The Protective Effect of Kefir and Vitamin C on Azoxymethane Induced Toxicity and Induction of Metallothionein in Mice

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Summary

The present study was conducted to investigate whether vitamin C or kefir (a milk-based fermentation product) protected Swiss albino mice from azoxymethane (AOM) toxicity. We also investigated the effect of AOM administration on the induction of metallothionein (MT) expression in mice tissues. 40 12-week-old male/female (20:20) Swiss albino mice with a mean weight of 31.4 g were allocated into four groups. Animals in the first group were the control group. Animals in the other three groups were treated with AOM (5mg/kg body weight) subcutaneously twice weekly for a total of 7 weeks. Animals in the second group were treated only with AOM. Those in the third group were allowed access to kefir (50% wt/vol) *ad libitum*. Those in the fourth group received vitamin C subcutaneously (500 mg/kg) per day for 7 weeks. Six weeks after the final AOM treatment, all animals were sacrificed and necropsied. AOM administration caused severe liver lesions including enlarged hepatocytes (megalocytes) and many contained enlarged nuclei. Vitamin C and kefir administration clearly reduced the severity of AOM induced liver lesions. Induction of MT expression was observed in the liver and kidneys, particularly in the centrilobular zones and renal cortex, mainly in the distal renal tubules, collecting tubules, Henle's loop, and medulla, respectively. In conclusion, vitamin C and kefir supplementation were found to be able to reduce the severity of hepatotoxic lesions.,

Introduction

The methylating carcinogen AOM [MeN=N(O)Me], a metabolite of 1,2-dimethylhydrazine, an isomer of the liver and kidney carcinogen, N-nitrosodimethylamine, which is also activated by methyl group hydroxylation (*Magee and Barnes, 1967*), is specifically toxic to hepatocytes (*Doering et al, 2002*) producing hepatocellular lesions including altered hepatic foci and liver cell neoplasms (*Tanaka et al, 1985*) as well as colonic crypt cells in rodents (*Zaidi et al, 1995*).

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Many antioxidants have been investigated for their potential as cancer chemopreventive agents. Vitamin C was targeted in this study, because several animal studies showed that vitamin C supplementation had varied effect on induced carcinogenesis and toxicity (*Netke et al, 1997*). The antioxidant vitamins have been suggested to exert their effect by scavenging the free radicals formed during oxidation reactions in carcinogenesis whereas vitamin C has been found to be most effective in protecting plasma lipids from oxidative damage (*Henson et al, 1991*).

Dietary factors clearly have a major impact on chronic diseases as well as cancer development (*World Cancer Research Fund, 1997*). Kefir is a stirred beverage made from milk fermented with a complex mixture of bacteria, including various species of lactobacilli, lactococci, leuconostocs,

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acetic acid bacteria, and yeasts (both lactose fermenting and nonlactose fermenting). Kefir differs from yogurt and other fermented milks in that kefir grains (small clusters of microorganisms held together in a polysaccharide matrix) or mother cultures from grains are added to milk and cause fermentation (*Hallé et al, 1994*). Despite the lack of data, recent studies suggest antibacterial, immunological and antitumor effects of kefir in animals (*Furukawa et al, 1991; Zacconi et al, 1995*).

Metallothioneins (MTs) are a family of small (61 amino acids), cysteine-rich (20 residues), low molecular weight (6-7 kDa) proteins playing an important role in various physiological processes (*Hamer, 1986*). Their synthesis can be induced by many factors, including heavy metals, hormones and chemical and physical stress (*Davis and Cousins, 2000*) indicating their role in cellular proliferation and differentiation as well as in cellular defence mechanisms. MTs are often associated with rapidly proliferating tissues, such as fetal and neonatal liver (*Banerjee et al, 1982*), and influence tumor growth by affecting both cell proliferation and death (*Kägi, 1991*). To the best of our knowledge, the significance of MT expression in AOM toxication has not previously been assessed.

The purpose of the present study was to investigate the effects of both kefir and vitamin C on AOM-induced toxicity by using a monoclonal anti-MT (I and II isoforms) antibody and compare these findings with histopathological findings in the various tissues of Swiss albino mice.

