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Enhanced oxidative stress in the skin of vitamin E deficient mice exposed to semisynthetic metal working fluids

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Abstract

Metal working fluids (MWFs) are widely used in industry for metal cutting, drilling, shaping, lubricating, and milling. Many occupational health concerns have arisen for workers exposed to MWFs. It has been reported earlier that occupational exposure to MWFs causes allergic and irritant contact dermatitis. Previously, we have shown that dermal exposure of female and male B6C3F1 mice to 5% MWFs for 3 months resulted in accumulation of mast cells and elevation of histamine in the skin. Topical exposure to MWFs also resulted in elevated oxidative stress in the liver of both sexes and the testes in males. The goal of this study was to evaluate whether preexisting oxidative stress in the skin exacerbated mast cell influx after MWFs treatment. Oxidative stress in the skin of B6C3F1 mice was generated by dietary vitamin E deprivation. Mice were given vitamin E deficient (5–10 IV/kg of vitamin E) or basal (50 IV/kg of vitamin E) diets for 34 weeks. Topical treatment with MWFs (100 μl, 30%) started after 18 weeks of alimentary vitamin E deprivation. Histology of the skin after 16 weeks of exposure to MWFs revealed a 53% increase in mast cell accumulation in vitamin E deficient diets compared to mice given a vitamin E sufficient diet. Total antioxidant reserve in skin of vitamin E deprived mice treated with MWFs was decreased by 66% as compared to those mice given a vitamin E sufficient diet. GSH and protein thiols in the dermis of vitamin E deprived mice exposed to MWFs were also decreased 39 and 42%, respectively, as compared to mice given basal diet. This study clearly delineates the role of oxidative stress in enhancing mast cell accumulation caused by topical exposure to MWFs.

Keywords: Metal working fluids; Oxidative stress; GSH; Vitamin E; Inflammation; skin

1. Introduction

One of the leading causes of occupational injury in the United States is skin disease. The incidence of occupational skin disease is highest in industrial workers exposed to metal working fluids (MWFs) (DOL, 1993; NIOSH, 1998). The spectrum of these occupational skin ailments extends from irritant/contact dermatitis to cancers of the skin and many other skin disorders (Alomar, 1994; De Boer et al., 1988, 1989a,b; Rycroft,

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1990). Various properties and components present in MWFs, such as alkalinity, metals like nickel and chromium, biocides, surfactants, non-corrosives, and a host of other components, are believed to play a role in the development of these skin ailments (Zugerman, 1986). These are present to increase the useful life of MWFs without deterioration, to improve and sustain lubrication, to protect the materials from excessive heat, and to remove any swarf from metal cutting or machinery operations (Sprince et al., 1994, 1996).

In our earlier work with the semisynthetic MWFs, we have shown that dermal exposure of female and male B6C3F1 mice to 5% MWFs for 3 months resulted in increased accumulation of mast cells and elevation of histamine content in the skin. Topical exposure also resulted in elevated oxidative stress in the liver of both sexes and the testes in males (Al-Humadi et al., 2000a,b). Published reports strongly indicate that oxidative stress plays an important role in the causation of irritant/contact dermatitis, burns, psoriasis, and carcinogenesis (Fuchs and Kern, 1998; Boh, 1996; Nachbar and Korting, 1995; Applegate et al., 1994; Picardo et al., 1992 Cetinkale et al., 1999; Freitas et al., 1998; Rezazadeh and Athar, 1997; Passi et al., 1998).

The complex nature of MWFs readily produces reactive oxygen species in the skin resulting in damage to the integrity of proteins, lipids, and the nucleic acids of the skin. This results in the recruitment of macrophages and neutrophils, which enhance the damage done to the skin. Skin is very sensitive to oxidative stress (Fuchs et al., 1989; Darr and Fidovich, 1994; Shindo et al., 1993). We hypothesized that depletion of major antioxidants in the skin will cause an increase in its susceptibility to injury and the production of reactive oxygen species. Therefore, we investigated the effect of dietary depletion of vitamin E followed by dermal exposure to MWF on the skin of B6C3F1 mice.

