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The effect of natural clinoptilolite on the serotonergic receptors in the brain of mice with mammary carcinoma

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Abstract

The ex vivo effect of tribomechanically micronized zeolite (MZ) on the binding of ^{3}H-8-OH-DPAT to 5-HT$_{1A}$ and ^{3}H-5-HT to 5-HT$_{1B}$ receptors was investigated in the brain of nontumorous (control) and mammary carcinoma bearing female mice. During 14 and 28 days mice were fed with standard food, standard food supplemented with 25% of MZ, or standard food supplemented with 25% of non tribomechanically micronized zeolite (non-MZ). A reduced binding of ^{3}H-8-OH-DPAT to 5-HT$_{1A}$ receptors in mammary carcinoma bearing mice was found when compared to control mice fed with standard food for 28 days, suggesting a time dependent alteration of 5-HT$_{1A}$ receptors in mammary carcinoma. The addition of MZ for 28 days in these mice abolished the decrease in 5-HT$_{1A}$ receptors binding, indicating a possible beneficial effect of MZ, at least on 5-HT$_{1A}$ receptors in mammary carcinoma bearing mice. The preliminary data show that MZ administered as a food supplement (25%) for 14 days induced a transient decrease in the binding of ^{3}H-5-HT to brain 5-HT$_{1B}$ receptors only in control, but not in tumor-bearing mice, that disappeared after 28 days of MZ-supplemented food administration. The mechanism of the indirect action of MZ on the brain serotonergic receptors might be achieved by the alterations in the electrolytes balance, and/or by the regulation of the immune system.

Keywords: Tribomechanically micronised zeolite; Mammary carcinoma; 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors; Mouse brain

Introduction

Natural zeolites are the hydrated microporous crystals, consisting of aluminosilicates, used mainly as inorganic cation-exchangers, adsorbents, catalysts, and detergent builders in different industries (Harvey

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et al., 1993; Colella, 1996). In the medical practice clinoptilolite, a main compound of the natural zeolites, is effective as an antidiarrheic drug (Rodriguez-Fluentes et al., 1997). Since clinoptilolite is a glucose adsorbent, it could be also used as an additional therapy in diabetes mellitus (Concepcion-Rosebal et al., 1997). Recently, antimetastatic and immunostimulatory properties of tribomechanically micronized natural zeolite clinoptilolite (MZ) were reported, suggesting that MZ could be used as a novel potential adjuvant in anticancer therapy (Pavelić et al., 2001; Pavelić et al., 2002). MZ consisted of mostly clinoptilolite, it does not contain fibers and its particles are negatively charged (Pavelić et al., 2001), making it a nontoxic and noncarcinogenic compound.

The role of serotonin (5-hydroxytryptamine, 5-HT) in the etiology and treatment of cancer and cancer-induced cachexia (Laviano et al., 2002) is not clear. Investigations of peripheral serotonergic biochemical markers have found that cancer patients have decreased platelet 5-HT concentration (Iwagaki et al., 1997; Deb et al., 2001), probably because of the decreased platelet 5-HT uptake (Pawlak et al., 2001), increased platelet monoamine oxidase (MAO) activity (Prasad et al., 1987), and increased free plasma 5-HT levels (Pawlak et al., 2001). Previous clinical studies have found that patients with cancer frequently develop comorbid depression (Jenkins et al., 1998; McCoy, 1996; Prasad et al., 1987), that might worsen the state of the patients and prognosis of the disease. Etiopathogenesis and treatment of depression are related to the altered function of the central serotonergic system (Errikson and Humble, 1990; Maes and Meltzer, 1995), and 5-HT regulates immune activity (Grimaldi and Fillion, 2000) and different physiological functions (appetite, sleep and pain) that could be altered in depression (Stahl, 1998), but also in cancer (Blaha et al., 1998; Laviano et al., 2002).