Materials and Methods

Kefir Grains

Kefir grains were obtained from the Department of Food Hygiene and Technology, School of Veterinary Science, University of Kafkas, Kars, Turkey. In the laboratory, they were propagated at 22ºC for 20 h with twice- of thrice-weekly transfers in sterilized cow milk and kept at 4ºC for short-term storage. Kefir was prepared daily with sterilized cow milk inoculated with 5% kefir grains and incubated at 22ºC for 20 h. At the end of fermentation, the milk was filtered to remove the kefir grains (*Marshall & Cole, 1985*).

Animals and Treatment

40 inbred BABL/c Swiss albino mice (University of Celal Bayar, Faculty of Medicine, Department of Histology and Embryology, Manisa, Turkey) aged 12 weeks and weighing on average 31.4±3.9 g were randomly assigned to four groups (five male and five female mice for each group kept in separate cages) housed in stainless-steel wire-mesh cages. The animals were housed in a well-ventilated, temperature-controlled room (23±2 °C), at 55 % relative humidity under a 12 h light/dark cycle. They were allowed access to laboratory rodent chow (Korkuteli Feeds, Antalya, Turkey) and tap water *ad libitum* up to the time of sacrifice. The first group (control group) received only saline subcutaneously as the vehicle control. Animals in the other three groups were treated with AOM (5mg/kg body weight) subcutaneously in a vehicle of normal saline twice weekly for a total of seven weeks. Of those animals in the second group were treated with AOM only and allowed free access to tap water and standard pellet diet *ad libitum*. Those in the third group were allowed to access kefir (50% wt/vol) *ad libitum* instead of water, and those in the fourth group received vitamin C subcutaneously (500 mg/kg) per day for seven weeks. As scheduled, the mice were sacrificed six weeks after the final AOM treatment. Immediately after sacrifice, individual segments of colon (proximal, medial and distal) and small intestines were removed, gently flushed with saline solution to remove fecal content, opened longitudinally and fixed in 10% buffered formalin. The liver, kidneys, spleen, lungs, heart, and pancreas were removed, the liver and spleen weighed, cut into thinner sections and placed in fixative. After fixation, sections were embedded in paraffin. Tissue sections were prepared and stained with H&E (Hematoxilyn&Eosin) for histological examination.

Immunohistochemistry

Sections from all the tissue samples were to cut

4μm and processed for immunohistochemical examination by a streptavidin-biotin-peroxidase method. Tissue sections were placed on 3-aminopropyltriethoxysilane (Sigma, St. Louis, Montana, USA) coated slides, dewaxed and hydrated. Antigen retrieval was facilitated by heating in citrate buffer (pH 6.0) for 20 min in a microwave oven with a power of 600 watts. The slides were then dipped in freshly prepared absolute methanol containing hydrogen peroxide 3% v/v for 20 min to block endogenous peroxidase activity. A mouse monoclonal antibody that reacts with human and rabbit MT (Clone: E9, Dako Corporation, Carpinteria, USA, code M0639) was used at a dilution of 1:600 for 60 min in this study. This antibody reacts with both MT-1 and MT-2. After washing with phosphate-buffered saline (PBS), the slides were incubated with biotinylated rabbit anti-mouse immunoglobulin G (Dako Corporation, Carpinteria, USA) diluted 1:300 in PBS for 60 min at room temperature. Sections then were incubated with streptavidin peroxidase complex (ABC; Dako Corporation, Carpinteria, USA) diluted 1:300 in Tris-buffered solution (TBS) for 60 min at room temperature. The slides were then treated for 5 min at room temperature with 3,3′ diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, Montana, USA) in distilled water (0.5 mg DAB/ml) containing hydrogen peroxide 30% v/v. Finally, sections were counterstained with Mayer's hematoxylin, dehydrated and mounted. Negative control tissue sections were incubated with normal rabbit serum. The specifity of the MT monoclonal antibody was tested by pre-absorption techniques in which the antibody was preabsorbed with an excess (100 μmol) of the initial antigen (horse MT; Sigma, St Louis, Montana, USA).

The percentage of the total area of the MT positive cells was assessed quantitatively under a light microscope with an ocular with grids and a 40X objective. A total of 10 high-power fields were randomly chosen. The findings were categorized as follows: (-) no positively staining cells; (+) 5-25%; (++) 26-50%; (+++) 51-75%; (++++) >75% of cells positive. The intensity of MT staining was assessed semiquantitatively for the cytoplasm and nucleus of the cells separately as follows: (-) none; (+) weak; (++) moderate; (+++) intense immunolabeling.