2. Materials and methods

Fatty acid-free human serum albumin (hSA), luminol, sodium dodecyl sulfate (SDS), and glutathione were purchased from Sigma Chemicals Co. (St. Louis, MO). Methanol, ethanol, chloroform, hexane (HPLC grade), and water (HPLC grade) were purchased from Aldrich Chemical Co. (Milwaukee, WI). ThioGlo-1™ was obtained from Covalent Inc. (Wobum, MA). 2,2′-azobis(2-aminodinopropane)-dihydrochloride (AAPH) was purchased from Wako Chemicals USA Inc. (Richmond, VA).

2.1. Experimental design

Animals: B6C3F1/Hsd male mice ($n = 120$) were obtained from Harlan (Indianapolis, IN). Each mouse was housed in an individual ventilated cage maintained at 70 °F in an AAALAC accredited facility. After 2 weeks of acclimatization, the mice were randomly divided into four groups. Groups one and two were placed on vitamin E deficient diet and groups three and four were given basal diet. The basal diet (Basal Diet™ 5755, Test Diet, Purina Mill, Richmond, IN) is a purified, synthetic diet that is said to provide all the essential nutrients to support maintenance, growth, gestation and lactation in laboratory mice and rats (Table 1). Vitamin E deficient diet was Basal Diet™ 5755 from which α-tocopherol was removed. Animals were daily supplied with fresh diets refrigerated at 4 °C. The α-tocopherol levels in basal and vitamin E deficient diets were 50 IV and less than 10 IV/kg of diet, respectively. The mice were kept on the respective diets for 34 weeks. Before treatments, the backs of mice were shaved (area 1.5×2.0 cm^2) with a hair clipper (Oster Professional Products, McMinnville, TN). After 18 weeks from the start of the study the mice from groups one and four were painted on the dorsal skin with unused MWF (100 μl, 30%, pH 7.8) once a day, 5 days a week for 16 weeks. Groups 2 and 3 (controls) were painted with saline (100 μl) at the same schedule.

2.2. Necropsy

After 34 weeks of the experiment the mice were euthanized by inhalation of an excess of carbon dioxide. The dorsal skin from the cervical to the sacral region (1.5×2.0 cm) was excised and equal

Table 1
Composition of diets

Typical analysis	%	Ingredients	%	Chemical composition[1]		Minerals		Vitamins	
				Nutrients[2]					
Protein	19.3	Casein-vitamin free	21.00	Protein, %	19.3	Calcium, %	0.6	Thiamin hydrochloride, ppm	20.0
Fat	10.0	Sucrose	5.00	Fat, %	10.0	Phosphorus, %	0.4	Riboflavin, ppm	20.0
Fiber[1]	4.3	Non-nutritive fiber (solka-floc)	3.00	Fiber (crude), %	4.3	Potassium, %	0.4	Nicotinic acid, ppm	90.0
Carbohydrate	60.6	Lard	5.00	Carbohydrate, %	60.6	Magnesium, %	0.065	Pyridoxine hydrochloride, ppm	20.0
		Dextrin	43.65	Gross energy, kcal/gm	4.1	Sodium, %	0.2	D-Calcium pantothenate, ppm	60.0
		DL-Methionine	0.15			Chlorine, %	0.2	Folic acid, ppm	4.0
		RP vitamin mixture[2]	2.00			Fluorine, ppm	5.0	Biotin, ppm	0.4
		Choline chloride	0.20			Iron, ppm	60.0	i-Inositol, ppm	200.0
		RP mineral mixture #10[1]	5.00			Zinc, ppm	20.0	Vitamin B_{12}, mcg/kg	20.0
		Total	100.00			Manganese, ppm	65.0	Menadione dimethylpyrimidinol bisulfite, ppm	20.0
						Copper, ppm	15.0	Vitamin A acetate, IU/gm	22.0
						Cobalt, ppm	3.2	Vitamin D_3, IU/gm	2.2
						Iodine, ppm	0.6	DL-α Tocopheryl acetate, IU/kg (sufficient diet)	50.0
						Chromium, ppm	3.0	DL-α Tocopheryl acetate, IU/kg (deficient diet)	<10.0
						Molybdenum, ppm	0.8		
						Selenium, ppm	0.2		

[1] Based on the latest ingredient analysis information.
[2] Nutrients expressed as percent of ration except where otherwise indicated.

pieces taken, with care that the same area of mouse skin was collected for histology and biochemical assays from each mouse.

