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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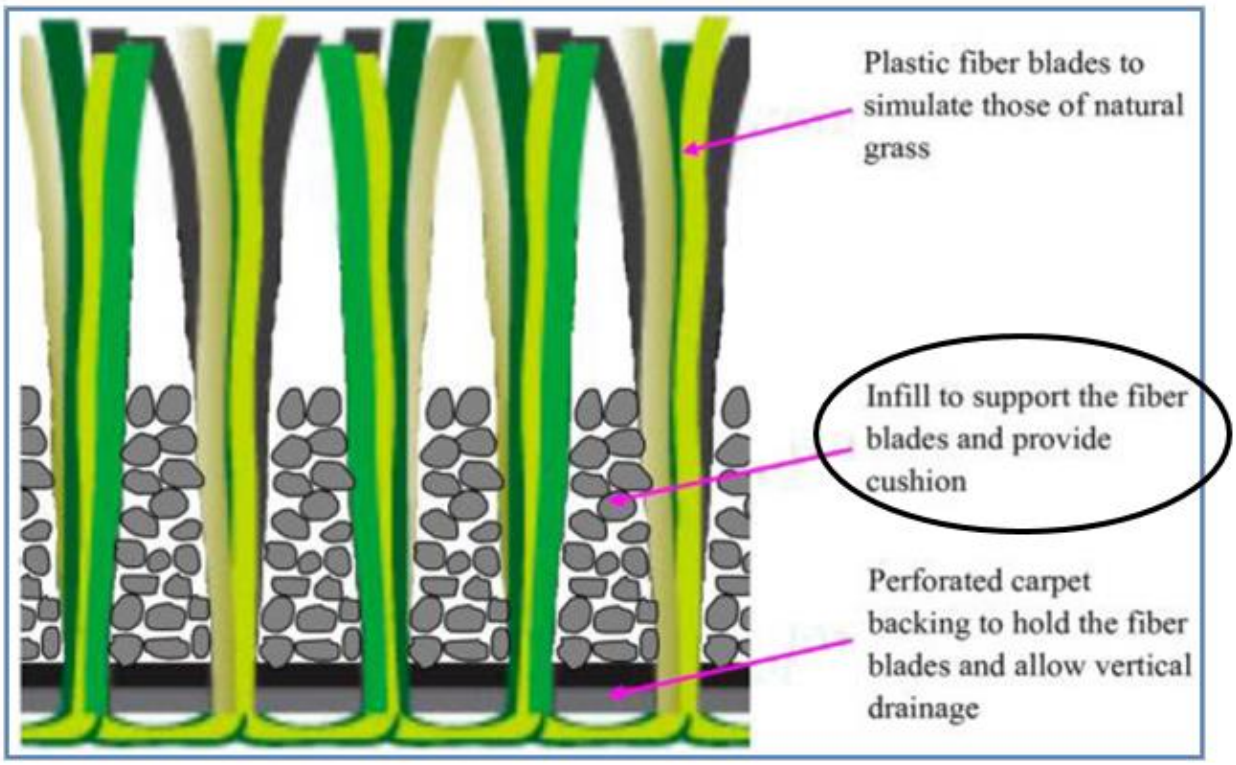
Abstract

