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DERMAL AND SYSTEMIC TOXICITY AFTER APPLICATION OF SEMISYNTHETIC METAL-WORKING FLUIDS IN B6C3F1 MICE

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About 10 million industrial workers of both sexes are exposed to metal-working fluids (MWFs) via inhalation, skin or both. Our preliminary results, following dermal application of 200 µl of 50% unused (neat) semisynthetic MWF (pH 7 or pH 9.7) to the unshaved backs of 6-wk-old B6C3F1 mice, twice a week for 6 wk, produced significant increase in weights of the liver of both sexes. The purpose of the present study was to determine if this weight change was related to oxidative stress subsequent to MWF exposure and also to determine whether ethanol intake influences this effect. Therefore, 6-mo-old mice of both sexes were exposed to MWFs following the protocol just described, except that the topical application was with 5% MWFs (pH 7 and 9.7, 5 d/wk) with or without adding 5% ethanol to their drinking water (7 d/wk) for 13 wk. The skin histamine levels and mast-cell numbers were significantly increased in the female group treated with 5% MWF (pH 7). The ascorbic acid levels in the liver (both sexes) (all groups except 5% MWF pH 9.7 males) and testes were reduced significantly. Malondialdehyde levels in the male liver were significantly increased with topical MWF exposure. Glutathione levels were reduced significantly in both male and female liver after 5% MWF (pH 7). Alcohol dehydrogenase activity of the male liver increased significantly after MWF (pH 7). These results suggest that MWFs are absorbed through the skin and produce toxicity in the liver of both sexes and in the male gonads. This may represent an important health risk to MWF-exposed industrial workers, and ethanol may exacerbate this risk.

Metal-working fluids (MWFs) are commonly used in manufacturing industries that perform machining, grinding, forming, or treating operations. MWFs are used to prolong the life of machine tools, carry away metal chips, and protect or treat the surfaces of the material being processed (Key et al., 1983).

MWFs are complex mixtures of eight or more chemicals. Four major types of MWFs are available for use in the workplace: the traditional straight oils, soluble fluids, semisynthetic fluids, and synthetic fluids (no oil). Semisynthetic MWFs contain the following: water, mineral oils, emulsifiers, chelating agents, coupling agents, antiweld agents, surfactant wetting agents, antifoaming agents, alkaline reserve, dyes, corrosion inhibitors (antirust), biocides (bioresistant compounds), and extreme pressure additives (Key et al., 1983; Howell, 1996).

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Workers of both sexes and of different ages who are involved in operating various types of machinery and equipment may be exposed to MWFs. The two main routes of exposure to MWFs are inhalation from aerosolization, and dermal from splashing of these fluids during machining, processing, and other operations. The dermal toxicity of semisynthetic MWFs has not been completely elucidated. Potentially irritating ingredients and additives have also not been completely studied with respect to their combined effects. The objective of this study was to investigate whether the significant increase in the liver weight seen in our earlier studies (Al-Humadi et al., 2000) was due to oxidative stress caused by MWFs treatment. Ethanol is a well-known, well-studied hepatotoxicant (Amini et al., 1996; Rouach et al., 1997). It was postulated that if the effects of MWF on liver are due to oxidant injury, the inclusion of ethanol should exacerbate this injury.

MATERIALS AND METHODS

MWF

For this study, MWFs were used at a concentration of 5% (v/v) at either pH 9.7 (diluted with water, normal dilution used in industries) or pH 7 (corrected with 0.2 IN HCl) for dermal exposure. Unused commercial semisynthetic metal working fluids with the following components were used: water (95%), mineral oils (1%), emulsifiers (0.7%), coupling agents (0.2%), antifoaming agents (0.15%), alkaline reserve (0.2%), corrosion inhibitors (antirust) (0.5%), biocides (bioresistant compounds) (0.74%), and lubricity agent (1.35%) (Key et al., 1983; Howell, 1996).

