

# A Multi-Mineral Natural Product Inhibits Liver Tumor Formation in C57BL/6 Mice

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**Abstract** C57BL/6 mice were maintained for up to 18 months on high-fat and low-fat diets with or without a multi-mineral supplement derived from the skeletal remains of the red marine algae *Lithothamnion calcareum*. Numerous grossly observable liver masses were visible in animals on the “western-style” high-fat diet sacrificed at 12 and 18 months. The majority of the masses were in male mice (20 out of 100 males versus 3 out of 100 females;  $p=0.0002$ ). There were more liver masses in animals on the high-fat diet than on the low-fat diet (15 out of 50 on high-fat versus 5 out of 50 on low-fat;  $p=0.0254$ ). The multi-mineral supplement reduced the number of liver masses in mice on both diets (3 out of 25 male mice in the low-fat diet group without the supplement versus 1 out of 25 mice with supplement; 12 of 25 male mice in the high-fat diet group without the supplement versus 3 of 25 mice with supplement [ $p=0.0129$ ]). Histological evaluation revealed a total of 17 neoplastic lesions (9 adenomas and 8 hepatocellular carcinomas), and 18 pre-neoplastic lesions. Out of eight hepatocellular carcinomas, seven were found in unsupplemented diet groups. Steatosis was widely observed in livers with and without grossly observable masses, but the multi-mineral supplement had no

effect on the incidence of steatosis or its severity. Taken together, these findings suggest that a multi-mineral-rich natural product can protect mice against neoplastic and pre-neoplastic proliferative liver lesions that may develop in the face of steatosis.

**Keywords** Calcium · Hepatocellular carcinoma · Liver disease · Minerals · Trace elements

## Introduction

Overnutrition in the form of a high fat or high caloric intake is highly associated with fat infiltration in the liver or steatosis. While steatosis alone is typically well-tolerated, some individuals further develop inflammation (steatohepatitis) and fibrosis, culminating in cirrhosis. The spectrum of overnutrition-associated liver changes and metabolic alterations are clinically described as non-alcoholic fatty liver disease (NAFLD). NAFLD has recently been shown to increase the risk of hepatocellular carcinoma [1–3]. Mice on a long-term “Western-style” diet, with high caloric and dietary fat content, develop at least some of the features of NAFLD, specifically steatosis, and in some instances, peri-venular fibrosis [4–8]. Additionally, long-term high-fat diets have been demonstrated to increase neoplastic and pre-neoplastic liver lesions in mice [9–12].

Although the typical Western style diet has been epidemiologically and experimentally linked to liver disease, the specific dietary components that most contribute to these changes are still under investigation. In addition to a high content of saturated fat and sugar, the typical Western-style diet is deficient in calcium [13–15] and possibly other trace minerals that occur in conjunction with natural sources of calcium. We previously demonstrated that a calcium-rich,

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multi-mineral-rich marine algae-derived dietary supplement reduced the formation of adenomatous colonic polyps in mice on a high-fat Western diet (HFWD) [16]. The effects of mineral supplementation on hepatic disease have not been previously evaluated to our knowledge. In the present study, we assessed the development of pre-neoplastic and neoplastic liver lesions in healthy mice and determined whether the mineral supplement may have a protective effect against proliferative liver lesions.

## Materials and Methods

### Mineral-Rich Natural Product

The multi-mineral-rich material used in this study was obtained from the skeletal remains of the red marine algae, *Lithothamnion calcareum* (Pallas), also known as *Phymatolithon calcareum* (Pallas) [17]. The mineral-rich algae product is approximately 12% calcium by weight, and 1% magnesium, but also contains measurable levels of 72 other trace minerals. The mineral product is sold as a food supplement under the name Aquamin® (GRAS 000028) and is used in various products for human consumption in Europe, Asia, Australia, and North America [Marigot Ltd, Cork, IR]. The complete mineral composition of the batch used in the present study is shown in Supplement Table 1.