Public health concern for playing on synthetic turf fields with crumb rubber (CR) infill has increased in recent years. CR manufactured from recycled tires contains potential 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 improve the understanding of potential human exposure and health impacts following CR exposure. The objectives of the in vitro study were to determine the leachability and biological effects (cytotoxicity) of CR using human keratinocytes (HaCaT cells) and peripheral lung (HPL-1D) cells to reflect dermal and inhalation routes of exposure. CR (100 mg/ml) was incubated in cell type-specific culture medium for 3 hr, 1, 4 or 7 days at RT, 37ºC or 60ºC to allow for the leaching of chemical constituents from the CR into the medium. CR-conditioned medium (CRCM) was then sterile filtered and serially diluted to 50, 25, 12.5 and 6.25 mg/ml for cell exposures. HaCaT or HPL-1D cells were exposed to CRCM (or control medium) for 24 hr and cell viability was measured using a cell proliferation (MTS) assay. CRCM-induced cytotoxicity was observed for both cell lines and was concentration-dependent, although the cytotoxic effect was most potent with HPL-1D cells. For HaCaT cells, 100 and 50 mg/ml CRCM were cytotoxic at 24 hr for CR incubation times of 1, 4 or 7 days at 60ºC. For HPL-1D cells, 100, 50 and 25 mg/ml CRCM were cytotoxic at 24 hr for all CR incubation times at 60ºC. For both cell types, cytotoxicity was also observed following incubation of CR at 37ºC and RT but was most pronounced at 60ºC. CRCM was cytotoxic to human small intestinal (FHs-74-Int) cells, which were used to reflect oral exposure, but not human hepatocytes (HepaRG cells). To determine if the cytotoxicity of CRCM was an artifact of CR incubation in (serum-rich) 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 ultraperformance liquid chromatography-mass spectrometry (UPLC-MS) was used to characterize the chemical composition of CRCM and CR-conditioned PBS or ALF. Principal component analysis showed segregation of chemical features present in ‘cytotoxic’ CRCM from ‘non-cytotoxic’ CR-conditioned PBS and ALF. Chemical compounds used in the vulcanization of rubber (such as 2-mercaptobenzothiazole, N,N’-diphenylguanidine and 1,2-benzisothiazoline-3-one) were found to leach from CR; however, these compounds were identified not only in CRCM but also in CR conditioned PBS and ALF, and thus likely did not contribute to the cytotoxic effect with HPL-1D cells. In conjunction with chemical characterization and in vivo testing of CR, this in vitro study will contribute to what is known about potential human health effects of playing on synthetic turf fields made from recycled tires.

Introduction

In recent years, there has been a rise 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 these initial reports was less than the expected rate for Washington state residents. Despite this, the level of public concern, extent of potential exposure and the nature of the material have warranted further investigation. Synthetic turf fields are widely used in the US and their use is continuing to expand. Synthetic turf installation is a common choice in areas where sustaining a grass field is logistically challenging due to cold or dry conditions, allowing sports to be played year-round. These fields have historically been installed in professional sports stadiums, but are also increasingly popular in recreational areas and public parks used by people of all ages, leading to the potential for life-time exposure.

Synthetic turf fields have been used for decades, and throughout this time the technology and composition of the fields have evolved. In attempts to lower injury rates and mimic play experienced on grass fields, infill materials were added for cushioning and traction. Most recently, crumb rubber (CR) has been employed as the infill material. CR is manufactured by shredding recycled automotive tires into rubber particles. Due to the fact that recycled tires are known to contain numerous potential carcinogenic and toxic substances, research efforts investigating potential health effects of playing on synthetic turf fields have focused on CR. Several federal and state research efforts are being undertaken to evaluate potential risks from CR exposure. The Federal Research Action Plan (FRAP) consisting of several federal agencies aims to identify and fill important knowledge gaps, characterize constituents of recycled tire crumb and identify ways that humans are exposed to tire crumb based on field activities. The California Office of Environmental Health Hazard Assessment (OEHHA) is currently conducting studies to evaluate exposure scenarios, characterize CR from new and in-field CR and develop biomonitoring protocols. In 2015, OEHHA nominated synthetic turf/CR to the NTP to conduct short-term in vivo and in vitro studies to enhance understanding of potential health impacts of chemicals released from synthetic turf, with an emphasis on CR. The NTP research program has focused on addressing uncertainties regarding potential human exposure and health risks following contact with CR. The approach (both in vivo and in vitro) has considered potential routes of human contact with CR and investigated which exposure conditions within an experimental laboratory setting might influence the risk of developing adverse health outcomes.



Cheng *et al.* (2014). Environmental and health impacts of artificial turf: a review. *Environmental Science and Technology*. 48(4): 2114-29.

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 exposure.

Cell line	**Cell type**	**Exposure route**	**Base medium**
HaCaT	**Skin keratinocyte**	**Dermal**	**DMEM**
HPL-1D	**Peripheral lung epithelial**	**Inhalation**	**F12**
FHs-74-Int	**Small intestine epithelial**	**Oral**	**Hybri-Care**
HepaRG	**Hepatocyte**	**n/a**	**MHPIT**

Experimental Design

CR was provided by OEHHA. The material was received from 2 facilities and manufactured either via the ambient or cryogenic process. A total of 3 lots of CR were received. All material was combined to produce one lot for all NTP studies.