2.3. Tissue processing

The skin was processed after fixation in 10% neutral buffered formalin, following the standard operating procedures of our laboratory. For regular histology, the histology slides were stained with hematoxylin and eosin. To detect mast cells, skin sections were stained with 0.1% toluidine blue. Using a light microscope (Olympus BX40) with a high dry objective ($40\times$), 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.

2.4. Homogenate preparation

The skin was excised promptly after the mice were sacrificed, and samples for biochemical analysis were frozen at -80 °C until processed. The skin homogenates were prepared from frozen tissues with phosphate buffered saline (pH 7.4) using a tissue tearer (model 985-370, Biospec Products Inc., Racine, WI). Homogenates were stored at -80 °C until processed further.

2.5. HPLC assay of α-tocopherol in the skin homogenates

α-Tocopherol from the above skin homogenates was extracted using the procedure described by Lang et al. (1986). A Waters HPLC system with an HP ODS Hypersil column (5 μm, 200×4.6 mm^2) was employed to measure α-tocopherol (Waters Associates, Milford, MA). A Waters HPLC system with a 717 auto sampler, Waters 600 controller pump and an 474 fluorescence detector was used. The wavelengths employed in the assay were 292 nm (excitation) and 324 nm (emission). Eluent was methyl alcohol (CH_3OH) and the flow rate was 1 ml/min. Under these conditions, the retention time for α-tocopherol was 8.5 min. The data acquired were exported from the Waters 474 detector using Millennium 2000 software for further analysis (Waters Associates).

2.6. Fluorescence assay of GSH and protein sulfhydryls

Total protein sulfhydryl concentration in homogenates of skin was determined using ThioGlo™-1, a maleimide reagent which produces a highly fluorescent product upon reaction with SH-groups (Shvedova et al., 2000). A standard curve was established by addition of GSH (0.04–4.0 mM) to 0.1 M phosphate buffer (pH 7.4) containing 10 μM ThioGlo™-1. GSH content was estimated from the immediate fluorescence response registered upon addition of ThioGlo™-1 to a tissue homogenate. Total protein sulfhydryls were determined from the augmentation of the fluorescence response after addition of SDS (4 mM) to the same homogenate. A spectrofluorophotometer (Shimadzu RF-5000 U, Kyoto, Japan) was employed in the assay (excitation $\lambda=388$ nm and emission $\lambda=500$ nm).

2.7. Chemiluminescence measurement of total antioxidant reserve in skin homogenates

A water soluble azo-initiator, AAPH, was used to produce peroxyl radicals at a constant rate (Niki, 1990). Oxidation of luminol by AAPH-derived peroxyl radicals was assayed by the chemiluminescence response to luminol. A delay in the chemiluminescence response, which is caused by interaction of endogenous antioxidants with AAPH-derived peroxyl radicals, is observed upon addition of homogenates. Based on the known rate of peroxyl radical generation by AAPH, the amount of peroxyl radicals scavenged by endogenous antioxidants can be calculated. The incubation medium contained 0.1 mM phosphate buffer (pH 7.4 at 37 °C), AAPH (50 mM), and luminol (400 μM). The concentration of protein in samples was 5–7 mg/ml. The reaction was started by addition of AAPH. A microplate luminometer (LB 96V, EG&G Berthold, Gaithersburg, MD) was employed for determinations.

2.8. Protein assay

Measurements of protein in homogenates of mouse skin were conducted using a Bio-Rad protein assay kit, cat. # 500-0006 (Richmond, CA).

2.9. Statistics

Data were expressed as the mean with ± standard error of the mean for each group. One-way ANOVA was employed to compare the responses between treatments. Statistical significance was set at $P < 0.05$.

3. Results

Body weight gain and food intake did not vary significantly between both groups given the basal and vitamin E deficient diets (Table 2).

Skin histology of mice given basal or vitamin E deficient diets for 34 weeks and topically treated with saline showed normal mouse skin structure (Fig. 1A and C). The covering squamous epithelium is one cell thick. Skin histology of mice given basal or vitamin E deficient diets, topically treated with MWFs for 16 weeks showed two cell thick covering squamous epithelium for the major portion (Fig. 1B and D). In both MWF treatment groups (Fig. 1B and D), the sebaceous glands and the epithelium look larger than those seen after saline treatment (Fig. 1A and C). Increased numbers of mast cells are seen in the dermis (Fig. 1B and D). This increase in mast cells was 80% in mice fed basal diets and treated with MWF compared to a 168% increase in similarly treated mice fed a vitamin E deficient diet (Fig. 2). The mast cell numbers were 53% higher in the skin of mice treated with MWFs and fed vitamin E deficient diet than in the mice fed basal diet and similarly treated with MWFs.