Among numerous 5-HT receptors (Hoyer et al., 2002), 5-HT_3 receptors have an important role in the chemotherapy-induced emesis (Cubeddu, 1996). Chemotherapeutic drugs release 5-HT from the enterochromaffine cells in the gastrointestinal tract, that acts on 5-HT_3 receptors, and induces the emetic response (Jones and Blackburn, 2002). In line with this hypothesis, high levels of urinary 5-hydroxyindolacetic acid (5-HIAA) were found in rats chronically treated with cisplatin (Cubeddu, 1996). The 5-HT_{1A} receptors are located in raphe nuclei as somatodendritic autoreceptors that inhibit the neuronal firing rate. The other 5-HT_{1A} receptors are located postsynaptically and are involved in the hyperpolarization of the postsynaptic neurons (Hoyer et al., 2002). The 5-HT_{1B} receptors are located on the nerve terminals in the basal ganglia, striatum and frontal cortex. Their main function is to control the release of 5-HT (Grimaldi and Fillion, 2000).

Since the data regarding 5-HT_{1A} and 5-HT_{1B} receptors in the brain of tumor bearing mice are missing, and because recently clinoptilolite has been shown to possess some favourable effects as an adjuvant in anticancer therapy (Pavelić et al., 2001; Pavelić et al., 2002), the aim of the study was to elucidate whether mammary carcinoma would alter the characteristics of 5-HT_{1A} and 5-HT_{1B} receptors in the mice brain, and to investigate ex vivo binding of ^{3}H-8-OH-DPAT to 5-HT_{1A} and ^{3}H-5-HT to 5-HT_{1B} receptors in the brains of control and tumor bearing mice after treatment with MZ (natural zeolite clinoptilolite).

Materials and methods

Clinoptilolite

The natural zeolite was a fine powder, originated from clinoptilolite-rich tuff from Slovakia, consisted of a mixture of about 80% of clinoptilolite and 20% of mordenite, montmorillonite, and

sliced materials (Pavelić et al., 2002). Chemical composition of clinoptilolite was reported previously (Pavelić et al., 2002), with particle-sized distribution curves taken by Masterize XLB (Malvern) laser light-scattering particle size analyzer. Tribomechanical micronization was done with a machine that "centrifuged" zeolite powder, particles gained a great acceleration because of the great spin, and transported their energy to each other. Since particles were fricted and minced, the activity of zeolites was changed, and therefore zeolite powder could adsorb up to 50% more of phospholipid or protein molecules. This type of micronization increased the outside surface area, and changed the energetic state above and under the surface layer of the particles. After tribomechanical micronization maximum frequency of particles (approximately 13%) appeared at 1.5 μm with size of 2.9 μm. In 25% of particles the size was up to 1.5 μm, in 50% of 2 μm, and in 75% up to 3 μm (Pavelić et al., 2001). Using small- and wide-angle X-ray scattering spectroscopy at the Austrian high-flux scattering beamline of the 2 GeV electron storage ring ELLETTRA, nano- and crystal structures of the particles were determined, showing similar dimension and the density of the voids between tribomechanically micronized and non-tribomechanically micronized zeolites (B. Pivac, unpublished). Tribomechanically micronized natural zeolite or MZ (25%), or non-tribomechanically micronized zeolite (non-MZ), were added to the mouse standard food (Animal Nutrition Institute, Domžale, Slovenia).

Experimental animals

CBA/HZgr female mice, 3–4 months old, 21–26 g body weight, were used in the study. Mice were bred in the animal facility at Ruđer Bošković Institute, and were kept under standard conditions: 3 per cage, light/dark cycle 12/12 h, temperature 22 °C, humidity 55% and with free access to food and water. Tumor (a transplantable mammary carcinoma) was induced by intramuscular injection of 1×10^5 mammary carcinoma cells suspended in 0.2 ml of Hanks' solution in the right thighs of mice (Poljak-Blaži et al., 1981).

Nontumorous (control) mice (10 per group) and tumor (one day after inoculation) bearing mice (5 per group) were fed with: 1) standard food; 2) mixture of standard food (75%) and 25% of non-MZ; 3) mixture of standard food (75%) and MZ (25%). Non-MZ was used as an additional control to determine if 25% replacement of the standard food would affect general condition of the mice. Each mouse ate about 7 g food daily, i.e. approximately 1.75 g of MZ or non-MZ.

Fourteen or 28 days after the beginning of the experiment mice were sacrificed by cervical dislocation under the light ether anesthesia. After 14 days the tumor volume was about 700 mm^3, and after 28 days about 1200 mm^3, respectively. The brains were removed and immediately frozen in isopentane. Tissue was stored at −80 °C until the preparation of the membrane for the binding experiments.