Animals

Two-week-old B6C3F1 mice of both sexes from Charles River Laboratories were aged to 6 mo (male 33–47 g and females 35–43 g) and divided into 6 groups ($n = 5$) (Table 1). Mice were housed (one per cage) at 75 ± 5°F (23.9°C), in an AAALAC-accredited facility (West Virginia University) with 40–60% relative humidity and a 12-h-light/dark cycle. Municipal water in bottles (with or without 5% ethanol; the consumed volume of the latter has been monitored with a graded cylinder once a week) and feed (Harlan Teklad rodent diet number 8604, Indianapolis, IN) were given with no restrictions, and Sani Chip Bedding (Harlan Teklad, Indianapolis, IN) was used and changed once a week. The average daily consumption of ethanol per mouse given in drinking water in our study was 4.4 ± 1.2 ml/d. All mice including two control groups (with or without ethanol in their water) were not shaved. MWFs were dispensed on the unshaved back skin from the cervical to sacral region (Al-Humadi et al., 2000). The fluids were evenly distributed with the help of curved stainless steel gavage needle throughout this region of the back. Animals were weighed soon after arrival, every 2 wk thereafter, and at sacrifice.

TABLE 1. Skin Application of Metal-Working Fluid to B6C3F1 Mice: Experimental Design

Group	Treatment[a]	Number of mice[b]	
		Females	Males
1	Control[c]	5	5
2	5% MWF/H_2O[d] pH 9.7	5	5
3	5% MWF/H_2O pH 7[e]	5	5
4	5% MWF/H_2O pH 9.7 + 5% ethanol	5	5
5	5% MWF/H_2O pH 7 + 5% ethanol	5	5
6	5% Ethanol only	5	5

[a]Exposure was 5 d/wk with 200 µl of the respective fluid dispensed on the unshaven back for 3 mo.
[b]B6C3F1 mice.
[c]Exposed to air only.
[d]Distilled water.
[e]The pH corrected with 1 *N* HCl.

Tissues

Animals were euthanized with carbon dioxide and exsanguinated. Complete necropsy was done on each mouse. The liver, testes, kidneys, lungs, spleen, and thymus were weighed immediately. The liver and testes were divided into several pieces and frozen immediately in liquid nitrogen. The skin was divided into two areas for consistency. One part was kept in the freezer at –80°C and used for total histamine measurements, and the second part was immersed in Davidson fixative and used for histology along with the above tissues. After fixation the tissue samples were embedded and sectioned at 5 µm. The sections were stained with hematoxylin and eosin. To detect mast cells, skin sections were stained with 0.1% toluidine blue. Mast cell counts were recorded using a light microscope (Olympus BX 40) with a high dry objective (40×). Five random fields were examined for mast cells. Cumulative counts from these five fields were recorded as the relative number of mast cells for the sample. The epidermis was clearly within the field of observation.

Chemical Analyses

Histamine Analysis Total skin histamine was measured by enzyme-linked immunosorbent assay (ELISA) as per the manufacturer's instructions (Immunotech, Inc., Westbrook, ME). Briefly, wet skin (40 mg) was extracted with 10 µl of 0.2 *N* perchloric acid per mg of tissue. Samples were homogenized using a sonifier cell disrupter (model 350, Branson Sonic Power Company, Danbury, CT) with a frequency of 20 Hz and an output of 40% maximum, 25 pulses (75 pulses/min), on ice. Samples were then centrifuged at 10,000 × g for 5 min at 4°C. Supernatant samples were collected and neutralized (pH 6.8) by addition of an equal volume of 1 *M* potassium borate (pH 9.25; 400 µl of 1 *M* potassium borate

per 400 µl of 0.2 *N* $HClO_4$). Samples were then centrifuged at 10,000 × g for 1 min at 4°C. Supernatants were collected and diluted (1:2000) with phosphate-buffered saline (PBS, pH 7.4). Analysis was performed using a Dynatech immunoassay system at a detection wavelength of 405 nm (Dynatech Inc., Chantilly, VA).

