Life Sciences 92 (2013) 903–910

Contents lists available at SciVerse ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Dietary zeolite supplementation reduces oxidative damage and plaque generation in the brain of an Alzheimer's disease mouse model

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ARTICLE INFO

Article history:
Received 18 September 2012
Accepted 15 March 2013

Keywords:
Zeolite
Oxidative stress
Brain

ABSTRACT

Aim: Oxidative stress is considered one of the main events that lead to aging and neurodegeneration. Antioxidant treatments used to counteract oxidative damage have been associated with a wide variety of side effects or at the utmost to be ineffective. The aim of the present study was to investigate the antioxidant property of a natural mineral, the tribomechanically micronized zeolite (MZ).
Main methods: Cell death and oxidative stress were assessed in retinoic acid differentiated SH-SY5Y cells, a neuronal-like cell line, after a pro-oxidant stimulus. In vivo evaluation of antioxidant activity and amyloidogenic processing of beta amyloid have been evaluated in a transgenic model of aging related neurodegeneration, the APPswePS1dE9 transgenic mice (tg mice) after a five-month long period of water supplementation with MZ.
Key findings: The study showed that 24 h of cell pretreatment with MZ (1) protected the cells by radical oxygen species (ROS)-induced cell death and moreover (2) induced a reduction of the mitochondrial ROS production following a pro-oxidant stimulation. Looking for an antioxidant effect of MZ in vivo, we found (3) an increased activity of the endogenous antioxidant enzyme superoxide dismutase (SOD) in the hippocampus of tg mice and (4) a reduction in amyloid levels and plaque load in MZ treated tg mice compared to control tg mice.
Significance: Our results suggest MZ as a novel potential adjuvant in counteracting oxidative stress and plaque accumulation in the field of neurodegenerative diseases.

Introduction

Zeolites are hydrated natural or synthetic microporous crystals with well-defined structures containing AlO_4 and SiO_4 tetrahedra, joined together in various regular arrangements to form an open crystal structure. Properties of zeolites, such as inorganic cation-exchangers, adsorbents and active reservoirs for metal-catalyzed reactions have earned them extensive industrial applications. Zeolite use in medicine is a relatively recent subject of interest, nevertheless its use in medicine has reached significant improvement in the last few years. Recently, Mück-Seler and Pivac (2003) reported that tribomechanically micronized natural zeolite (MZ)-supplemented food administration in mammary tumor bearing mice leads to a modulation of serotonin receptors in the brain of mice with tumor. Moreover, in vivo antioxidant and immunostimulatory properties of MZ have been recently reported in various tumor types, suggesting that zeolite acts as an immune system modulator (Pavelic et al., 2001, 2002).

Given these recent findings, the first aim of the present study was to investigate whether the reported MZ ability to induce the modulation of the oxidative stress in various in vivo tumor models was also present in neuronal cells. To this purpose, we tested zeolite on SH-SY5Y human neuroblastoma cell line treated with retinoic acid as an in vitro model of neuronal-like cells. Our results demonstrated the ability of zeolite to counteract radical oxygen species (ROS) production in mitochondria, one of the main sources of oxidative stress in the cells. Since oxidative damage in mitochondria has been reported to be the causative event that leads to neurodegenerative processes, we investigated the antioxidant property of zeolite in an in vivo experimental model of aging-related neurodegeneration, the APPswePS1dE9 transgenic mice. Our results demonstrated that a chronic oral administration of MZ in tg mice is able to increase the endogenous antioxidant system and to counteract Aβ accumulation in the mouse brain. To our knowledge this is the first time that MZ was used in an in vivo model of neurodegenerative diseases. Our data highlight MZ as a novel potential adjuvant in the field of neurodegenerative diseases with no reported side effect over a long-term treatment.

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http://dx.doi.org/10.1016/j.lfs.2013.03.008

Material and methods

Cell culture and treatment

The human neuroblastoma cell line SH-SY5Y was cultured in Ham's F12 and Dulbecco modified Eagle's medium (DMEM) in a ratio of 1:1, supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 50 μg/ml penicillin and 100 μg/ml streptomycin and kept at 37 °C in humidified 5% CO_2/95% atmosphere. For differentiation, cultured cells were treated for one week with 10 μM all trans retinoic acid (Sigma-Aldrich, USA). To obtain reproducible results, SH-SY5Y cells ranging from passage 18 to passage 25 were used for all the experiments. Unactivated zeolite or MZ, tested at different concentrations (0.6, 1.25 and 2.5 ng/ml), have been added into the cell media for 24 h before each experiment. Zeolite was produced by Panaceo International. In our experiments we referred to unactivated zeolite or MZ according to manipulation used to produce zeolite powder. When zeolite powder was processed twice by tribomechanical activation it resulted in powder micronization and we referred to it as MZ, conversely when zeolite was obtained without tribomechanical activation we referred to it as zeolite. The mean particle size of zeolite that was used in our experiments was 20.86 μm and 7.37 μm for zeolite and MZ, respectively.