Tissue preparation

Brains (mean weight 0.42 ± 0.01 g) were homogenized in 30 volumes of cold Tris HCl buffer (50 mM, pH = 7.2 at 22 °C) and centrifuged at 20,000 × g for 20 min. The pellet was resuspended in 20 volumes of the same buffer and incubated at 37 °C for 30 min to remove endogenous 5-HT. The pellet was centrifuged and washed twice by resuspension in cold Tris HCl buffer and centrifugation. The final pellet was resuspended in the incubation buffer (50 mM Tris HCl, contains 0.1% ascorbic

acid, 5.8 mM $CaCl_2$ and 10 μM pargyline) to obtain about 2.5 mg of protein per ml of the membrane preparation.

Binding assay

The binding of ^{3}H-8-OH-DPAT to 5-HT_{1A} receptor was performed using ^{3}H-8-Hydroxy-2-(di-n-propylamino) tetralin (^{3}H-8-OH-DPAT, NEN, specific activity 124.9 Ci/mmol) as described previously (Mück-Šeler and Peričić, 2002). Each tube contained 0.1 ml (or 0.25 mg protein) of the membrane preparation, 0.89 ml Tris HCl buffer and 0.01 ml of ^{3}H-8-OH-DPAT (4 nM). The binding of 5-hydroxy-[^{3}H]-tryptamine creatinine sulphate (^{3}H-5-HT; NEN, specific activity 24.0 Ci/mmol) to 5-HT_{1B} receptor was performed as described by Mück-Šeler and Peričić (1993). Briefly, 0.1 ml of the membrane preparation, 0.87 ml Tris HCl buffer and 0.01 ml of ^{3}H-5-HT (15 nM) were incubated in the presence of 100 nM of 8-OH-DPAT (to mask 5-HT_{1A} receptors) and 1 μM mesulergine (to mask 5-HT_{2C} receptors). Nonspecific binding was determined in the presence of 10 μM of cold 5-HT and accounted for 10 to 25% of total binding.

Samples were incubated at 23 °C for 1 hour. The incubation was terminated by a rapid filtration through Whatman GF/C filters (presoaked overnight in 1% polyethylenimine) to separate bound from free radioactivity. Filters were rapidly rinsed four times with 3 ml ice cold Tris HCl buffer and transferred to the vials. Three ml of scintillation cocktail (PPO, POPOP in toluen) was added to filters and radioactivity was counted by liquid scintillation spectroscopy at 47% efficiency. The specific binding was determined as the difference between total and nonspecific binding. All tubes were run in triplicate and each experiment was done two to three times. Protein concentration was determined by the method of Lowry et al. (1951).

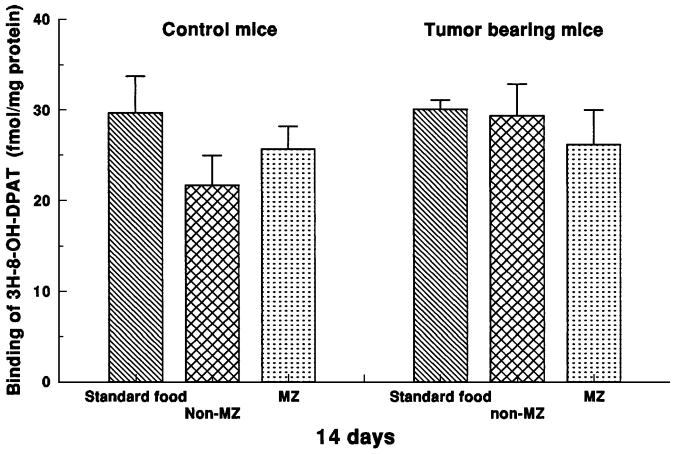

Fig. 1. Ex vivo binding of ^{3}H-8-OH-DPAT to 5-HT_{1A} receptors in the mouse brain. Control and tumor bearing mice were fed for 14 days with standard food, and with the mixture of standard food with 25% of non-MZ, or 25% of MZ. Bars represent means ± SEM.