### Diet Groups

Four diet groups were included in this study, the AIN 76A (low-fat) rodent diet, a high-fat, western-style diet (HFWD), which is a variant of AIN 76A, and the same two diets supplemented with the mineral-rich algae product. AIN 76A contains 5% fat from corn oil. The HFWD was prepared according to the formulation of Newmark et al. [18] and designed to mimic the diet consumed by many individuals in Western society. It contains 20% fat from corn oil. On a per weight basis, the percentage of calories from fat in this diet is 37.8% as compared to 11.5% in the AIN 76A diet. Although sucrose is reduced in the HFWD relative to AIN 76A, the overall calories provided in the HFWD is 4,767 versus 3,902 kcal% in AIN 76A. In the HFWD, the calcium level is reduced from 5.25 to 0.41 g/kg. Both diets contain a mix of essential trace elements including potassium, magnesium, manganese, chromium, copper, iron, selenium, and zinc. For half of the animals in the present study, diets were supplemented with the mineral-rich natural product at 62 g%, providing comparable dietary calcium to what is in the AIN 76A diet. Diets were formulated and provided by Research Diets Incorporated (New Brunswick, NJ). The compositions of the four diets are presented in Supplement Table 2.

### Treatment Protocol and Necropsy

Separate cohorts of male and female C57BL/6 mice were maintained for 5, 12, or 18 months on their respective diet. Food was provided ad libitum. Animals were monitored at 2-day intervals throughout the maintenance phase and were weighed every 2 weeks. Animals were euthanized at each time point and subjected to necropsy. After the abdominal cavity was opened and the liver exposed, it was photographed in situ, if there was any gross lesion. Then, the liver was removed, weighed, and fixed in 10% buffered formalin for histology. Tissue sections from selected normal-appearing areas and from all grossly visible lesions (35 lesions in all) were stained with hematoxylin and eosin and examined at the light microscopic level by a board-certified veterinary pathologist (I.B).

Liver lesions were classified according to recently revised, standardized guidelines established by the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) project [19]. This project represents consensus criteria for histopathological lesions in rodents as established by the North American, European, British, and Japanese Societies of Toxicologic Pathology. Proliferative lesions were classified as foci of cellular alteration (FCA), non-regenerative hyperplasia (NRH), regenerative hyperplasia (RH), hepatic adenoma (HA), or hepatocellular carcinoma (HCC). These can be considered as a spectrum of pre-neoplastic and neoplastic lesions. The pre-neoplastic lesions (foci of cellular alteration, non-regenerative, and regenerative hyperplastic nodules) have been used as early markers of hepatic carcinogenesis in the mouse in short- and medium-term carcinogenicity studies [20].

Blood was obtained at the time of necropsy. A clinical chemistry profile including serum levels of albumin, globulin, total protein, alkaline phosphatase (ALKP), alanine aminotransaminase (ALT), cholesterol, and glucose was obtained for a subset of the animals (i.e., male mice at month 18). Serum calcium levels were assessed in the same samples. Values were determined using the Vet-Test dry chemistry analyzer (Idexx Laboratories, Westbrook, ME). Calcium levels in long bones were determined in parallel. Long bones (one femur and one tibia from each animal) were carefully separated from the surrounding connective tissue. Bones from all animals were “pooled” in order to obtain a sufficient quantity of bone, and the bone tissue converted to ash. Calcium levels were then determined by atomic adsorption spectroscopy (Advanced Laboratories, Inc., Salt Lake City, UT). C-reactive protein was assessed in serum using an enzyme-linked immunosorbent assay (ELISA) kit (Life Diagnostics Inc., West Chester, PA). Finally, a panel of cytokines was measured in serum samples

using the Bio-Plex bead-based cytokine assay from Bio-Rad Laboratories (Hercules, CA).

