Evaluation of 4-methylcyclohexanemethanol in a Combined Irritation and Local Lymph Node Assay

Victor J. Johnson[1], Scott S. Auerbach[2], Michael I. Luster[1], Suramya Waidyanatha[2], Scott A. Masten[2], Mary S. Wolfe[2], Florence G. Burleson[1], Gary R. Burleson[1], Dori R. Germolec[2]

[1]Burleson Research Technologies, Inc. Morrisville, NC, USA

[2]Division of National Toxicology Program, National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC, USA

Introduction

4-Methylcyclohexanemethanol (MCHM) is a flotation reagent used in fine coal beneficiation. On January 9, 2014 crude MCHM (~88.5% MCHM) was inadvertently released into the Elk River in Charleston, WV resulting in temporary contamination of 15% of the state's tap water and causing significant dermal exposure. These studies were undertaken to determine whether MCHM[1] or crude MCHM has the potential to produce dermal irritation and/or sensitization. Female BALB/c mice were treated daily for 3 consecutive days by direct epicutaneous application of 25 μL of various concentrations of MCHM or crude MCHM to the dorsum of each ear. A mouse ear swelling test, used to determine irritancy potential, was conducted in combination with the Local Lymph Node Assay (LLNA) to determine dermal sensitizing potential. Dermal exposure to MCHM caused irritation of the skin at the application site at concentrations above 20% and overt toxicity at the 100% concentration, but did not induce sensitization. Mice treated with ≥75% crude MCHM also showed evidence of dermal irritation, although weaker when compared to MCHM. Overt toxicity was also observed in mice treated with 100% crude MCHM, although the severity was less than MCHM. Dermal application of crude MCHM resulted in increased lymphocyte proliferation in the draining lymph node at concentrations ≥5%. The Stimulation Index (SI), a measure of sensitization, was significantly increased in mice treated with ≥20% crude MCHM, relative to the vehicle control group. The SI was above 3, the threshold for positive sensitization potential, following dermal application of ≥40%. These results indicate that crude MCHM has the potential to cause dermal sensitization at exposure concentrations that are non-irritating.

[1]>99% MCHM; referred to as MCHM

Approach and Methods

- Female BALBc mice were purchased from Taconic Biosciences Inc, (Hudson, NY) and were 8 weeks of age at the start of treatment.
- For dermal irritation assessment, mouse ear swelling was measured on Day 3 after the final application of test article and again at study termination on Day 6.
- The local lymph node assay (LLNA) was used to evaluate the sensitization potential. The treatment procedure and method was selected on the basis of previously published data (ICCVAM, 2011).
- Two independent studies were conducted due to the observation of overt clinical signs of toxicity in mice treated with MCHM[1]or crude MCHM in Study 1. Study 2 was conducted to confirm the LLNA findings with crude MCHM.

Study	Vehicle	Test Article (concentration)	Positive Control
Study 1	AOO	MCHM[1] (100/50[2], 20, 2%) crude MCHM (100/80[3], 40, 20, 5, 2, 1%)	DNFB (0.15%)
Study 2	AOO	crude MCHM (75, 50, 25, 5, 1%)	DNFB (0.15%)

AOO = Acetone Olive Oil (4:1 v:v); DNFB = 1-fluoro-2,4-dinitrobenzene

[1]>99% MCHM; referred to as MCHM

[2]Concentration reduced to 50% on Days 2 and 3 due to excessive clinical signs observed on Day 1.

[3]Concentration reduced to 80% on Days 2 and 3 due to excessive clinical signs observed on Day 1.