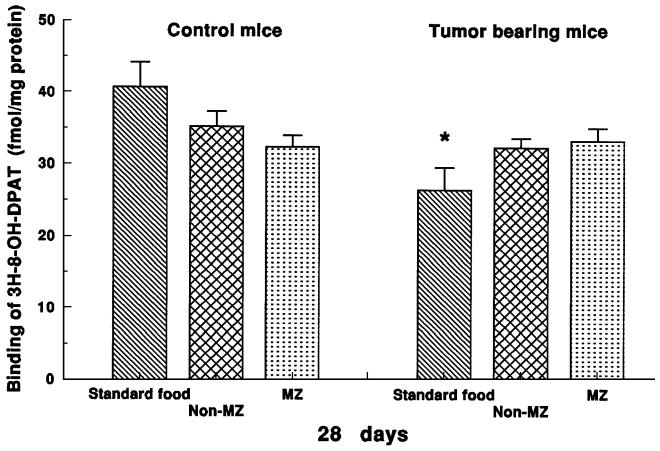

Fig. 2. Ex vivo binding of ^{3}H-8-OH-DPAT to 5-HT$_{1A}$ receptors in the mouse brain. Control and tumor bearing mice were fed for 28 days with standard food, and with the mixture of standard food with 25% of non-MZ or 25% of MZ. Bars represent means ± SEM. * $p < 0.05$ vs. control mice fed with standard food (ANOVA and Newman Keuls' test).

Statistical analysis

Data are presented as means ± SEM. Statistical evaluation of the results was done by one-way analysis of variance (ANOVA), followed by multiple comparison Newman Keuls' test. To evaluate the significance of the effect of several variables: effect of treatment (standard food, MZ and non-MZ), and the effect of time (14 or 28 days), on the binding of ^{3}H-8-OH-DPAT on 5-HT$_{1A}$ and of ^{3}H-5-HT on 5-

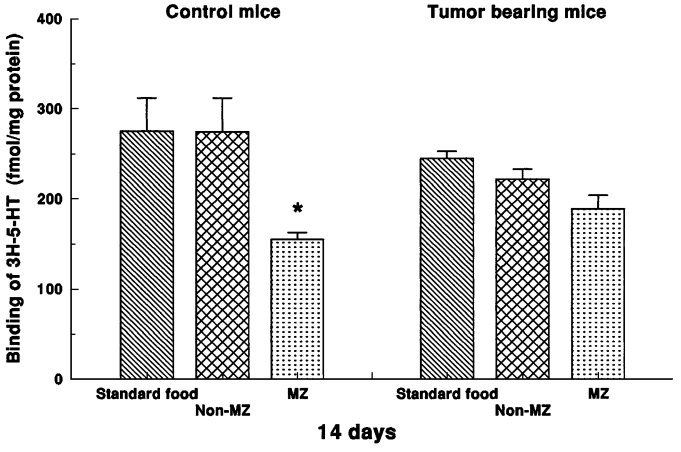

Fig. 3. Ex vivo binding of ^{3}H-5-HT to 5-HT$_{1B}$ receptors in the mouse brain. Control and tumor bearing mice were fed for 14 days with standard food, and with the mixture of standard food with 25% of non-MZ or 25% of MZ. Bars represent means ± SEM. * $p < 0.05$ vs. control mice fed with standard food and with standard food supplemented with non-MZ (ANOVA and Newman Keuls' test).

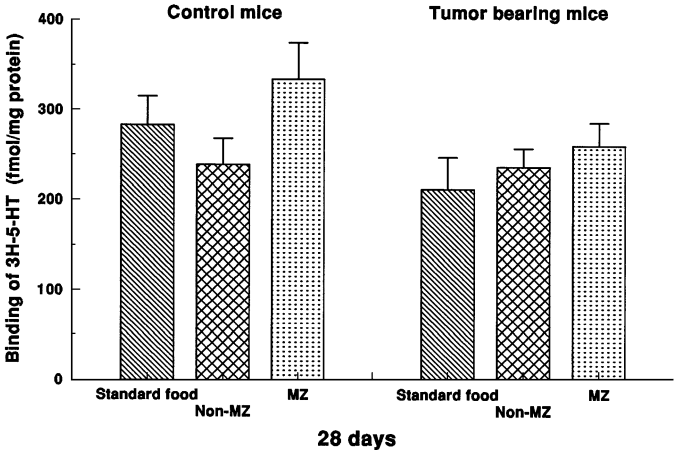

Fig. 4. Ex vivo binding of ^{3}H-5-HT to 5-HT$_{1B}$ receptors in the mouse brain. Control and tumor bearing mice were fed for 28 days with standard food, and with the mixture of standard food with 25% of non-MZ or 25% of MZ. Bars represent means ± SEM.