All of the procedures involving animals were reviewed and approved by the University Committee on Use and Care of Animals at the University of Michigan.

### Statistical Evaluation

Differences in the incidence of liver lesions among diet groups were assessed for statistical significance using the Fisher Exact Test (two-tailed). Biochemical data were evaluated statistically by ANOVA followed by paired group comparisons. Differences were considered significant at the  $p < 0.05$  level.

## Results

### Suppression of Liver Injury with the Mineral-Rich Natural Product: Gross Findings

Male and female mice were maintained on their respective diets for 5, 12, and 18 months. At all three time points, mice on the HFWD diet were heavier than animals on the low-fat diet. However, the presence or absence of the mineral-rich supplement in the diet had no effect on weight gain in either sex. Average weights for each group at the end of the 18-month time point are shown in Supplement Table 3.

Livers obtained at necropsy were examined in situ and after removal. While all livers appeared normal at the 5-month time point, distinct liver masses were observed in 20 male mice (out of 100 animals) at the 12- and 18-month time points (combined) as compared to only 3 lesions in 100 female mice ( $p = 0.0002$ ). Figure 1 demonstrates a large tumor in the liver of a male mouse while the female mouse has a normal-appearing liver.

Among male mice, there was a higher incidence of liver mass formation in the high-fat diet group than in the low-fat diet group (15 out of 50 males on high-fat versus 5 out of 50 on low fat;  $p = 0.0254$ , Fig. 2). Inclusion of the mineral-rich supplement in the diet was associated with a reduction in liver mass formation among male mice. In the high-fat diet group, 12 of 25 mice without the supplement had liver masses versus 3 of 25 mice with the supplement ( $p = 0.0129$ ) (Fig. 2). There was also a lower incidence of liver mass formation with the mineral supplement in the low-fat diet. However, with such a small number of lesions to begin with, there was no statistical difference (Fig. 2). With the high-fat and low-fat diet groups combined, the difference was significant (16 of 50 without the supplement versus 4 of 50 with the supplement ( $p = 0.0054$ ).

### Histological Findings

Liver masses identified grossly were histologically classified as pre-neoplastic or neoplastic proliferative lesions according to the guidelines established by the INHAND project [19]. Table 1 summarizes lesion histology among male mice as a function of diet group and Fig. 3 presents representative histology. Seventeen lesions in male mice were classified as neoplasms, with nine lesions identified as adenomas (benign tumors) and eight as hepatocellular carcinoma (malignant tumors). Thirteen of these 17 lesions occurred in animals without the dietary mineral supplement. Six lesions were identified as FCA, all in animals without the mineral supplement (Table 1). The remaining lesions were identified as RH or NRH. Among female mice, a total of three lesions were characterized histologically. All were non-malignant in nature (regenerative or non-regenerative hyperplasia). With so few lesions, not much can be said about diet influence.

In addition to replicative abnormalities, we also assessed livers for fatty infiltration (steatosis) and for inflammation. Virtually every animal (both males and females) at the 12- and 18-month time points demonstrated some degree of fatty infiltration. Even in livers with no grossly visible anomalies, steatosis was observed. Steatosis was more extensive in males than in females. The incidence was higher in mice on the high-fat diet than on the low-fat diet, but there was little difference between mice with the mineral-rich supplement and controls (not shown).