Results

Subcutaneous AOM and vitamin C administration and oral consumption of kefir had no apparent effects on body-weight gain on all groups including the control group, and all animals survived the 7-week exposure period. There was not any sex-related significant difference between male and female mice in all the experimental groups. The means of the body and organ weights are presented in the Table 1.

Table 1. Initial and final average body (BW) and final organ weights (W) of 12-week-old mice. Each animal was subcutaneously injected with azoxymethane (AOM; 5 mg/kg body weight) twice a week for a total of seven weeks. The experiment lasted for 13 weeks and it was terminated six weeks after the final AOM treatment.

	Groups							
	Control (1st week)	Control (13th. week)	AOM (1st week)	AOM (13th. week)	AOM+Vit C (1st week)	AOM+Vit C (13th. week)	AOM+Kefir (1st week)	AOM+Kefir (13th. week)
BW (g)	31.1±3.2	32.9±4.4	30.3±3	34.9±3.1	30.4±4.6	33.5±4.5	32±3.3	28.8±8
Liver W	-	1.8±0.1	-	1.9±0.2	-	1.9±0.2	-	1.5±0.4
Spleen W	-	0.2±0.06	-	0.2±0.04	-	0.2±0.05	-	0.2±0.07

Gross pathological changes

In all groups, the primary lesions were observed in the liver. The gross assessment of the livers at necropsy showed that the livers of all the animals were enlarged and pale yellow to tan in colour in all groups compared to controls. However, the changes were less severe in the vitamin C and kefir groups. AOM exposure did not cause any significant macroscopical change in any of the other organs examined.

Histopathological studies

The severity of histopathological lesions was graded as minimal, mild, moderate and severe. The AOM exposure caused severe liver damage in all animals in the AOM group. The damage was composed of marked hepatocellular atypia, including hepatocellular anisocytosis, megalocytosis, megakaryocytosis, occasional binucleated hepatocytes (Fig. 1), scattered individual hepatocyte necrosis, apoptosis and multilobular degeneration. The volume of the affected hepatocytes was increased with an enlarged single nucleus. The nuclear membrane was also stained strongly with scant and fragmented chromatin. The nucleolus was enlarged and intranuclear spherical one or two membrane bound inclusions formed by invagination of cytoplasm into the nucleus, were often seen. The cytoplasmic volume was increased with sharp and condensed margins. The enlarged cells were closely apposed making sinusoids less evident. Associated lesions included mild cholestasis, lymphocyte aggregates with presence of neutrophils, mild proliferation of bile ducts within the parenchyma and in the portal triads. The lesions were only mild or moderate in the vitamin C (Fig. 2) and kefir groups (Fig. 3) with reduced number of intranuclear inclusions and mild hepatocellular anisocytosis.

For the kidneys the histopathological findings in each exposure group were very similar. They exhibited no significant abnormalities in the glomeruli. Renal tubuli occasionally showed hyaline casts in their lumens. Mild focal interstitial nephritis was also present, characterized by interstitial focal aggregates of lymphocytes.

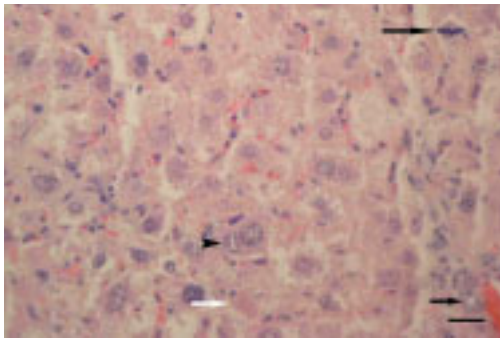

Figure 1. Liver section from a mouse given AOM. Enlargement of hepatocytes, involving nucleus and cytoplasm (megalocytosis), and hepatocellular atypia. Note binucleated hepatocytes (arrow head) with nuclear inclusions and proliferating bile ductules (short arrow). A mitotic figure is present (long arrow). Apoptosis is reflected by the small, densely eosinophilic anuclear mass (white arrow). H&E; Bar = 25 μm.