HT$_{1B}$ receptors in control ad tumor bearing mice, and to test the interaction between them, multifactorial three-way ANOVA was used.

Results

The binding of ^{3}H-8-OH-DPAT to 5-HT$_{1A}$ receptors was similar (one-way ANOVA; $F_{5,30} = 1.04$, $p = 0.41$, NS) in control and tumor bearing mice fed for 14 days with standard food, or standard food supplemented with MZ, or non-MZ (Fig. 1).

When mice were fed for 28 days with standard food and food supplemented with MZ, or with non-MZ (Fig. 2), a significant difference (one-way ANOVA; $F_{5,30} = 3.99$, $p = 0.007$) in the binding of ^{3}H-8-OH-DPAT to 5-HT$_{1A}$ receptors was observed between control and tumor bearing mice. Tumor bearing

Table 1
Three-way ANOVA for the binding of ^{3}H-8-OH-DPAT to 5-HT$_{1A}$ receptors (main effect) in nontumorous and tumor bearing mice (tumor), fed for 14 and 28 days (time) with standard food, the standard food supplemented with 25% of MZ, or the standard food supplemented with 25% of non-MZ (treatment)

Source of variance	Df	F-value	Significance (p)
MAIN EFFECT	1,3	3.72	0.008
Tumor	1,3	0.38	0.548 n.s.
Time	1,3	13.21	0.0006
Treatment	1,3	0.65	0.524 n.s.
2-FACTOR INTERACTIONS	1,3	2.99	0.017
Tumor × time	1,3	3.38	0.073 n.s.
Tumor × treatment	1,3	7.47	0.008
Time × treatment	1,3	0.59	0.558 n.s.

n.s. = nonsignificant.

Table 2
Three-way ANOVA for the binding of ^{3}H-5-HT to 5-HT$_{1B}$ receptors (main effect) in nontumorous and tumor bearing mice (tumor), fed for 14 and 28 days (time) with standard food, or with mixture of standard food and MZ (25%), and mixture of the standard food and non-MZ (25%) (treatment)

Source of variance	df	F-value	Significance (p)
MAIN EFFECT	1,3	2.31	0.067 n.s.
Tumor	1,3	4.01	0.049
Time	1,3	4.34	0.041
Treatment	1,3	0.45	0.638 n.s.
2-FACTOR INTERACTIONS	1,3	3.45	0.008
Tumor × time	1,3	1.06	0.306 n.s.
Tumor × treatment	1,3	0.32	0.727 n.s.
Time × treatment	1,3	7.77	0.001

n.s. = nonsignificant.

mice fed for 28 days with standard food had significantly ($p < 0.05$; Newman Keuls' test) decreased binding of ^{3}H-8-OH-DPAT to 5-HT$_{1A}$ when compared to control mice fed with standard food for 28 days (Fig. 2.).

Fig. 3. shows a significant difference (one-way ANOVA; $F_{5,30} = 4.10$, $p = 0.006$) in ^{3}H-5-HT binding to 5-HT$_{1B}$ receptors between control and tumor bearing mice fed for 14 days with standard food or standard food with different supplements. Significant differences ($p < 0.05$; Newman Keuls' test) were detected in control mice fed with MZ, as compared with control mice fed with standard food, and with standard food supplemented with non-MZ (Fig. 3).

One-way ANOVA revealed no significant differences ($F_{5,30} = 1.90$, $p = 0.12$, NS) in the binding of ^{3}H-5-HT to 5-HT$_{1B}$ receptors between the groups of control and tumor bearing mice fed for 28 days with standard food, food supplemented with MZ, or non-MZ (Fig. 4).

To further evaluate the net effects of MZ of ^{3}H-8-OH-DPAT binding to 5-HT$_{1A}$ receptors, three-way ANOVA was used (Table 1) with factors: presence of tumor, time and treatment (standard food, MZ or non-MZ). Significant main effects (i.e. binding), and a significant effect of time, but no significant effect of the addition of MZ as food supplement, was found between control and tumor bearing mice (Table 1). There was a significant two-factor interaction between tumor and treatment (Table 1).