CR (100 mg/ml) was incubated (with vortexing) in cell type-specific culture medium (10 ml) for 3 hr, 1, 4 or 7 days at RT, 37ºC or 60ºC to allow for the leaching of chemical constituents from the CR into the medium (the CR particles were too large for direct exposure of the cells). Control medium was incubated without CR at the same temperature for the same amount of time (HPL-1D cells were cultured in Ham's F12 medium + 1% FBS + supplements).

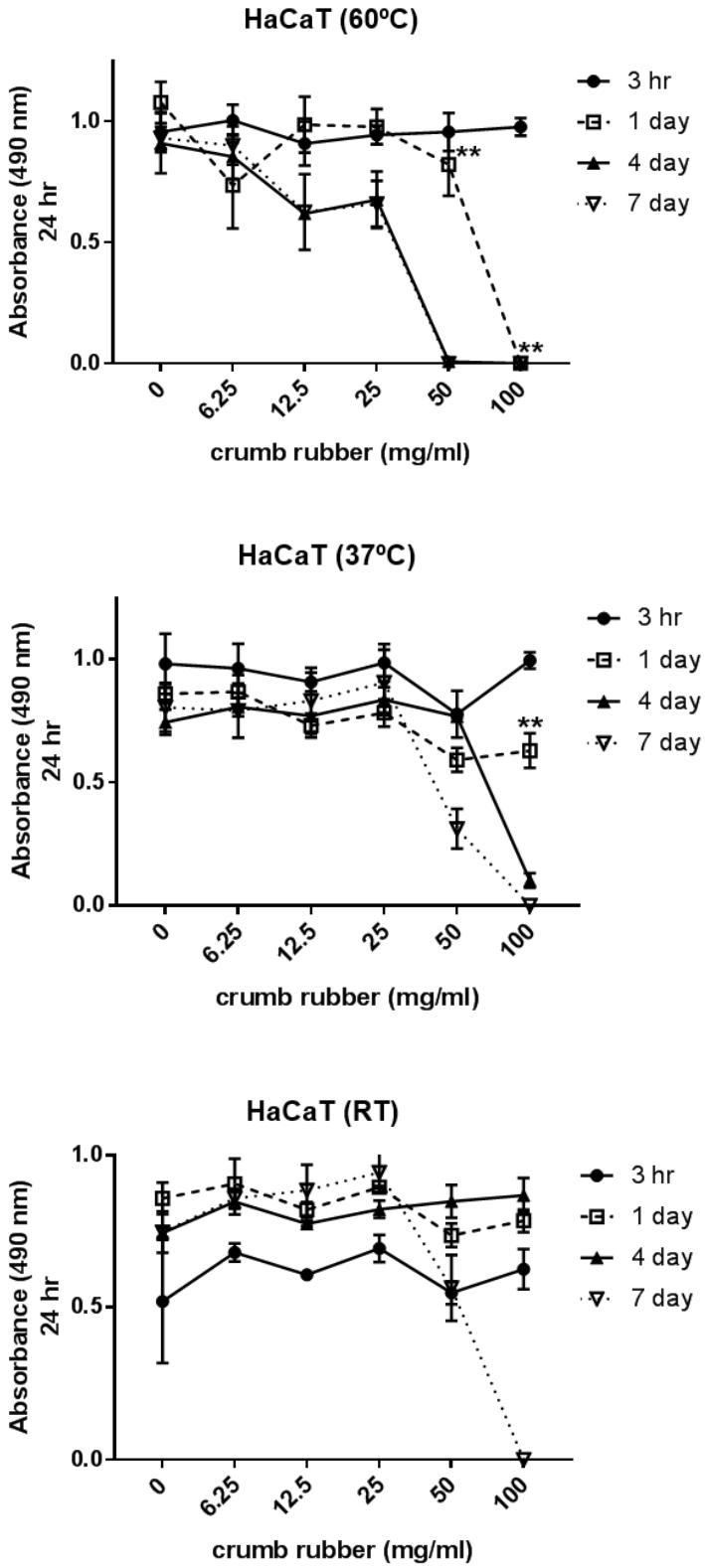
After centrifugation, the CR-conditioned medium (CRCM) was sterile filtered and then serially diluted with control medium to 50, 25,12.5 and 6.25 mg/ml for cell exposures. All CR-conditioned and control medium used for cell exposures were negative for microbial (bacterial and fungal) contamination (data not shown).