Immunohistochemistry

AOM treatment caused MT expression in both the liver and kidneys of the mice. Vitamin C and kefir

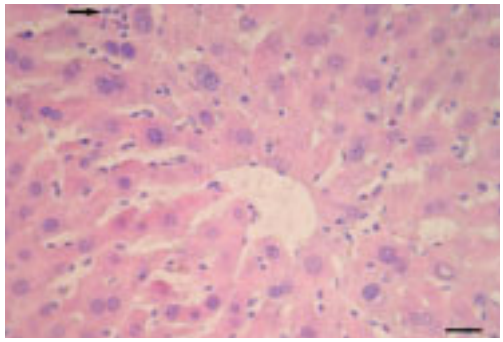

Figure 2. Liver sections of a mouse after exposure to AOM and application of vitamin C. The changes are minimal with mild infiltration of lymphocytes in the midzonal region (arrow). The central vein is located in the centre. H&E; Bar = 25 μm.

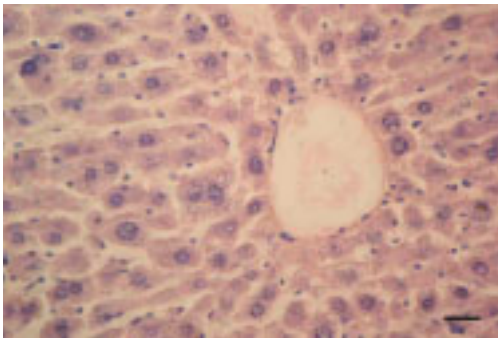

Figure 3. Liver sections of a mouse after exposure to AOM and oral administration of kefir showing centrilobular sinusoidal dilatation and mild vacuolar change in the cytoplasm of hepatocytes. The hepatocellular atypia is minimal. H&E; Bar = 25 μm.

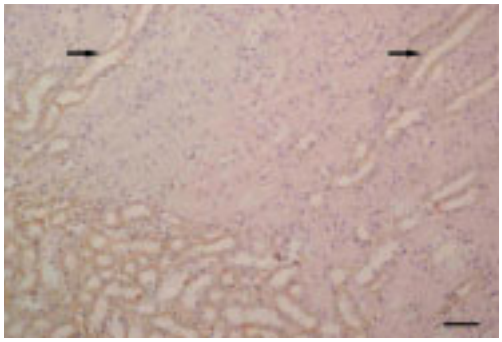

Figure 5. Immunohistochemical staining of MT in the kidney of a mouse from the vitamin C group. AOM induced renal MT is detected in pars radiata (Henle's loop; arrows) as well as in the renal medulla (bottom left). Peroxidase avidin-biotin complex. Bar = 50 μm.

administrations did not alter the MT immunostaining caused by AOM application. MT was detected in isolated cells of the hepatic parenchyma, and in hepatocytes around the central veins, presenting a moderate to intense pattern of staining in the cytoplasm while sparing the nuclei (Fig. 4). Cytoplasmic patterns of staining for MT were observed in hepatocytes located in the vicinity of inflammatory infiltrations. Kupffer cells did not show any MT immunoreactiviy during the course of the experiment.

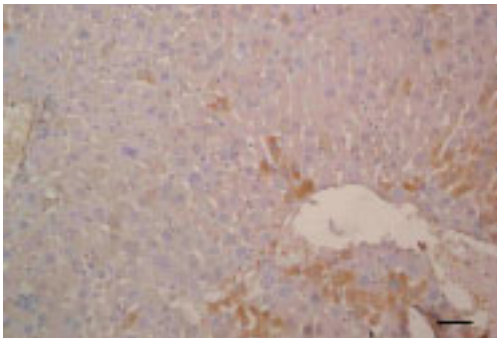

Figure 4. MT immunoreactivity in the cytoplasm of the centrilobular hepatocytes in liver sections of the mouse in the vitamin C group. Peroxidase avidin-biotin complex. Bar = 50 μm.

No MT immunohistochemical staining was seen in the kidney of control mice. However, AOM exposure caused moderate to intense MT immunoreactivity in the cytoplasm of the tubular epithelium of the renal cortex, mainly in the distal renal tubules, collecting tubules and Henle's loop in both the kefir and vitamin C (Fig. 5) administered groups. The area of MT labeling in the cortex increased from deep cortex to medulla. MT-positive granules were demonstrated in the lumina of some of the tubules. No MT labeling was observed in the glomeruli.

Weak MT immunoreactivity was very occasionally demonstrated in the alimentary tract in some of the crypts of the small and large intestines without any significant difference between control and experimental groups. Diffuse and moderate MT immunoreactivity was also demonstrated in the pancreatic acinar cells in both control and experimental groups. Details about other organs are given in Table 2.