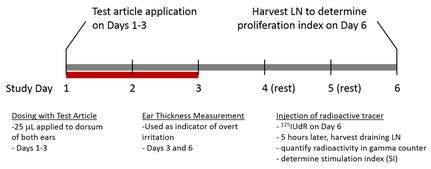

Figure 1

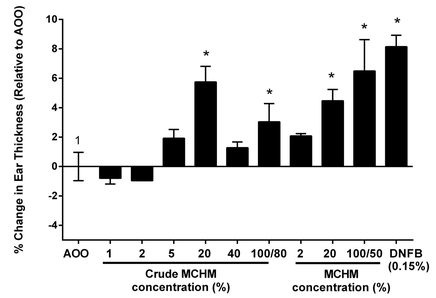

Figure 1. Treatment with MCHM and crude MCHM caused dermal irritation in BALB/c mice (Study 1; changes in ear thickness on Day 3). Data are expressed as relative change from the vehicle control group mean ear thickness. Each bar represents the mean ± SE (N=5 except 100/50% MCHM where N=3). *Significantly different from AOO vehicle control (P<0.05, Dunnett's T-Test). [1]Significant trend for MCHM and crude MCHM (*P*<0.05, Jonckheere's Trend Test).

Figure 2

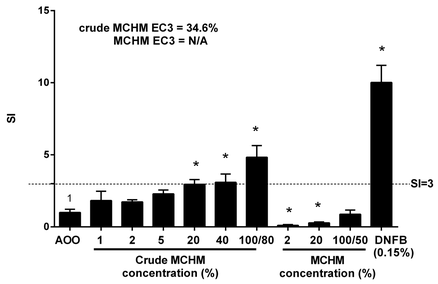

Figure 2. Dermal treatment with ≥ 20% crude MCHM increased lymphocyte proliferation in the draining lymph node indicative of sensitization (Study 1; Day 6). Lymphocyte proliferation in the draining lymph node was determined by quantifying the incorporation of ^{125}IUdR into the DNA of proliferating cells. The Stimulation Index (SI) was determined for each animal relative to the mean lymphocyte proliferation in the vehicle control group. Each bar represents the mean +/- SEM (N=5 except 100/50% where N=3). *Significantly different from AOO vehicle control (*P*<0.05, Dunnett's T-Test). [1]Significant trend for crude MCHM (*P*<0.05, Jonckheere's Trend Test).

Figure 3

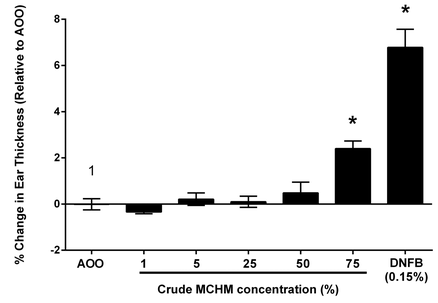

Figure 3. Treatment with 75% crude MCHM caused mild dermal irritation in BALB/c mice (Study 2; changes in ear thickness on Day 6). Treatment induced changes in ear thickness were measured on Day 6. Data are expressed as relative change from the vehicle control group. Each bar represents the mean ± SE (N=8). *Significantly different from AOO vehicle control (P<0.05, Dunnett's T-Test). [1]Significant trend (*P*<0.05, Jonckheere's Trend Test).

Figure 4

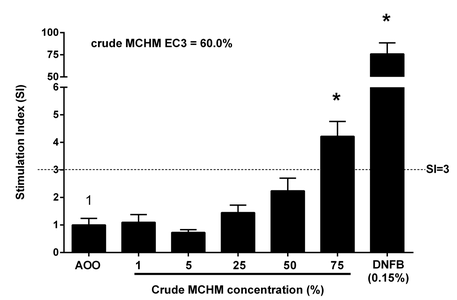

Figure 4. Dermal treatment with 75% crude MCHM increased lymphocyte proliferation in the draining lymph node indicative of sensitization (Study 2; Day 6). Lymphocyte proliferation was determined by quantifying the incorporation of ^{125}IUdR into DNA. The Stimulation Index (SI) was determined for each animal relative to the mean lymphocyte proliferation in the vehicle control group. Each bar represents the mean +/- SEM (N=8). *Significantly different from AOO vehicle control (*P*<0.05, Dunnett's T-Test). [1]Significant trend (*P*<0.05, Jonckheere's Trend Test).