After 34 weeks of vitamin E deprivation, the level of α-tocopherol in skin of vitamin E deprived mice was decreased by 99% compared to those mice given basal vitamin E sufficient diet (Table 3). Sixteen weeks of exposure to MWFs resulted in a 51 or 94% decrease in α-tocopherol level in skin of mice given basal or vitamin E deficient diets, respectively, as compared to those in saline treated controls.

Addition of ThioGlo™-1 to skin homogenates produced an instantaneous increase in fluorescence due to the formation of the GSH–ThioGlo™-1 reaction product (Fig. 3). The intensity of the response did not change further unless SDS was added to the incubation system to unfold protein SH-groups at which point a slow increase of fluorescence was observed which leveled-off after approximately 45–60 min (Fig. 4). This latter fluorescence response was due to the interaction of protein SH-groups with ThioGlo™-1 (Shvedova et al., 2000). As shown in Figs. 3 and 4, the levels of GSH and protein thiols in the skin of B6C3F1 mice given vitamin E deficient diet for 34 weeks were significantly decreased by 70 and 64%, respectively, compared with saline treated mice given basal diet. We observed that exposure of mice to MWFs produced a significant 63% reduction in GSH concentration and a 56% de-

Table 2
Weight of B6C3F1 mice during the study (g)

Basal diet						Vitamin E deficient diet				
Treatment	Weeks					Weeks				
	0	4	8	12	16	0	4	8	12	16
Control	32.7 ± 1.6	35.0 ± 1.8	35.7 ± 1.6	36.8 ± 1.3	38.4 ± 1.5	29.0 ± 0.8	36.6 ± 1.6	33.6 ± 1.6	33.0 ± 1.6	29.6 ± 1.3
MWFs	32.6 ± 0.9	30.7 ± 1.5	31.8 ± 0.9	33.6 ± 0.8	36.4 ± 0.7	28.8 ± 0.8	34.8 ± 0.8	32.6 + 0.7	36.3 ± 0.9	29.0 ± 0.8

Values are means ± standard errors of 30 mice in each group.