Discussion

AOM has previously been shown to exert its toxic effects on the liver causing selective necrosis of hepatocytes sparing the liver sinusoidal endothelial

Table 2. Immunohistochemically detected MT in mice tissues. The percentage of the total area of the MT positive cells was assessed quantitatively as follows: (-) no positively staining cells; (+) 5-25%; (++) 26-50%; (+++) 51-75%; (++++) >75% of cells positive.

Organs	**Groups**			
	Control	AOM	AOM+Vit C	AOM+Kefir
Liver	-	+	+	+
Kidney	-	+	+	+
Pancreas	+++	+++	+++	+++
Spleen	-	-	-	-
Heart	-	-	-	-
Lungs	-	-	-	-
Jejunum	-	-	-	-
Ileum	-	-	-	-
Cecum	-	-	-	-
Colon	-	-	-	-

cells (*Doering et al, 2002*). In the present study, the administration of AOM in mice caused liver injury recognized histopathologically. It was also demonstrated that the expression of MT, in response to AOM-induced hepatotoxicity, increased significantly in the liver and kidneys of mice.

Past experience showed that a subcutaneous dose of 15 mg/kg has an acute toxic effect on mice and caused death within 48 hours. For that reason we reduced the AOM dose, based upon existing toxicity data for rodents (*NIOSH, 1985*) to 5mg/kg (twice weekly for the seven weeks). This experimental protocol was similar to those of (*Kroes et al, 1975*) and (*Oredipe et al, 1989*) in which s.c. doses of 10 and 15 mg/kg/week were applied for 16 and three weeks, and the experiments terminated four and 32 weeks following the last injection of AOM, respectively. In the present study, the duration of AOM application was deliberately limited to seven weeks and the experiment was terminated six weeks after the last injection of AOM to permit delineation of the initiation phase and to detect the toxicological effects of AOM.

The liver lesions encountered were similar to those induced by other well-described hepatotoxins including pyrrolizidine alkaloids and aflatoxins (*Bull et al, 1968; Netke et al, 1997*). Toxic hepatitis is generally characterized by hepatic necrosis, presence of megalocytes, cellular atypia, bile duct proliferation and fibrosis. The hepatotoxic lesions observed in mice were attributed to the biotransformation of AOM by mixed function oxidases to more toxic intermediate reactive species such as methylazoxymethanol, and this readily yields the methyldiazonium ion, which in turn is capable of alkylating macromolecules by enzymatic (alcohol dehydrogenase or microsomal enzymes) and nonenzymatic processes in the liver and colon (*Fiala et al, 1984*).

In this study we did not observe hepatocellular carcinoma, similar to the work of (*Wargovich et al. 1991*). However, (*Tanaka et al. 1985*) reported finding many hepatocellular neoplasms in rats treated with subcutaneous injections of AOM. Similarly, previous studies also showed that AOM caused liver cell foci that have some characteristic changes recognized to be preneoplastic lesions and the precursor of liver neoplasms in the hepatocarcinogenesis (*Pitot & Sirica, 1980*). In the present study such lesions were not present in all the groups. Such differences are possibly due to the different dose of AOM and the duration of the experiment.

The present investigation was also designed to determine whether intake of vitamin C and kefir protected mice from AOM toxicity. From the present work, it is evident that although cellular damage and megalocytosis occurred during AOM treatment, vitamin C administration and kefir supplementation reduced these changes. Vitamin C has been reported to have a protective effect on rat liver injury (*Shiraishi et al, 1993*) and liver carcinogenesis (*Iverson et al, 1988*) induced by cadmium and aflatoxin, respectively. The mechanism of action proposed for the protective effect of vitamin C during carcinogenesis is similar to the other antioxidants (*Hendrich & Pitot, 1987*) scavenging of free radicals or neutralizing reactive oxygen species (*Chen, 1988*) that can damage DNA (*Wiseman & Halliwell, 1996*). In the present study the oral administration of kefir similarly reduced the severity of the liver lesions. The most well known benefits of orally administered kefir is inhibiting tumor growth and inducing the apoptotic form of tumor cell lysis in mice, by stimulation of host immune systems, (*Liu et al, 2002*) and its antioxidative features (*Hoolihan, 2000*), which are possibly derived from the biological activity of kefir microflora (*Guven et al, 2003*).