Cells (1×10^4 per well for the HaCaT, HPL-1D and FHs-74-Int cells; 1×10^5 per well for the HepaRG cells) were seeded overnight in 96 well culture plates and then exposed to 100 µl CRCM or control (0 mg/ml CR) medium in at least triplicate for 24 hr at 37ºC. A cell proliferation (MTS) assay was used to measure cell viability (Ab 490 nm).

In some experiments with HPL-1D cells, CR (100 mg/ml) was incubated in PBS or artificial lung fluid (ALF) at 60ºC for 1 day. After centrifugation, the CR-conditioned PBS or ALF was sterile filtered and then diluted with culture medium 1:2 to 50 mg/ml or 1:4 to 25 mg/ml for HPL-1D cell exposures. PBS or ALF incubated without CR (60ºC, 1 day) was diluted 1:2 or 1:4 with culture medium to serve as controls (ALF contained 10 mM magnesium chloride, 150 mM sodium chloride, 4 mM potassium chloride, 1 mM disodium phosphate, 5 mM sodium sulfate, 25 mM calcium chloride, 7 mM sodium acetate, 24 mM sodium bicarbonate, 3 mM sodium citrate and 0.20% (w/v) dipalmitoyl lecitin (a component of pulmonary surfactant) in water).

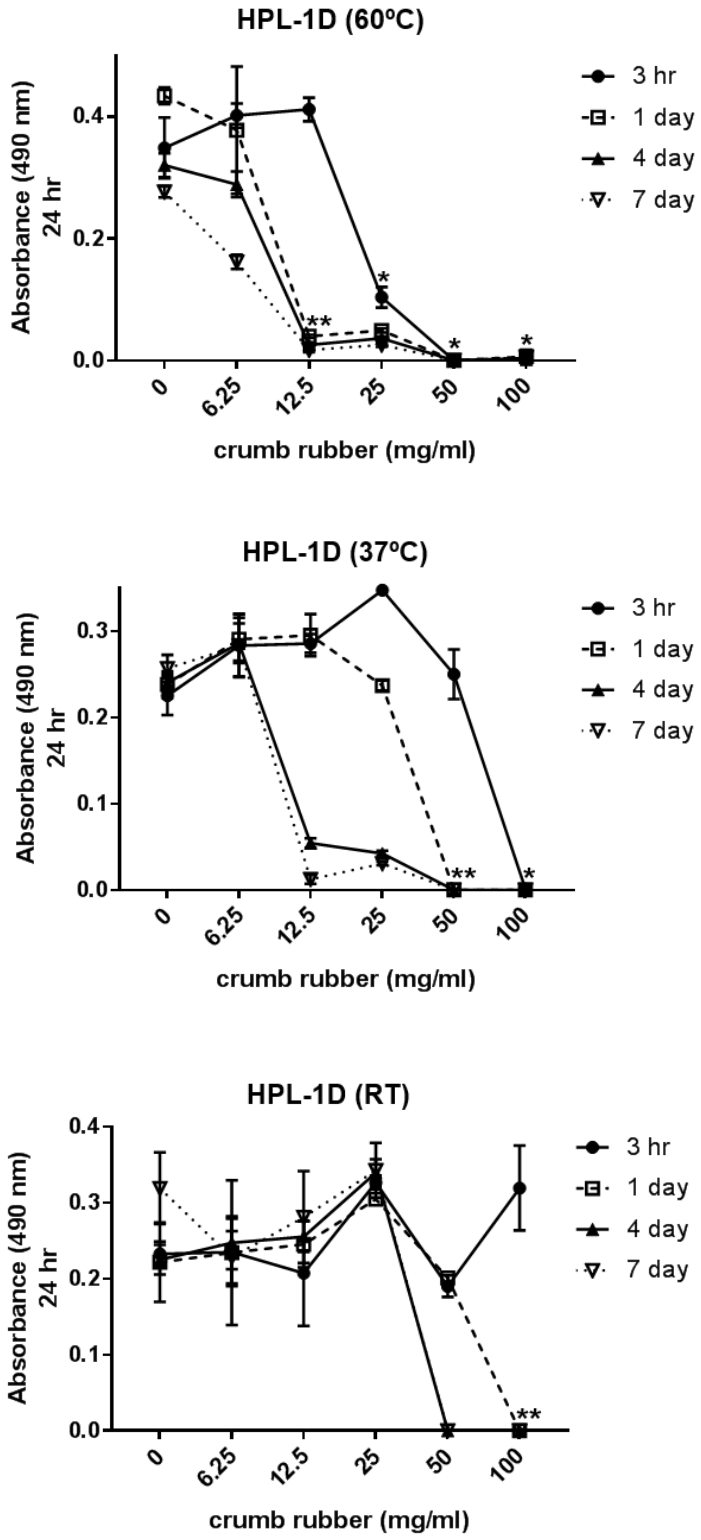
Chemicals present in the 100 mg/ml (60ºC, 1 day) CRCM and CR-conditioned PBS or ALF samples (in triplicate) were tentatively identified by untargeted UPLC-MS (Thermo Vanquish UPLC system with Thermo Hypersil GOLD aq C18 column (100 x 2.1 mm, 1.9 mm particle size) coupled to Thermo Q Exactive Plus MS with electrospray ionization source). Data acquisition was done using Thermo Xcalibur 4.0. Untargeted data analysis used Compound Discoverer 2.1.