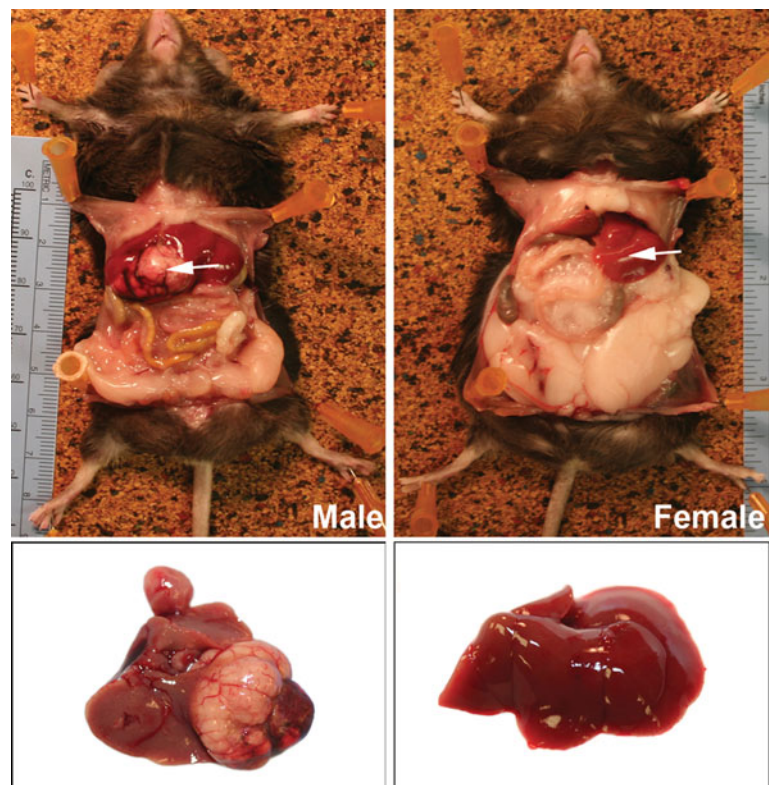
None of the animals euthanized at 5 or 12 months (male or female on any diet) showed significant inflammatory changes. At the 18-month time point, wide-spread liver inflammation was observed in three of the male mice on high-fat diet and some degree of inflammatory changes was also seen in four others.

### Metabolic Parameters, Cytokines, and Calcium Levels in Male Mice

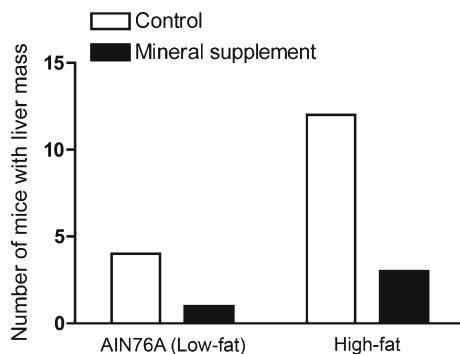
A number of metabolic markers reflective of liver function were assessed in serum of male mice at the 18-month time point. For several parameters, no diet-specific differences were detected (not shown). Not surprisingly, when animals with liver masses were compared to those without overt disease, differences were observed. Specifically, alkaline phosphatase and alanine aminotransaminase levels were increased in tumor-bearing mice (Table 2). Serum cytokine levels showed similar trends. That is, there were no diet-dependent differences, but there were differences between animals with liver tumors versus those without (Table 2).

Calcium levels were assessed in male mice at 18 months. Serum calcium levels were not statistically different between mice in any diet group, irrespective of mineral

**Fig. 1** In situ appearance of livers in a male and female mouse on the HFWD for 18 months. In the male mouse (left), a large liver mass is visible (arrow). In the female mouse, the liver is normal in appearance (arrow). A large amount of fat in the peritoneal wall of the female mouse can be seen, but this is not seen in the male mouse



supplementation (Supplement Table 4). Additionally, no difference in serum calcium levels between male mice with tumors and those without were seen (Table 2). Finally, bone reserves of calcium were also compared. Differences were slight (Supplement Table 4). Since bones from all mice in a group were pooled to obtain sufficient sample for analysis, it was not possible to compare animals with tumor versus those without.



