-infused synthetic turf fields. The NTP project team is investigating potential health effects of playing on synthetic turf fields made from recycled tires.

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