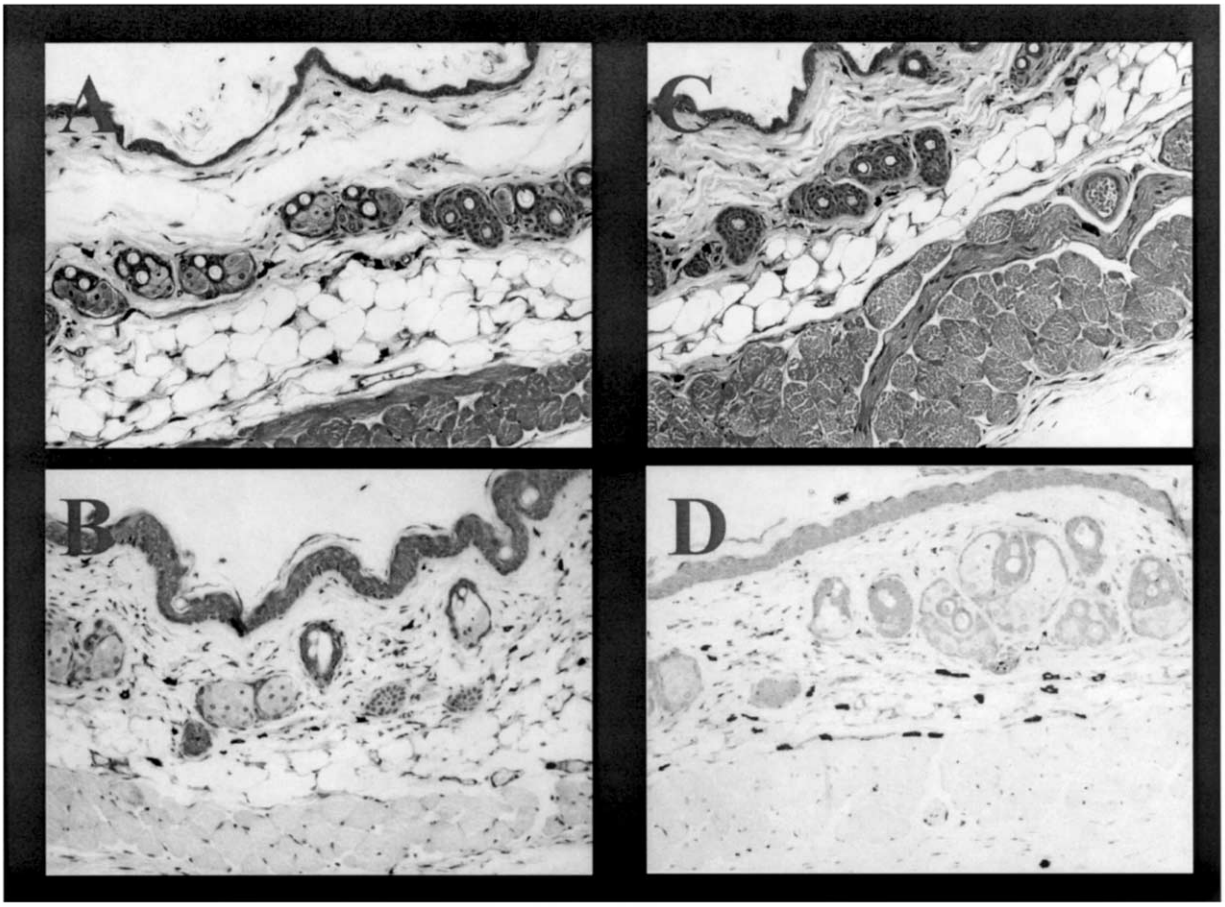

Fig. 1. Skin histology of B6C3F1 mice given basal or vitamin E deficient diets and treated with saline or MWF. (A) Basal diet, topically treated with saline (100 μl). (B) Basal diet, topically treated with MWF (100 μl, 30%, pH 7.8). (C) Vitamin E deficient diet, topically treated with saline (100 μl). (D) Vitamin E deficient diet, topically treated with MWF (100 μl, 30%, pH 7.8). Histology slides were stained with hemotoxylin and eosin. Magnification 200 ×. *Conditions:* saline or MWF (100 μl, pH 7.8) was topically applied to the shaved mouse back.

crease of protein sulfhydryls in skin homogenates of mice given basal diet (Figs. 3 and 4). The vitamin E deficient mice treated with MWFs produced significant decrease in GSH levels (39%) and protein thiols (42%) in the skin compared to the effect of MWFs in mice given basal diet.

Exposure to toxic insults resulted in the accumulation of biomarkers of oxidative stress when antioxidant defenses are compromised. To investigate total antioxidant reserves in the skin of mice, we employed a luminol-enhanced chemiluminescence assay. We used a water-soluble azo-initiator, AAPH, to produce peroxyl radicals at a constant rate (Niki, 1990). Interaction of peroxyl radicals generated by AAPH with luminol in phosphate buffer (pH 7.4 at 37 °C) resulted in a characteristic chemiluminescence response. Addition of skin homogenates to the oxidation system produced a lag period during which the chemiluminescence response was not observed. The duration of the lag period produced by homogenates from mice given basal diet and treated with saline was significantly greater than that observed for the homogenates from mice given vitamin E deficient diet and treated with saline (Fig. 5). The lag period is caused by the competition of all endoge-

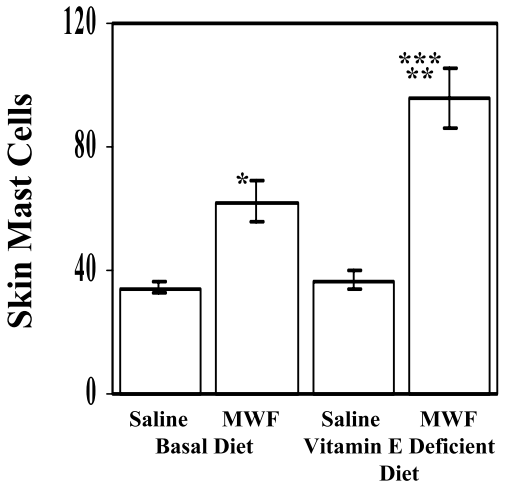

Fig. 2. Mast cells accumulation in the skin of B6C3F1 mice given vitamin E deficient or basal diets and treated with saline or MWF. Values are means ± SEM of 8 mice in each group. *$P < 0.05$ vs mice given a basal diet and topically treated with saline, **$P < 0.05$ vs mice given a basal diet and topically treated with MWF, ***$P < 0.05$ vs mice given a vitamin E deficient diet and topically treated with saline.