**Fig. 2** Distribution of liver masses in male mice by diet group. Values shown represent the number of male mice at 12 and 18 months (combined) with overt liver masses at necropsy. Differences in the incidence of liver mass formation were assessed for statistical significance using the Fisher exact test (two tailed). Differences between high-fat and AIN 76A (low-fat) were significant at  $p=0.0254$ ; differences between unsupplemented (high-fat alone) and mineral-supplemented (high-fat) were significant at  $p=0.0129$ ; differences between unsupplemented and mineral-supplemented (high-fat and low-fat combined) were significant at  $p=0.0054$

## Discussion

A high-fat diet is known to produce fatty changes in the liver. Fatty changes are common and not considered serious, but more destructive liver disease including steatohepatitis, cirrhosis/fibrosis can follow. In addition, proliferative changes, leading, eventually, to hepatocellular carcinoma occur in a subset of individuals with pre-existing fatty changes [7, 21–23]. While dietary fat is clearly linked with liver disease, other components of the typical Western-style diet including high carbohydrate content and a deficiency of methyl group donors (i.e., certain B vitamins, methionine, and choline) also appear to contribute to deleterious changes in the liver [24].

In addition to a high content of saturated fat and sugar and a lack of methyl group donors, the typical Western-style diet also has a deficiency in calcium [13–15]. Possibly, other trace minerals that occur in conjunction with calcium are deficient in the same diet, as well. What role a deficiency in calcium and/or other inorganic minerals play in liver disease is not known. What is known is that another consequence of a Western style diet (i.e., adenomatous colon polyps) is clearly linked to an inadequate level of calcium. This has been demonstrated in humans in both epidemiological [25–30] and interventional [31, 32] studies. Experimental animal studies have shown that calcium alone reduces polyp formation in healthy rodents maintained on a high-fat diet [18, 33, 34] and our own recent studies have shown that a

**Table 1** Histological characterization of liver masses in male mice

Group	Histological description				
	FCA	NRH	RH	HA	HCC
AIN 76A (Low-fat)	3	1	0	2	3
AIN 76A (low fat)+mineral supplement	0	1	0	2	0
HFWD	3	6	1	4	4
HFWD+mineral supplement	0	2	1	1	1

A total of 35 distinct liver masses were observed in 20 male mice. Each lesion was characterized by light microscopy of hematoxylin and eosin-stained sections and classified according to the INHAND project (19)

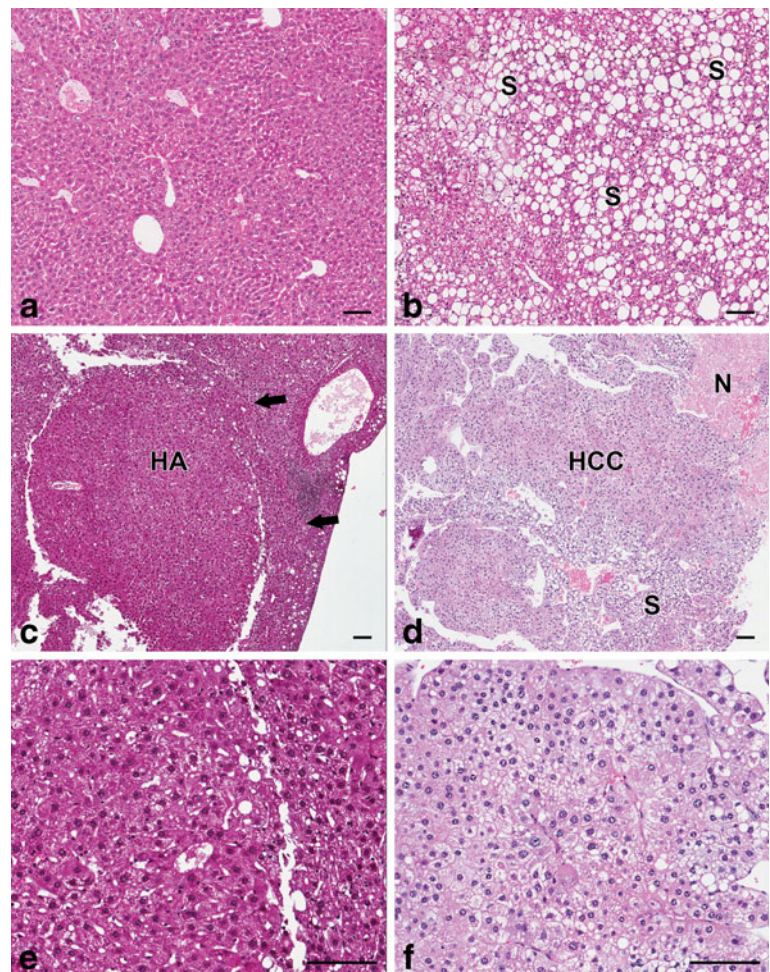
*FCA* focus of cellular alteration, *NRH* non-regenerative hyperplasia, *RH* regenerative hyperplasia, *HA* hepatic adenoma, *HCC* hepatocellular carcinoma