Evaluation of cell viability

Cell viability was measured through the quantitative colorimetric assay MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) that shows mitochondrial activity in living cells. Differentiated SH-SY5Y neuronal cells, seeded at 5×10^4 cells/well into 96-microwell culture plates, were treated for 24 h with either medium alone or MZ treated medium at different concentrations (0.6, 1.25 and 2.5 ng/ml). Then cells were challenged with H_2O_2 1 mM for 5 min, before adding 500 μg/ml MTT in each well and incubating cells at 37 °C for additional 1.5 h. MTT was removed and cells were lysed with dimethyl sulfoxide. Then the purple formazan precipitate was solubilized in DMSO and the intensity of colored reaction was measured at 595 nm using a Bio-Rad 3350 microplate reader. The experiments were performed in triplicate. Control cells were treated in the same way without H_2O_2 stimulation, and the optic density values were expressed as percentage of control cell death.

Reactive oxygen species detection in mitochondria

After differentiation of SH-SY5Y neuroblastoma cells in neuronal-like phenotype, mitochondrial ROS production was assessed according to Koopman et al. (2006) in real-time by a video-rate confocal microscopy-based method. Briefly, cells were plated in Petri with glass dish and 24 h later they were resuspended in physiological buffer containing the ROS Detection Reagent (CM-H_2CFDA) at the final concentration of 1 μM. To localize mitochondria, cells were loaded with a marker selective to mitochondria, the Mitotracker Deep Red 633 FM (100 nM), for 45 min in 5% CO_2 at 37 °C. Cells were then excited at increasing laser intensity (starting from 4% and up to 9%) and the ROS Detection Reagent oxidative conversion into CM-DCF was selectively recorded in mitochondria as fluorescence intensity. Fluorescence emission intensity was calculated as average green level value per pixel and corrected for background. Data are expressed as percentage of baseline excitation (4% of laser intensity).

Animal housing and treatment

Male hemizygous B6C3F1/J congenic mice expressing a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9), were obtained from The Jackson Laboratory breeding facilities. APPswePS1dE9 (tg) mice developed Aβ deposits in the brain by 6 to 7 months of age (Jankowsky et al., 2004) and are useful in studies of Alzheimer's disease, amyloid plaque formation and aging. Tg mice were housed in ventilated cages maintained at a constant temperature (21 ± 1 °C) with a 12-h light–dark cycle and ad libitum access to food and water. Seven month old mice were randomly divided into two experimental groups (n = 5 per group): (i) control tg mice watered with water and (ii) MZ-treated tg mice in which MZ was dissolved in drinking water at the concentration of 1 mg/ml, according to the manufacturer's instructions. Water bottles were changed every three days and treatment was carried on for 5 months. In order to measure water consumption per mouse, animals were housed individually for all the duration of the experiment. Each mouse drank about 5 ml of water daily, which means that it assumes approximately 5 mg of MZ per day. Over the 5 months of treatment there was no difference in water consumption and gain in body weight between the MZ treated tg mice and age-matched control tg mice.

All experiments were carried out in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Research Committees of the University of Brescia.

Superoxide dismutase activity

Twelve month-old tg mice were anesthetized and brain areas were rapidly collected and stored at −80 °C until analysis. Samples were homogenized by sonication in lysis buffer (50 mM Tris pH 7.6, 5 mM EDTA, 150 mM NaCl, 0.5% of NP40 and a cocktail of protease and phosphate inhibitors). Protein concentration of each sample was determined by Bradford method (Bio-Rad, USA). Total SOD enzyme activity in tissue homogenates was measured based on the ability of the enzyme to inhibit epinephrine oxidation. Epinephrine is a colorless substrate and its oxidation, other than generating superoxides (O_2^-) that are necessary for epinephrine auto-oxidation, converts epinephrine in an orange/pink product that is measured spectrophotometrically at 480 nm. The increase in epinephrine oxidation was inhibited by the presence of SOD, as SOD enzyme sequesters O_2^- slowing thus oxidizing epinephrine. SOD activity was measured using a buffer containing 0.05 M glycine, 0.1 M NaOH and 0.1 M NaCl at pH 10.3. After epinephrine addition to the samples, SOD enzymatic reaction was read in a microplate reader at 480 nm. Enzyme activity was expressed as milliUnits of SOD activity per μg of protein.