Ascorbic Acid Determination Samples (liver and testes) were weighed and placed into centrifuge tubes. A modified method for ascorbic acid extraction was used (Dhariwal et al., 1991). Briefly, phosphate buffer solution (liver = 250 µl; testes = 150 µl) and 90% methanol + 1 m*M* ethylenediamine tetraacetic acid (EDTA; liver = 500 µl; testes = 250 µl) were added to the samples. Samples were homogenized with a cell disrupter (see the histamine assay) and centrifuged at 5300 × g for 10 min at 4°C. Supernatants were filtered through 0.2-µ*M* nylon filters and 150 µl of the filtrate was used for the high-performance liquid chromatography (HPLC) analysis. A Waters Millennium 3.2 HPLC system and an ESA electrochemical detector (5010 model) were used. By using a Vandyke C18 column with a mobile phase of 0.05 *M* KH_2PO_4 (pH 2.5) at a flow rate of 0.7 ml/min, ascorbic acid peaks were detected (with limits of detection of 12.5 ng) at the potentials of E1 = –125 and E2 = 150.

Malondialdehyde Determinations The Bioxytech LPO-586 colorimetric assay (R&D Systems Inc., Minneapolis, MN) for lipid peroxidation was used to determine malondialdehyde levels in the liver and testes samples. Samples, standards or blank (water) (200 µl), were added to a tube containing 10 µl of 0.5 *M* butylated hydroxytoluene and 650 µl of 10.3 m*M* *N*-methyl-2-phenylindole (in acetonitrile). The mixture then was vortexed and 150 µl of 37% (12 *N*) HCl was added and mixed. The tubes then were stoppered and incubated for 60 min at 45°C. Turbid samples were centrifuged at 15,000 × g for 10 min to obtain a clear supernatant, and absorbance was measured at 586 nm.

Glutathione (GSH) Determinations The total protein sulfhydryl concentrations in liver homogenates were determined using ThioGlo TM1, a maleimide reagent, which produces a highly fluorescent product upon its reaction with SH groups (Langmuir et al., 1996).

Alcohol Dehydrogenase The method of Vallee and Hoch (1955) was used to determine the reaction velocity in which the rate of absorbance increase at 340 nm resulting from the reduction of NAD was measured. Pyrophosphate buffer at pH 8.8 (500 or 250 µl) was added to the liver or the testes samples, and then homogenized and centrifuged at 14,700 × g for 14 min (4°C). Liver supernatant was diluted 1/5 with sodium pyrophosphate buffer. Cuvettes were prepared by adding 1.5 ml of 0.032 *M* pyrophosphate buffer, pH 8.8, 0.5 ml of 2.0 *M* ethanol, and 1.0 ml of 0.025 *M* NAD. At zero time, 100 µl of the sample was added and absorbance readings were taken at 340 nm every 30 s for 6 min. The activity was calculated in units per milligram protein, equal to (ΔA_{340}/min)/(6.22 × mg protein/ml reaction mixture).

Data Analysis

Data were analyzed using Sigma Stat (version 2.0, Jandel Scientific Software, San Rafael, CA) statistical software for Windows 95, NT, and 3.1. An analysis of variance (ANOVA, Tukey test) and Student's *t*-test were conducted. Values of $p \leq .05$ were considered statistically significant.

RESULTS

Although dermal mast-cell numbers tended to increase in all female mice treated with MWF, a significant increase was seen in the skin of female mice from group 3 only (Table 2) with a concomitant increase in the dermal histamine levels. Skin histamine levels tended to be higher in female mice treated with MWF and given 5% ethanol in the drinking water compared to those treated with MWF with no ethanol in the drinking water (group 2 vs. 4); however, the mast-cell numbers were not increased beyond the MWF treatment level (group 2 vs. 4). The numbers of mast cells and the skin histamine levels were generally lower in male compared to female mice, and the effects of MWF were less striking.

Ascorbic acid (AA) was significantly decreased in the livers of female mice after treatment with either pH 9.7 or 7 MWF (Figure 1). In the male, similar decreases in the liver AA level were seen in the MWF (pH 7.0) group. Ethanol by itself reduced the level of liver AA in both female and male mice. Liver AA levels in the MWF plus ethanol treatment were not significantly different from treatment with either MWF or ethanol alone. The AA levels of testes of mice treated with MWF (pH 7 or pH 9.7) with or without ethanol decreased significantly.

The liver malondialdehyde (MDA) levels of treated (MWF, either pH, with or without ethanol) male mice was increased significantly (Table 3). In male mice the increase was statistically significant in both liver and testes.