Several mechanisms of inhibition of tumor development by probiotics have been proposed. The local tumor suppression trait of lactic acid bacteria was attributed to the peptidoglycan structure composed of muramyl peptides present in the bacterial cell wall (*Bogdanov, 1973*). The beneficial effects of these bacteria have also been associated with the ability to modulate immune parameters, including T cell, natural killer cells and macrophage activity, important for hindering tumor development (*Ouwehand et al, 1999*). In addition (*Liu et al. 2002*) have shown that kefir can increase endogenous IgA production, and so may reduce the risk associated with exposure to food antigens, possibly due to the polysaccharide in the kefir.

Our results revealed similar beneficial effects of kefir and vitamin C for reducing the effects of early stages of toxicity induced by AOM, although the proposed mechanisms differ. Thus, while kefir acts by enhancing the immune response of the host (*Liu et al, 2002*), vitamin C acts by free radical scavenging and neutralisation of reactive oxygen species (Chen, 1988).

MT is a small cysteine-rich protein, thought to be mainly involved in metal regulation and detoxification, and implicated also in cell growth and differentiation (*Webb, 1987*). The accumulation of MT differs between tissues, being most pronounced in the liver and kidney because of their physiological role as detoxifiers (*Hunziker & Kagi, 1985*). In the present study, MT immunoreactivity was demonstrated in the cytoplasm of the hepatocytes, but sparing the nuclei. Similarly, MT is also detected as a cytoplasmic protein in rat-liver hepatocytes after partial hepatectomy (*Margeli et al, 1994*). It has been reported that cytoplasmic MT expression protects against the cytotoxicity of heavy metals, whereas nuclear MT protects against the cytotoxicity of mutagenic agents (*Woo and Lazo, 1997*). It has been shown that, in cases of hepatocellular proliferation, an induction of liver MT occurs (*Tohyama et al, 1993*). The toxic effects of AOM on the liver are theorized to involve free radicals (*Slauson & Cooper, 1990*) and MT can scavenge free radicals in vitro (*Thomas et al, 1986*).

The MT immunoreactivity observed in the kidneys was similar to the findings in the developing rat kidney (*Nishimura et al, 1989*). Adult rat kidney showed less intense immunolabeling than did developing rat kidney, suggesting a possible involvement of MT in cell proliferation and differentiation in the kidney (*Nishimura et al, 1989*). Cadmium injection in rats caused MT staining mainly in the cytoplasm, the brush border, or lumen of proximal tubular cells (*Tohyama et al, 1988*). However, in the present study, no MT immunoreactivity was observed neither in proximal tubular cells nor in glomeruli or interstitial cells. The distribution of MT was observed mainly in distal portions of convoluted tubules, collecting tubules and loops of Henle. Similarly, in rat kidneys, repeated cadmium injections caused more MT staining in the

ascending thick segment of Henle's loop and in distal convoluted and descending thin segment of Henle's loop (*Tohyama et al, 1988*). These results indicate that some MT-related processes are important for understanding both toxicity and its protective effect on kidney tubules. It is still unclear whether the MT synthesized in the kidney acts as a protective or as a toxic agent to the tubular cells. However, the appearance of MT in the regenerating cells in the kidney and liver may indicate another possible role of MT in cellular regeneration and proliferation (*Tanimoto et al, 1999*).

In the present study, AOM caused a significant increase in the MT expressions in the liver and kidneys of all experimental groups without any sex difference. However, our results did not show any important difference in terms of MT expression among the experimental groups. We speculate that the induction of MT in hepatic lesions may be a common event, resulting in resistance to apoptosis and the formation of hepatic tumors. MT immunoreactiviy was demonstrated in the glandular epithelium of the pancreas in all groups including the controls without any difference between groups in terms of staining intensity and distribution. This suggests that MT is greatly involved in homeostasis of the pancreas and synthesis of pancreatic hormones (*Milnerowicz et al, 2004*). It also accords with previous reports suggesting a possible involvement of MT in the secretion of metals such as zinc (*Nishimura et al, 1990*).

In conclusion, the present results showed that orally administered kefir as a fermented milk product and parenteral vitamin C administration both caused a reduction of hepatotoxic lesions indicating a protective effect of kefir and vitamin C on AOM-induced liver injury in mice. Furthermore, present findings revealed a clear increase of MT expression in the liver and kidney associated with AOM-induced toxicity and vitamin C and kefir applications did not alter the level of MT expression in the mice.

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