Catalase activity

Catalase activity was carried out following the method of Goth (1991). Briefly, this method is based on the formation of a yellow complex after the reaction of ammonium molybdate with H_2O_2 that was measured spectrophotometrically. Tissue homogenates were incubated with 65 μM H_2O_2 in 6 mM of phosphate buffer, pH 7.4, for 60 s. Reaction was stopped by adding 32.4 mM of ammonium molybdate and reading the reaction in a microplate reader at 405 nm. Two blanks were prepared as described: (i) Blank1 containing 65 μM H_2O_2 in 6 mM of phosphate buffer without enzyme, and (ii) Blank2 containing 6 mM of phosphate buffer without enzyme and without substrate. Catalase activity expressed in milliUnits/microliter was calculated as follows:

$$(\text{OD sample} - \text{OD Blank1}) / (\text{OD Blank1} - \text{OD Blank2}) * 271,$$

where OD is the optical density at 405 nm and 271 is a factor derived by Goth (1991).

3-NT determination

Brain cerebral areas were incubated with Laemmli sample buffer in a 1:2 ratio (0.125 M Trizma base pH 6.8, 4% sodium dodecyl sulfate, 20%

glycerol) for 30 min as previously described (Buizza et al., 2012). Proteins (5 μg) were loaded onto the slot dot apparatus and standard immunochemical methods were followed to detect 3-NT. Nitrocellulose membrane was incubated overnight at 4 °C with a primary anti-3NT antibody (Sigma-Aldrich, USA), followed by 1 h of incubation at room temperature with an infrared secondary antibody (Li-cor Biosciences, USA). Fluorescence signals were acquired and analyzed with Odyssey FC, a near-infrared fluorescence detection system equipped with a CCD camera and supplied with software (Image Studio Software, Li-cor Biosciences, USA) able to analyze and quantify the spots. Data were normalized on α-tubulin (Sigma-Aldrich, USA) expression level and expressed as percentage of control.

In vivo evaluation of amyloid protein levels

Aβ (x-42) levels were determined using a highly sensitive sandwich ELISA (Wako, Chemicals GmbH, Germany) according to the manufacturer's protocol. This kit was designed to recognize both Aβ and Aβ (x-42), the Aβ truncated or modified N-terminus. The detection sensitivity of this assay ranges from 0.1 to 20.0 pmol/l. Briefly, samples were added in the wells coated with a monoclonal antibody whose epitope is Aβ (11–28) and let incubate overnight at 4 °C. The following day the solution was removed and the wells were washed. The second monoclonal antibody labeled with Horse-Radish Peroxidase and directed against the C-terminus of Aβ (x-42) was added and let incubate for 1 h. After addition of 3,3,5,5-tetramethyl benzidine (TMB) solution positive sample developed a blue color. The reaction was stopped within 30 min and read at 450 nm. Data are expressed as pg of Aβ for ml of sample solution.

In vivo evaluation of plaque burden

Congo red staining (Sigma-Aldrich, USA) was performed on coronally cryosectioned brain slices of 30 μm thickness. Six 20× magnification fields were acquired on five previously selected brain sections (at +1.94, +0.74, −0.58, −1.82 and −3.08 mm posterior to bregma) and a total of 30 fields per mouse were used to quantify plaque number and plaque area. Stained areas (Congo red positive pixels) were measured using ImageJ software (NIH, USA) and expressed as positive stained pixels/total field assessed pixels. Data are indicated as percentage of Congo red stained area. An Olympus BX41 microscope (Olympus, Japan) equipped with a camera was used for image acquisition.

Statistical analysis

For the in vitro experiments data were presented as mean ± SD and analyzed with two-way Anova followed by Bonferroni post hoc test. For the in vivo results, data presented as mean ± SD were analyzed using two-tailed *t* test. A value of $p < 0.05$ was considered statistically significant. Graph Pad Prism 5 software was used for the statistical analysis.