TABLE 2. Histamine and Number of Mast Cells of B6C3F1 Mice, Skin

Group[a]	Treatment	Females, skin, histamine (n*M*/mg wet tissue ± SE)	Females, mast cells (number ± SE)	Males, skin, histamine (n*M*/mg wet tissue ± SE)	Males, mast cells (number ± SE)
1	Control	317.1 ± 5.3	51.0 ± 7.8	240.2 ± 30.1	32.6 ± 4.1
2	5% MWF pH 9.7	343.1 ± 23.5	74.3 ± 11.3	209.8 ± 15.4	32.4 ± 3.7
3	5% MWF pH 7	454.0 ± 32.7[b]	73.6 ± 4.3[b]	281.4 ± 29.9	36.4 ± 3.3
4	5% MWF pH 9.7 + ethanol	486.3 ± 53.9	72.2 ±7.8	197.0 ± 19.0	32.6 ± 4.2
5	5% MWF pH 7 + ethanol	464.4 ± 17.3	66.4 ± 7.2	394.6 ± 33.3	48.4 ± 6.0
6	5% Ethanol only	403.2 ± 37.2	59.0 ± 7.7	279.2 ± 39.6	29.8 ± 6.6

[a]Group 1 served as control for groups 2 and 3. Group 6 served as control for groups 4 and 5.
[b]Significantly different from group 1 at $p \leq .05$, *t*-test.

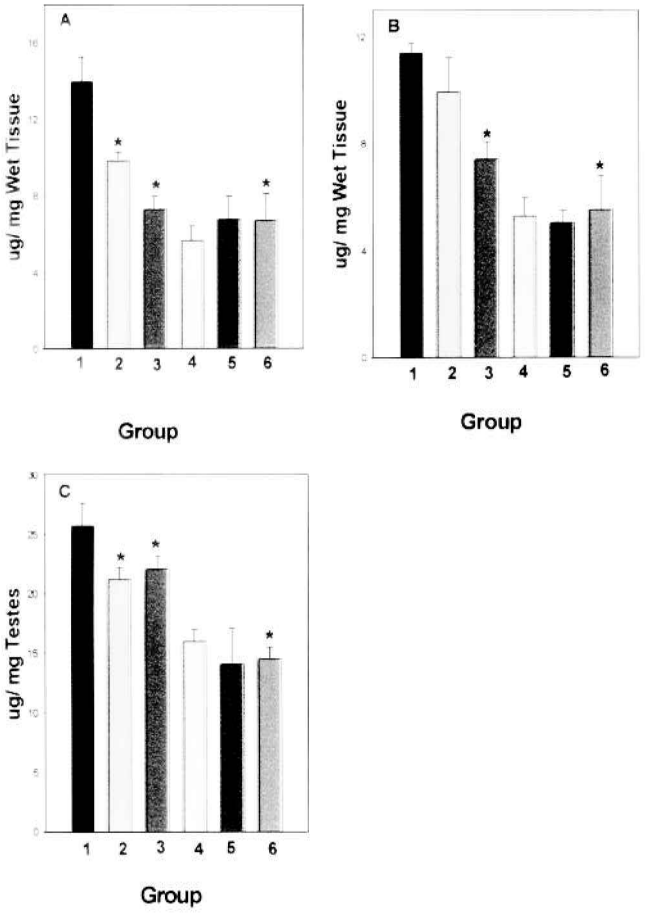

FIGURE 1. Ascorbic acid levels in the liver of (A) female and (B) male mice and (C) testes of B6C3F1 mice ($n = 5$). 1, Control; 2, 5% MWF/H_2O; 3, 5% MWF/H_2O, pH 7; 4, 5% MWF/H_2O + 5% ethanol; 5, 5% MWF/H_2O, pH 7 + 5% ethanol; 6, 5% ethanol only. Asterisk indicates significantly different from group 1 at $p \geq .05$. (Note: Group 1 served as control for groups 2 and 3. Group 6 served as control for groups 4 and 5).

Ethanol alone did not significantly alter liver MDA of control or MWF-treated male or female mice. Lipid peroxidation was significantly elevated in the testis after treatment with MWF (pH 7).