Figure 1. CRCM is cytotoxic to HaCaT cells



** = $p < 0.05$ vs. 0 (cytotoxic @ 1, 4 and 7 day)

Figure 2. CRCM is cytotoxic to HPL-1D cells



* = $p < 0.05$ vs. 0 (cytotoxic @ all incubation times)
** = $p < 0.05$ vs. 0 (cytotoxic @ 1, 4 and 7 day)

HPL-1D (CR; 60ºC, 1 day)

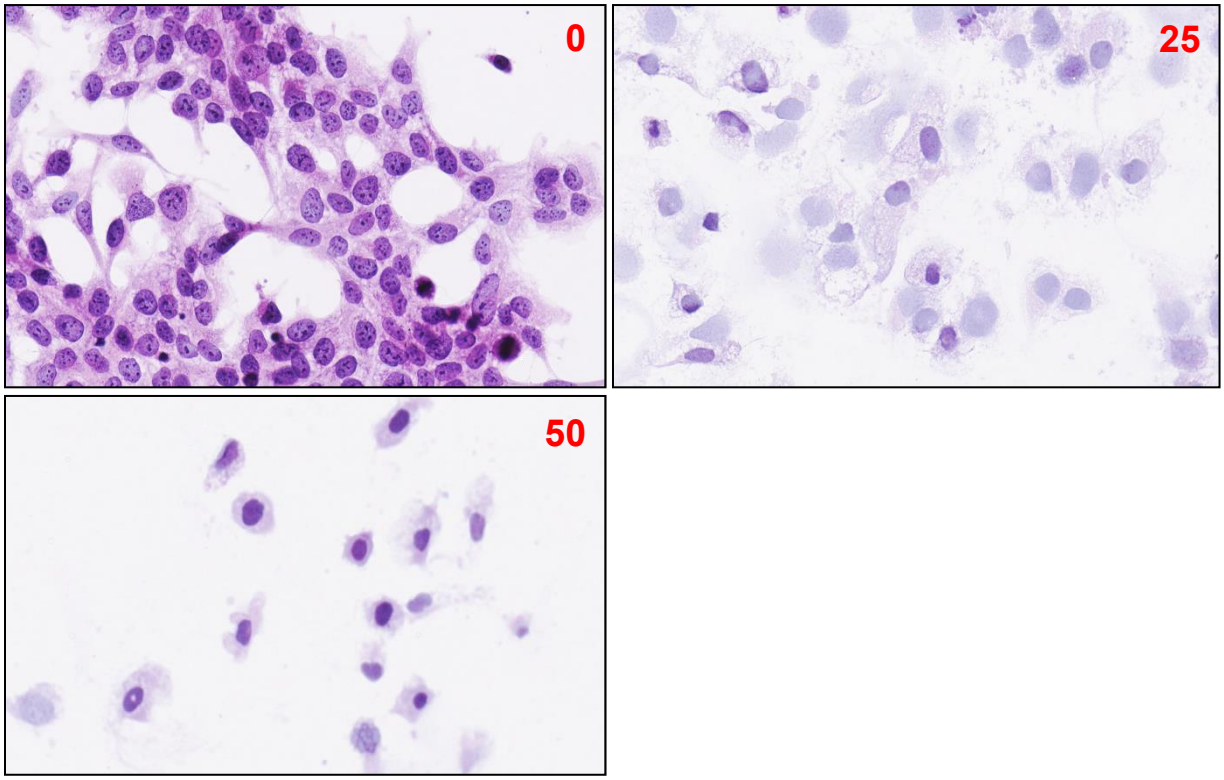
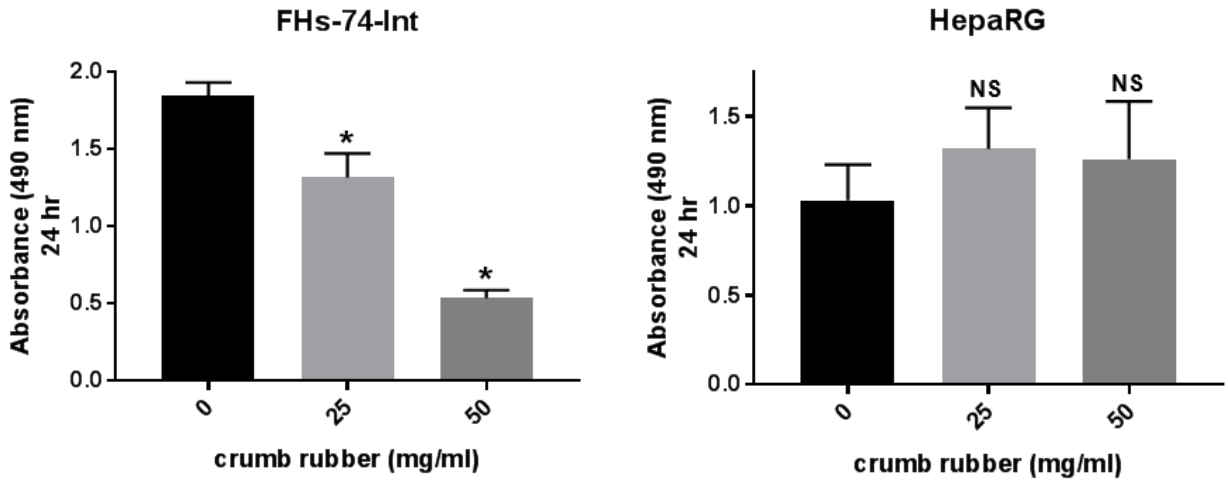


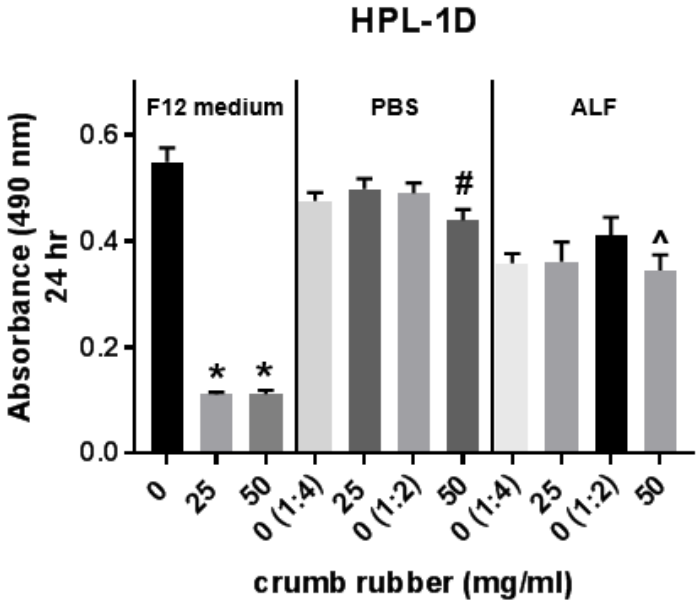
Figure 3. CRCM is cytotoxic to FHs-74-Int, but not HepaRG, cells