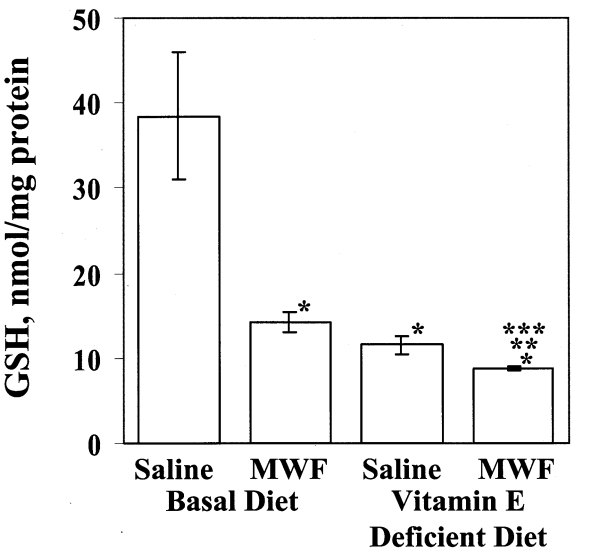

Fig. 3. Level of GSH in the skin of B6C3F1 mice given vitamin E deficient or basal diets and topically treated with saline or MWF for 16 weeks. Values are means ± SEM of 10 mice in each group. *$P < 0.05$ vs mice given a basal diet and topically treated with saline, **$P < 0.05$ vs mice given a basal diet and topically treated with MWF, ***$P < 0.05$ vs mice given a vitamin E deficient diet and topically treated with saline.

nous skin antioxidants with luminol for AAPH-derived peroxyl radicals (Kagan et al., 1998). The luminol-enhanced chemiluminescence assay revealed a significant 80% decrease in total antioxidant reserves of skin homogenates of mice kept on vitamin E deficient diet (Fig. 5). Total antioxidant reserves of skin of mice given basal and vitamin E deficient diets were reduced by 76 and 60% after MWF exposure, respectively, compared to saline treated controls. Total antioxidant reserves in skin of vitamin E deprived mice treated with MWFs was decreased by 66% as compared to those mice given vitamin E sufficient diet.

Table 3
Level of vitamin E in the skin of B6C3F1 mice given basal or vitamin E deficient diets and topically treated with MWF (pmol/mg protein)

Treatment	Basal diet	Vitamin E deficient diet
Saline	286.3 ± 58.9	3.0 ± 2.6*
MWF	139.3 ± 20.0*	0.8 ± 0.5*,**,***

Values are means ± SEM of 10 mice in each group.

* $P < 0.05$ vs mice given a basal diet and topically treated with saline.

** $P < 0.05$ vs mice given a basal diet and topically treated with MWF.

*** $P < 0.05$ vs mice given a vitamin E deficient diet and topically treated with saline.

4. Discussion

Plethora of evidence indicates intimate association with antioxidant inadequacy by depleted dietary vitamin E and an increase in ROS leading to cell injury, and subsequent disorders in the affected tissues including the skin (Nachbar and Korting, 1995). The keratinocytes of the epidermis provide the first line of defense against oxidative damage induced by environmental factors as they contain many enzymes and low molecular weight antioxidants, such as tocopherol, glutathione and ascorbic acid, essential for protection against reactive oxygen species. A decrease in tissue indices, such as malondialdehyde, conjugated dienes and antioxidants, can be considered

as evidence of oxidative stress. Our results strongly indicate a significant reduction of these primary antioxidants in the skin of mice given MWFs and maintained with a basal diet or vitamin E deficient diet, with the latter showing more marked effects.