The liver GSH is decreased significantly in female and male mice treated with MWF pH 7 (Table 4). Ethanol alone decreased liver GSH of male but not female mice. The liver GSH levels for groups 2 and 4 were not determined.

The liver alcohol dehydrogenase (ADH) activities of treated male and female with or without ethanol are similar with respect to the same-sex controls with the exception of the males treated with MWF at pH 7 (Group 3), which showed a significant increase (Table 5).

TABLE 3. Malondialdehyde Levels in the Liver of B6C3F1 Mice

Group[a]	Treatment	Females, liver (n*M*/mg wet tissue ± SE)	Males, liver (n*M*/mg wet tissue ± SE)	Testes (n*M*/mg wet tissue ± SE)
1	Control	11.0 ± 2.0	4.4 ± 1.5	10.5 ± 1.4
2	5% MWF pH 9.7	17.4 ± 7.1	10.8 ± 1.7[b]	13.0 ± 5.0
3	5% MWF pH 7	14.4 ± 3.2	11.2 ± 1.9[b]	23.9 ± 6.1[b]
4	5% MWF pH 9.7 + ethanol	15.2 ± 7.6	14.4 ± 4.0[c]	9.7 ± 1.8
5	5% MWF pH 7 + ethanol	12.7 ± 2.9	17.8 ± 2.0[c,d]	13.7 ± 1.9
6	5% Ethanol only	15.5 ± 4.5	4.8 ± 1.8	22.0 ± 4.1[b]

[a]Group 1 served as control for groups 2 and 3. Group 6 served as control for groups 4 and 5.
[b]Significantly different from group 1 at $p \leq .05$, one-way ANOVA, Tukey test.
[c]Significantly different from group 6 at $p \leq .05$, one-way ANOVA, Tukey test.
[d]Significantly different from group 3 at $p \leq .05$, one-way ANOVA, Tukey test.

Microscopic examination of the back skin revealed hypertrophic sebaceous glands and increased numbers of mast cells in the dermis of groups 2, 3, 4, and 5. Other tissues (kidneys, liver, lung, spleen, and thymus) did not show any changes related to treatment.

DISCUSSION

Even in the normal physiological state, the production of oxygen free radicals in vivo is well recognized. These reactive oxygen species include superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen, among others (Aust et al., 1993; Kehrer, 1993; Shi et al., 1998). These cause oxidative degradation of important macromolecules such as unsaturated lipids of cell membranes, proteins, and nucleic acid (DNA) (Stadtman, 1992). These degradative changes have been implicated in the induction and evolution of many diseases like cancer and aging (Slaga, 1995; Shi et al., 1998; Lu et al., 1999; Shvedova et al., 2000). However, anti-

TABLE 4. Glutathione Levels in the Liver of B6C3F1 Mice

Group[a]	Treatment	Females, liver (μmol/mg protein ± SE)	Males, liver (μmol/mg protein ± SE)
1	Control	87.1 ± 6.0	18.3 ± 2.0
3	5% MWF pH 7	55.5 ± 9.5[b]	9.8 ± 2.5[b]
5	5% MWF pH 7 + ethanol	80.8 ± 6.4	6.3 ± 1.6
6	5% Ethanol only	92.2 ± 10.5	5.0 ± 1.2[b]

[a]Group 1 served as control for group 3. Group 6 served as control for group 5.
[b]Significantly different from group 1 at $p \leq .05$, one-way ANOVA, Tukey test.

TABLE 5. Alcohol Dehydrogenase Activities in the Liver of B6C3F1 Mice

Group[a]	Treatment	Females, liver (units/min/μg protein ± SE)	Males, liver (units/min/μg protein ± SE)
1	Control	1.1 ± 0.2	0.6 ± 0.1
2	5% MWF pH 9.7	1.3 ± 0.3	1.5 ± 0.5
3	5% MWF pH 7	1.3 ± 0.3	1.7 ± 0.7[b]
4	5% MWF pH 9.7 + ethanol	1.8 ± 0.1	1.0 ± 0.2
5	5% MWF pH 7 + ethanol	2.0 ± 0.3	1.0 ± 0.1
6	5% Ethanol only	1.5 ± 0.2	1.3 ± 0.4

[a]Group 1 served as control for groups 2 and 3. Group 6 served as control for groups 4 and 5.
[b]Significantly different from group 1 at $p \leq .05$, one-way ANOVA, Tukey test.

oxidant substances like glutathione, a hydrophilic antioxidant, and vitamins C and E either alone or in combination provide protection against the oxidative damages.