The effect of feeding with standard food, and food with the addition of MZ (25%), during 14 and 28 days in control and tumor bearing mice, on the binding of ^{3}H-5-HT to 5-HT$_{1B}$ receptors (Table 2) was also evaluated by three-way ANOVA (Table 2). Significant main effects, and a significant effect of time, but no significant effect of MZ were detected on the binding of ^{3}H-5-HT to 5-HT$_{1B}$ receptors (Table 2). The interaction between time and MZ was significant, while there were no interactions between MZ and presence of tumor (Table 2).

Discussion

The results of our study have shown a decreased binding of ^{3}H-8-OH-DPAT to 5-HT$_{1A}$ receptors in mammary carcinoma bearing mice when compared to control mice fed with standard food for 28 days, and a transient decrease in the binding of ^{3}H-5-HT to 5-HT$_{1B}$ receptors in control mice fed for 14 days

with MZ as compared with control mice fed with standard food and with standard food supplemented with non-MZ.

In the present study we have found that tumor bearing mice had altered brain 5-HT_{1A}, but not 5-HT_{1B} receptors. To our knowledge, this is the first report of a decreased binding of ^{3}H-8-OH-DPAT to 5-HT_{1A} receptors in mice bearing mammary carcinoma. This alteration is time dependent, since the change in the binding of ^{3}H-8-OH-DPAT to 5-HT_{1A} receptors was observed only 28 days after tumor inoculation.

The change in 5-HT_{1A} receptors could be connected with the levels of its natural agonist, i.e. 5-HT. Although we did not determine brain concentration of 5-HT, markedly elevated 5-HT concentrations in serotonergic cell bodies (raphe nuclei) and nerve terminals (midbrain, hypothalamus) in mice bearing chemically-induced fibrosarcoma (Banik and Lahiri, 2000), or in hypothalamus of the rats bearing methylcholantrene sarcoma (Blaha et al., 1998) were found. These results suggest that both somatodendritic and postsynaptical 5-HT_{1A} receptors could be desensitized in tumor bearing animals. The consequence of the desensitization of the 5-HT_{1A} receptors might be hypophagia and resultant anorexia. In the present study the alteration in 5-HT_{1A} receptors, observed in tumor bearing mice fed with standard food, was not observed in mice fed with MZ or non-MZ. Due to the small number of animals, we have found only a trend towards an increase in ^{3}H-8-OH-DPAT binding to 5-HT_{1A} receptors after 28 days feeding with MZ, that did not reach the level of statistical significance. However, three-way ANOVA revealed that addition of MZ abolished the decrease in the binding of ^{3}H-8-OH-DPAT to 5-HT_{1A} receptors in mice bearing mammary carcinoma, indicating a possible beneficial effect of MZ, at least on 5-HT_{1A} receptors. Two weeks of feeding with MZ reduced the binding of ^{3}H-5-HT to 5-HT_{1B} receptors. This effect was more pronounced in control nontumorous than in tumor bearing mice. On the other hand, the addition of MZ for 28 days abolished the decrease in 5-HT_{1B} receptor binding previously detected after 14 days.

At present it is difficult to explain the mechanisms by which MZ abolished the decrease in the binding of ^{3}H-8-OH-DPAT to 5-HT_{1A} receptors in mice bearing mammary carcinoma, and affected in a time dependent manner the binding of ^{3}H-5-HT to 5-HT_{1B} receptors in nontumorous mice. Since MZ is not resorbed from gastrointestinal system (Pavelić et al., 2002), its effects in vivo can not be due to a direct biochemical interaction. In line with the ion exchange properties of the natural zeolites (Colella, 1996), one explanation for the mechanism by which MZ affected the binding of 5-HT_{1A} and 5-HT_{1B} receptors in mouse brain might be sought in its effect on mineral homeostasis. It has been shown that clinoptilolite affected the release of natrium or calcium and was able to take up potassium and ammonium, probably in the gastrointestinal tract (Mumpton, 1999). In serum of control mice, a 20% increase of the potassium level and slight increase in total calcium and inorganic phosphorus, magnesium and zinc levels was detected after administration of MZ as a food supplement (Martin-Kleiner et al., 2001). In untreated tumor-bearing mice an increase in potassium and decrease in sodium and chloride levels was found, while MZ administration ameliorated sodium and chloride levels (Martin-Kleiner et al., 2001). These results suggest that clinoptilolite affects electrolytes balance (Colella, 1996), and therefore might influence second messenger system (adenylate cyclase), responsible for the 5-HT_{1A} and 5-HT_{1B} receptors regulated neuronal function. In addition, synaptic transmission of 5-HT is terminated by either its enzymatic degradation or by active transport into the presynaptic neuron. The activity of 5-HT transporter in the membrane of presynaptic nerve terminals is dependent on the natrium intracellular/extracellular gradient and may also require either potassium or chloride ions (Masson et al., 1999). Consequently, any disturbance in the electrolyte balance could induce alterations in 5-HT homeostasis.