The intrinsic reduction of vitamin E levels in the skin of mice fed a vitamin E deficient diet as seen in this study should be noted. Large differences of vitamin E levels of animal feed between and within different countries are well documented (Lehr et al., 1999). The major disadvantage of this is that the results obtained in established animal models may not be comparable or reproducible. The type of feed used in this study helps to avoid this vagary.

A spectrum of cutaneous disorders spanning from irritant and allergic contact dermatitis to squamous cell carcinoma has been associated with occupational exposures to MWFs (Alomar, 1994). The presence in the skin of mast cells and the hypertrophy of the sebaceous glands in mice dermally exposed to the MWF and maintained on a basal diet or vitamin E deficient diet confirmed our earlier observations of transdermal penetration of MWFs or some MWFs components (Al-Humadi et al., 2000a,b). The comparable body weight gains of the mice on basal and vitamin E deficient diets strongly suggests that the diet is adequate to maintain homeostasis of physiological processes, such as signaling pathways and responses to any stress inducing agents. This is in accord with earlier observations (Bei et al., 1996).

Our results convincingly show that the skin of B6C3F1 mice is more prone to injury when it is depleted of antioxidants, such as the micronutrient vitamin E. Indeed, when vitamin E depletion is superimposed with a further increase in ROS formation due to topical application of MWFs for 16 weeks, dermal injury is increased. The results of this study suggest that this oxidative stress is more pronounced when ROS formation exceeds the biochemical defenses of antioxidant reserves that have evolved to protect against oxidant-induced injury.

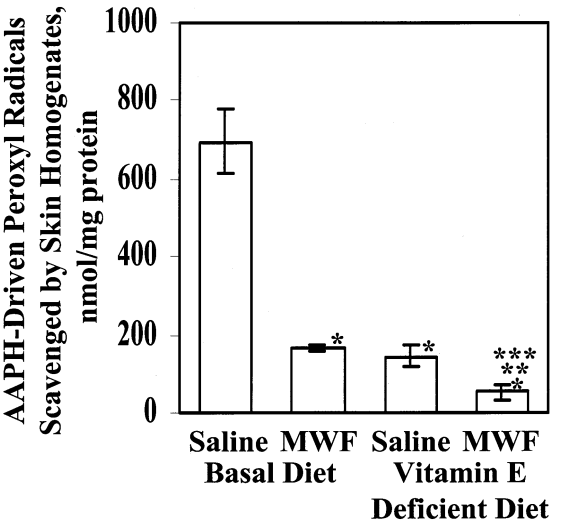

Fig. 5. Level of total antioxidant reserve in the skin of B6C3F1 mice given vitamin E deficient or basal diets and topically treated with saline or MWF. Values are means ± SEM of 9 mice in each group. $*P < 0.05$ vs mice given a basal diet and topically treated with saline, $**P < 0.05$ vs mice given a basal diet and topically treated with MWF, $***P < 0.05$ vs mice given a vitamin E deficient diet and topically treated with saline.

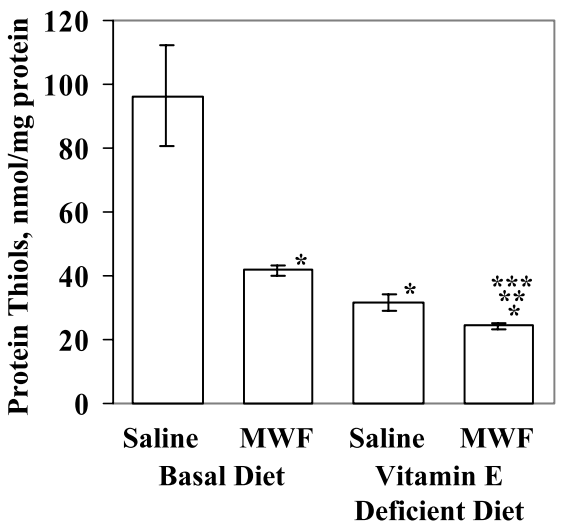

Fig. 4. Level of protein thiols in the skin of B6C3F1 mice given vitamin E deficient or basal diets and topically treated with saline or MWF. Values are means ± SEM of 10 mice in each group. $*P < 0.05$ vs mice given a basal diet and topically treated with saline, $**P < 0.05$ vs mice given a basal diet and topically treated with MWF, $***P < 0.05$ vs mice given a vitamin E deficient diet and topically treated with saline.

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