Our results indicate that the dermal application of MWF with or without ethanol given in drinking water resulted in increased oxidative damage in the liver of male mice as measured by the presence of malondialdehyde (MDA), a product of chain cleavage reactions that occur during degradation of fatty acids (Jauero, 1990) and prostaglandin synthesis (Marnett, 1994a). Lipid peroxidation is a substantial threat to the integrity of lipid membranes affecting the structure and function, as well as forming DNA adducts (Stadtman, 1992). Furthermore, MDA has been shown to be mutagenic (Marnett, 1994b) and carcinogenic (Spalaling, 1988). Other studies have shown that many functions of hepatic subcellular organelles are impaired as a consequence of lipid peroxidation (Dianzani & Ugazio, 1978).

Ascorbic acid (AA) provides protection against lipid peroxidation. In our study, the levels of AA in the liver of both sexes and in the testes of the male were decreased by MWF. This suggests that there was a decrease in endogenous AA with a concomitant increase in endogenous MDA levels in the treated mice. This supports the idea that the levels of AA in the tissues examined are not adequate to exert antioxidant activity in these mice. Of particular concern is the low level of AA on the physiological functions of the spermatozoa, in view of the important role that AA plays in the development and maturation of spermatozoa and in some enzymic activities (Grattagliano et al., 1997). It has been suggested that ascorbate is important for spermatogenesis, and the decreased concentrations of ascorbic acid may play an important role in the functional alterations of the testes (Grattagliano et al., 1997). Ascorbic acid has been reported as an important cofactor in several enzymatic activities of GSH and vitamin E (Meister, 1992). Rosenblum et al. (1989) showed that severe germ-cell injury and seminiferous tubule atrophy resulted from lipid peroxidation. Morphologically, the testes did not show any abnormalities in our study.

This does not mean that there was no gonadal functional deficiency in these male mice. One should keep in mind that these mice were treated for 91 d. Perhaps prolonging the treatment time or increasing the dose level would elicit morphological changes in the testes that may indicate or predict functional alterations.

Data indicated a decrement of glutathione levels in the liver. It is possible that GSH levels in the testes may also decrease. Glutathione levels were 4.8 times higher in control female livers than in males, which might explain why MWF did not increase lipid peroxidation (MDA) in females. Cytoprotection against lipid peroxidation and other types of oxidative damage is accomplished by diverse enzymatic and nonenzymatic means. The selenoenzyme glutathione peroxidase is one of the enzymes that can reduce and detoxify H_2O_2 and various organic hydroperoxides at the expense of GSH (Thomas et al., 1990). Phospholipid hydroperoxide glutathione peroxidase (PHGPX) plays an important role in the protection of the genetic material from harmful lipid hydroperoxides, particularly during periods of rapid cell proliferation and gametogenesis (Roveri et al., 1992).

The ingestion of ethanol with topical administration of MWF did not affect the ADH activity in the liver. Although ADH is said to be present in testes, the method used could not detect any ADH in the testes. This may be partly due to the level of ethanol in drinking water (the average daily consumption of ethanol per mouse in our study was 4.4 ± 1.2 ml/d). It has been reported that pregnant mice when exposed to ethanol (3 g/kg body weight intragastrically or to 15% in the drinking water) increased the generation of lipid peroxides and decreased glutathione, vitamin E, glutathione peroxidase, and superoxide dismutase activities in the liver (Amini et al., 1996). Ethanol has been reported to cause a significant decrease in the glutathione concentrations of the liver in male rats (Rouach et al., 1997) and in the rat fetuses (Addolorato et al., 1997). Our data confirmed that the effect of ethanol on the male rat liver also occurs in male mouse.

The antioxidants and oxidative products measured in this study suggest that when MWF is applied to the skin for 91 d, the liver may suffer from oxidative stress. It is proposed that supplementation of antioxidants may decrease oxidant injury due to MWF exposure. This hypothesis may be a useful avenue for future research.

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