calcium-rich, multi-mineral supplement provides almost complete protection [16]. The current study demonstrates that the same multi-mineral-rich natural product used for polyp prevention also reduces liver injury. Specifically pre-neoplastic and neoplastic (adenoma and hepatocellular carcinoma) lesions are decreased. In our study with C57BL/6 mice, male mice maintained for up to 18 months on a high-

fat diet had significantly more liver lesions than animals fed a low-fat diet. However, even on the low-fat diet, some animals developed liver lesions. In both groups of animals, the mineral supplement reduced both the incidence and severity of disease.

How supplementation of the diet with a mineral-rich product protects against liver injury is not understood. Previous short-

**Fig. 3** Representative histological sections of liver in male mice. **a** Normal liver appearance from a male mouse on the mineral-supplemented high-fat diet at 18 months. **b** Steatotic liver from a male mouse on the mineral-supplemented high-fat diet at 18 months. Mineral supplementation did not affect the number of mice with steatosis. **c** and **e** Hepatic adenoma (*HA*) from a male mouse on the high-fat diet without supplementation at 18 months. *Arrow* indicates compression of adjacent parenchyma. **d** and **f** Hepatocellular carcinoma (*HCC*) in a male mouse on the high-fat diet without supplementation at 18 months. Hepatocytes form sheets and thick trabeculae without normal architectural features. Fat accumulation (steatosis, *S*) and necrosis (*N*) are evident in some areas. Neoplastic (and pre-neoplastic) lesions were more frequent in non-supplemented male mice. All sections are hematoxylin and eosin (bars=100  $\mu$ m)



**Table 2** Blood chemistry and cytokine analysis in mice with and without liver tumors

		No liver tumor	With liver tumor
Blood chemistry			
Albumin	(g/dL)	2.8±0.4	2.7±0.8
Globulin	(g/dL)	3.2±0.3	3.5±0.4
T protein	(g/dL)	6.0±0.5	6.2±1.0
Cholesterol	(mg/dL)	157±38	150±103
Glucose	(mg/dL)	263±49	233±66
ALKP	(U/L)	77±25	136±87*
ALT	(U/L)	67±48	263±305*
Calcium	(mg/dL)	10.2±1.1	9.5±1.5
C-reactive protein	(ng/mL)	238±167	242±374
Cytokines			
IL-1 $\alpha$	(pg/mL)	0.09±0.19	0.66±0.82*
IL-6	(pg/mL)	6.8±14.3	16.5±25.9
TNF- $\alpha$	(pg/mL)	0.7±1.9	2.3±3.6
KC (IL-8)	(pg/mL)	30.7±21.5	41.4±23.6
MCP-1	(pg/mL)	146±102	172±94
RANTES	(pg/mL)	14.2±7.8	12.1±5.7

Values shown are means and standard deviations, based on  $n=8$  mice with liver masses and  $n=41$  mice without masses. Differences were evaluated using the Student's  $t$  test