Although natural zeolites were used as additives in various industries in poultry (Oguz et al., 2000) and animal breeding (Poulsen and Oksbjerg, 1995), only a few reports described the effects of

clinoptilolite for the novel use as a potential adjuvant in anticancer therapy (Pavelić et al., 2001, 2002), or as antidiarrheic drug for humans (Rodriguez-Fluentes et al., 1997). It has recently been reported that MZ affected intracellular pathways, leading to the regulation of gene expression (Pavelić et al., 2001). In several human tumor cell lines (mammary carcinoma, cervical carcinoma, mouse fibrosarcoma) the addition of MZ in medium inhibited cellular growth (Pavelić et al., 2001). In mice bearing mammary carcinoma MZ inhibited tumor growth, but the addition of MZ did not affect mice survival (Martin-Kleiner et al., 2001; Pavelić et al., 2001). The mechanism by which MZ reduced tumor cells growth was presumably achieved by its ability to decrease the activity of protein kinase B (or Akt protein), mediated via epidermal growth factor-triggered pathways, and by a slight decrease of MAPK activity (Pavelić et al., 2001), indicating that MZ induced programmed cell-death, i.e. apoptosis (Pavelić et al., 2001). On the other hand, clinoptilolite has been shown to induce a better cell growth in hybridoma cell cultures by reducing the ammonia in the medium (Capiaumont et al., 1995).

In addition, some other mechanisms might be responsible for the effect of MZ on brain 5-HT_{1A} and 5-HT_{1B} receptors. It has been reported that 5-HT system regulates the immune system, and serotonergic receptors are found on immunocompetent cells (Grimaldi and Fillion, 2000), while proinflammatory cytokines are responsible for the 5-HT release (Leonard, 2001). Immunostimulatory effects of MZ were recently described, showing that MZ caused local inflammation that resulted in activation of intestinal macrophages, with consequent release of cytokines, and activation of T-cell immunological response (Pavelić et al., 2002). MZ-induced regulation of the immune response was proposed to be responsible for the anticancer and antimetastatic effects of MZ (Pavelić et al., 2002). In line with that, MZ might have influenced 5-HT receptors also by acting as a nonspecific neuroimmunomodulator.

Conclusion

This study is, as far as we know, the first report showing the ex vivo effect of MZ, added to the standard food, on serotonergic receptors in the mouse brain. The limitation of our study is a lack of the kinetic characteristics of the serotonergic receptors. Our results show a reduced binding of ^{3}H-8-OH-DPAT to 5-HT_{1A} receptors in mammary carcinoma bearing mice when compared to control mice fed with standard food for 28 days, indicating time dependent alteration of 5-HT_{1A} receptors in mammary carcinoma. The addition of MZ for 28 days in these mice abolished the decrease in 5-HT_{1A} receptors binding, indicating a possible beneficial effect of MZ, at least on 5-HT_{1A} receptors in mammary carcinoma bearing mice. The preliminary data show that MZ administered as a food supplement (25%) for 14 days induced a transient decrease in the binding of ^{3}H-5-HT to brain 5-HT_{1B} receptors only in control, but not in tumor-bearing mice, that disappeared after 28 days of MZ-supplemented food administration. The mechanism by which MZ affects serotonergic receptors might be achieved by the effects of clinoptilolite on electrolytes balance, or neuroimunomodulatory effects, or some other unknown action.

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