Results – Study 1

- In Study 1, 2 of the 5 mice treated with 100% MCHM showed clinical signs of toxicity on Day 1 and were euthanized. The dose was subsequently lowered to 50% (the group is referred to as 100/50%).
- Mice treated with 100% crude MCHM demonstrated similar, although less severe, clinical signs of toxicity after the first day of dosing resulting in the concentration being lowered to 80% (the group is referred to as 100/80%).
- Crude MCHM significantly increased ear swelling on Day 3 at the 20% and 100/80% test concentrations. **(Figure 1)**
- MCHM significantly increased ear swelling at the 2%, 20%, and 100/50% test concentrations on Day 3 **(Figure 1)** (similar results on Day 6, Data not shown).
- Treatment with the positive control (0.15% DNFB), increased ear swelling on Day 3 **(Figure 1)** and Day 6 (data not shown).
- Treatment with MCHM did not increase DPM or SI values relative to the vehicle control indicating a negative LLNA response.
- Treatment with crude MCHM significantly increased the DPM and SI values in mice treated with ≥20% with a statistically significant dose-response trend observed **(Figure 2).**
- SI values >3.0 occurred in mice treated with crude MCHM at ≥40% test concentration with a maximum SI value of 4.83 in the 100/80% treatment group. **(Figure 2)**
- The EC-3 (extrapolated concentration resulting in SI = 3) for crude MCHM was determined to be 34.6%.
- Treatment with the positive control, 0.15% DNFB, resulted in highly significant increases in DPMs and the SI value (SI = 10.02). **(Figure 2)**

Results – Study 2

- Treatment with 75% crude MCHM caused a statistically significant increase in ear swelling on Day 3 (data not shown) and Day 6. **(Figure 3)**
- Treatment with the positive control DNFB resulted in significant ear swelling on Day 6.
- Treatment with 75% crude MCHM significantly increased the SI value while DPM values were significantly increased at ≥ 50% MCHM concentrations (data not shown). **(Figure 4)**
- Similar to Study 1, an SI value of 4.2 occurred in mice treated with crude MCHM at the 75% test formulation. **(Figure 4)**
- The EC-3 for crude MCHM was determined to be 60%. **(Figure 4)**

Conclusion

- Both MCHM and crude MCHM produced skin irritation, although crude MCHM appeared to be a considerably weaker irritant.
- Crude MCHM, but not MCHM, was identified as a skin sensitizer in the LLNA.
- Differences between the crude MCHM and MCHM in the LLNA response are postulated to be due to either variations in their isomers concentrations or the presence of sufficient concentrations of other chemicals in the crude MCHM mixture that may have the potential to cause sensitization.
- **The concentrations found to produce either irritation or sensitization were well above levels found in tap water following the Elk River spill.**

References and Acknowledgements

- CDC, 2014. http://emergency.cdc.gov/chemical/MCHM/westvirginia2014/mchm.asp
- Eastman Chemical Co. 1997. Toxicity studies with crude MCHM. http://www.eastman.com/Pages/Eastman-Crude-MCHM-Studies.aspx.
- ICCVAM. 2011. NIH Publication No. 11-7709. Research Triangle Park, NC: National Institute of Environmental Health Sciences.
- Whelton, AJ, et al., 2015.. Environ Sci Technol 49: 813-823.
- West Virginia Poison Center Fact Sheet. February 10, 2014. http://www.wvafp.org/wp/wp-content/uploads/2014/02/Charleston-WV-Health-Professionals-Factsheet.pdf
- [+]These studies were approved by the IACUC for adherence to the *Guide* and the applicable policies of the PHS Policies on Humane Care and Use of Laboratory Animals and were conducted in compliance with Nonclinical Laboratory Studies GLP Regulations (Title 21 of the Code of Federal Regulations, Part 58).
- Work support by NTP/NIEHS Contract HHSN273201400017C