CR; 60ºC, 1 day

* = $p < 0.05$ vs. 0
NS = not significant vs. 0

Figure 4. CR-conditioned PBS or ALF is not cytotoxic



* = p < 0.05 vs. 0 [F12 medium]
# = p < 0.05 vs. 0 (1:2) [PBS]
^ = p < 0.05 vs. 0 (1:2) [ALF]

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

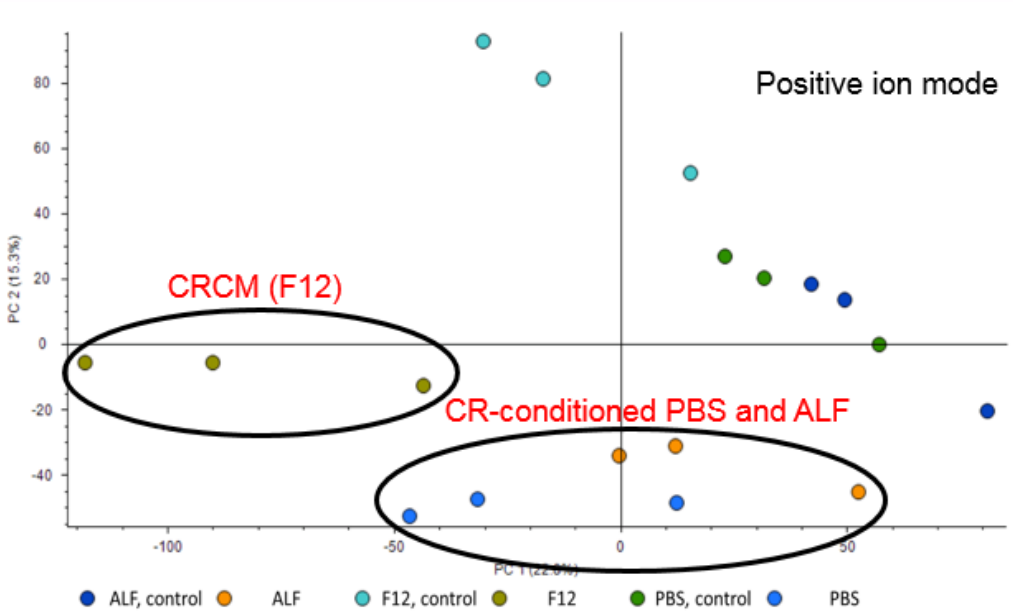


Table 1. Untargeted analysis by UPLC-MS

Chemical ID	% Match[a]	F12	PBS	ALF
PEG n12	89-94	5.8[b]	6.1	4.5
PEG n8	89-91	7.0	7.4	4.7
N,N'-Dicyclohexylurea	89-91	5.1	5.1	3.8
Atropine	89-91	3.4	3.4	2.3
PPG n7	87-88	1.9	3.1	1.9
1,2-Benzisothiazolin-3-one	84-89	10.7	11.4	8.2
PEG n7	82-90	6.5	7.5	4.9
Triisopropanolamine	81-92	6.1	8.2	8.1
PEG n6	79-86	5.9	7.0	4.2
9-Oxo-10(E),12(E)-octadecadienoic acid	76-79	6.5	6.8	3.4
PPG n6	74-82	5.4	5.1	4.9
Gelsemine	72-76	6.9	7.4	6.0
Kahweol	70-74	7.1	7.4	5.2
Cafestol	70-73	6.9	6.9	6.3
Methyl red	64-67	8.7	7.4	7.1
Testosterone	63-66	5.9	5.4	4.9
Pyridoxamine	60-75	4.2	6.4	5.0
CB-13	59-64	6.1	7.1	5.8
7-Aminonimetazepam	56-59	8.5	9.2	6.9
α-Piperidinobutiophenone	51-58	5.8	9.6	6.4
11β-Hydroxyandrosterone	51-57	5.5	7.4	6.2
PEG n11	89-93	5.7	5.9	-[c]
PEG n15	85-86	6.0	6.2	-
N,N'-Diphenylguanidine	84-85	11.5	13.0	-
Indole-3-acrylic acid	84	5.4	6.1	-
Harmine	78	9.1	9.1	-
2-Mercaptobenzothiazole	78	6.1	5.2	-
PEG n14	76-86	5.5	6.2	-
N,N'-Diphenylurea	76-78	3.7	3.4	-
15-Deoxy-Δ12,14-prostaglandin A1	76-77	6.6	6.7	-
PPG n9	75-84	3.4	3.0	-
Metyrapone	69-70	6.4	6.5	-
2-Aminobenzothiazole	67-68	8.8	9.5	-
Hexamethoxymethyl melamine	66-68	6.5	7.8	-
Acetyl norfentanyl	64-65	5.9	5.8	-
PPG n10	63-69	3.6	3.2	-
Tropicamide	63-64	4.6	4.5	-
7-Aminonitrazepam	59-66	5.5	7.5	-
3-Desoxy-3,4-methylenedioxy pyrovalerone	59-60	3.8	3.4	-
Dextromethorphan	58-78	7.4	7.7	-
Gabapentin	53-61	4.3	5.0	-
Glutethimide	52-54	3.1	2.8	-
PEG n5	65-67	-	5.3	4.0
11-Ketotestosterone	62-64	-	6.7	6.1
Methoxyphenamine	61	-	8.2	7.8
α-Linolenic acid	89	6.7	-	-
Eicosapentaenoic acid	79	6.7	-	-
PPG n11	67	4.0	-	-
Penbutolol	62	3.3	-	-
(+)-Evodiamine	61	2.9	-	-
3-(2-Hydroxyethyl)indole	55	1.9	-	-
DEET	52	5.1	-	-
N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-5-methyl-3-isoxazolecarboxamide	52	3.0	-	-
Exemestane	51	7.8	-	-