*ALKP* alkaline phosphatase, *ALT* alanine aminotransaminase, *IL-1  $\alpha$*  interleukin 1 alpha, *IL-6* interleukin 6, *TNF- $\alpha$*  tumor necrosis factor alpha, *KC* keratinocyte chemoattractant, *MCP-1* monocyte chemoattractant protein 1, *RANTES* regulated upon activation, normal T-cell expressed and secreted

\* $p<0.05$  significance

term (6 weeks) studies demonstrated that including high levels of chromium (to provide up to 80  $\mu\text{g}/\text{kg}$  of body weight) in the diet reduced liver injury in chronic cholestasis [35] as well as liver injury occurring in diabetic mice [36]. In another short-term study, sodium selenite (4 mg/kg of diet) reduced carcinogen-induced liver tumors in rats [37]. Mechanisms of action were not clearly delineated in these studies as multiple histo-pathological and biochemical changes were seen. Two other trace elements—zinc and copper—have also been shown to partially reverse liver injury in experimental animals [38]. An anti-oxidant effect was postulated to underlie protective activity. While all of these minerals are present in the multi-mineral-rich natural product used here, we do not believe that our results can be attributed to the activities of these important minerals alone. First of all, the amounts used in the previous, short-term studies were much higher than the levels found in the natural product (see Supplement Table 1). Second, both the low-fat diet (AIN 76A) and the high-fat diet—which is a variant of AIN 76A—contain significant amounts of essential minerals (including chromium, selenium, copper, and zinc) (see Supplement Table 2).

One possibility is that calcium in the multi-mineral-rich supplement is primarily responsible for protection from liver injury. In other tissues that have been studied in detail, including the colon, calcium promotes differentiation and, concomitantly, reduces epithelial cell proliferation [39–41]. Colon polyp chemopreventive activity is thought to reflect this property. The colon, of course, can be exposed to high levels of calcium and other minerals via the colonic fluid. The liver, on the other hand, relies on plasma calcium levels, which are tightly regulated. In our study, serum calcium levels differed by approximately 5% between male mice receiving the mineral-rich supplement in the high-fat diet and those on the high-fat diet without supplement. Whether this difference is sufficient to account for liver protection against tumor formation is not clear. Additional studies will be needed to address this issue.

Several of the minor trace minerals, especially members of the lanthanoid family of “rare earth” elements, are known to enhance calcium's epithelial cell growth-controlling activity. Lanthanoid elements bind to calcium-binding sites on regulatory molecules, including the extracellular calcium-sensing receptor (CaSR); in some cases, the affinity is higher than that of calcium itself [42–45]. Our own studies have shown that proliferation of epithelial cells can be suppressed by the multi-mineral-rich natural product under conditions in which equivalent amounts of calcium alone are not growth inhibitory [46]. Individual lanthanoid elements duplicate growth-inhibiting activity [47]. The capacity of the lanthanoid elements to suppress epithelial growth may be particularly important in tissues such as the liver, where exposure to calcium is limited by plasma concentrations. This may be especially important under conditions of relative (dietary) calcium deficiency.

Whether the multi-mineral-rich marine algae extract can be used under conditions needed to protect the liver will require clinical studies in humans. Given that in this animal study no measurable adverse effects were attributed to the supplement, a clinical study evaluating biomarkers of hepatic function may be informative.

In summary, mice on a high-fat diet with and without a multi-mineral supplement had similar degrees of steatosis, but mice without the supplement developed the majority of the pre-neoplastic and neoplastic lesions. This suggests that mineral supplementation, while it may not reverse fat accumulation in the face of a high-fat content diet, may provide protection against the more deleterious effects of such a diet. Given the clinical difficulties in limiting liver steatosis associated with human dietary habits and contributing metabolic conditions (diabetes mellitus), such a therapy may be very useful. Ongoing clinical studies related to polyp prevention could, perhaps, be modified to incorporate liver function biomarkers.

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