N1-(2-Pyridylmethyl)-2-aminobenzamide	50	6.4	-	-
PPG n8	83	-	3.6	-
Isotretinoin	74	-	6.1	-
5-Fluoro-3,5-AB-PFUPPYCA	73	-	6.3	-
2-[4-(Diethylamino)styryl]-5-nitrobenzonitrile	64	-	6.8	-
Cyanazine	63	-	4.3	-
1,3-di-o-Tolylguanidine	56	-	8.2	-
2-Methoxyestrone	56	-	5.0	-
Rimantadine	51	-	6.6	-
3,5-di-tert-butylbenzoquinone	n/a[d]	-	7.1	-
Triisopropanolamine cyclic borate	70	-	-	3.9
MDPBP	70	-	-	2.1
(S)-(+)-N-Boc-3-pyrrolidinol	64	-	-	2.6
Decanamide	64	-	-	1.6
Metolachlor morpholinone	59	-	-	6.9
4'-(Imidazol-1-yl)acetophenone	58	-	-	5.0

Associated with vulcanization of rubber

Increased only in 'cytotoxic' CRCM

[a]Fragmentation data from MS/MS scan matched to mzCloud database

[b]Log 2 fold increase over respective control = Log 2 (average peak area/average peak area of control)

[c]Not detected or not increased over respective control

[d]Simulated (not in mzCloud database)

Conclusions

CRCM was cytotoxic to multiple human cell types (skin, lung epithelial and small intestinal cells) in vitro representing different potential routes of CR exposure (via dermal contact, inhalation and ingestion). CRCM was not cytotoxic to human hepatocytes in vitro.

This biological effect may not be relevant in vivo or to human CR exposure because CR incubated in PBS or biofluid (ALF), which is more physiologically-relevant than the culture medium used for HPL-1D cells, was not cytotoxic to HPL-1D cells.

Principal component analysis showed segregation of chemical features present in 'cytotoxic' CRCM from 'non-cytotoxic' CR-conditioned PBS and ALF.

A number of chemicals were tentatively identified via untargeted analysis to be elevated in 'cytotoxic' CRCM, but not in CR-conditioned PBS or ALF, which may have contributed to CRCM-induced cytotoxicity.

Chemical compounds used in the vulcanization of rubber (such as 2-mercaptobenzothiazole*, N,N'-diphenylguanidine and 1,2-benzisothiazoline-3-one) were found (via untargeted analysis) to leach from CR; however, these compounds were identified not only in CRCM but also in CR-conditioned PBS (all three) and ALF (1,2-benzisothiazoline-3-one only), and thus likely did not contribute to cytotoxicity.