Results

In vitro neuroprotective effects of zeolite in an experimental model of ROS-induced cell death

SH-SY5Y neuroblastoma cells, differentiated in neuronal-like cells by treatment with retinoic acid, have been used as an in vitro model of ROS generating cells (Uberti et al., 2002). Previous experiments performed in our laboratory (Uberti et al., 2002) demonstrated that incubating SH-SY5Y cells with different H_2O_2 concentrations and for different time periods, led to ROS production and cell death. Such toxic effect was correlated with the concentration of H_2O_2 used and the time of exposure tested. In the present study, we thus chose to challenge SH-SY5Y with 1 mM H_2O_2 for 5 min, a concentration value known to induce cell death. Our first aim was to test whether zeolite pre-treatment was able to counteract H_2O_2 induced cell death in SH-SY5Y cells. Since it has been reported that zeolite activation could have a different behavior compared to unactivated zeolites, we tested two different types of zeolites, unactivated zeolite and tribomechanically micronized zeolite (MZ). These two types of zeolites differed in the size of the crystals and the outside surface area exposed, that was increased in the MZ thus changing the adsorbent properties between the two zeolites (Pavelic et al., 2001; Mück-Seler and Pivac, 2003). Different concentrations (0.6, 1.25 and 2.5 ng/ml) of unactivated zeolite or MZ were added into cell media and the cells were incubated for 24 h before being exposed to H_2O_2 insult. Cell death was analyzed by the MTT assay and data were expressed as percentage of cell death normalized to control death level. Cells cultured with medium alone have been used as control group. MZ treatment induced a dose-dependent reduction in cell death with a statistically significant 50% reduction already present at the concentration of 0.6 ng/ml. Notably, unactivated zeolite (Fig. 1) did not show any protective effects, demonstrating that zeolite activation has an important role in zeolite exerted prevention of ROS-induced cell death.

In vitro inhibition of mitochondrial ROS production by MZ

We next wondered whether the protective effect exerted by MZ treatment in reducing ROS-induced cell death was due to the reported ability of MZ to act as an antioxidant compound. To this purpose we used an in vitro experimental model of mitochondrial ROS production as previously described in Cenini et al. (2010). Briefly, SH-SY5Y cells have been exposed to progressively increasing laser intensity (starting from 4% laser power up to 9%) and the effect of the cytotoxic insult was measured by recording the oxidative conversion of 5-chloromethyl-2′,7′-dichlorodihydrofluorescein (CM-H_2DCF) into CM-DCF inside the mitochondria of living neurons. The results are expressed as percentage of fluorescence intensity compared to baseline intensity (4% of laser intensity). In our experiments the cells have been treated with different concentrations of MZ (0.6, 1.25 and 2.5 ng/ml) for 24 h before laser excitation. Cells cultured with medium alone have been used as control. Interestingly, we found that, at all the three tested MZ concentrations there was a significant reduction in ROS production and that such effect was higher at the concentration of 2.5 ng/ml, where we observed a steep reduction in mitochondrial ROS. Moreover, MZ mediated reduction in ROS levels was clearly observed starting from 5% of laser intensity excitation and was still present at higher laser intensity, up to 9% (Fig. 2).

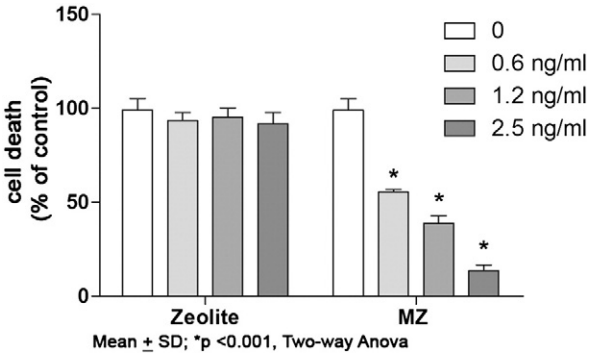

Fig. 1. Neuroprotective effects of MZ against H_2O_2-induced cell death. Retinoic acid differentiated SH-SY5Y cells were exposed to different concentrations (0.6, 1.25, 2.5 ng/ml) of zeolite or MZ (0.6, 1.25, 2.5 ng/ml) for 24 h before being exposed to 1 mM H_2O_2 for 5 min. Cell viability was evaluated 1.5 h later by MTT assay. Data represent mean ± SD of at least three different cell preparations. *$p < 0.001$ vs control treated cells, two-way Anova, followed by Bonferroni post-hoc test. MZ: tribomechanically activated zeolite.

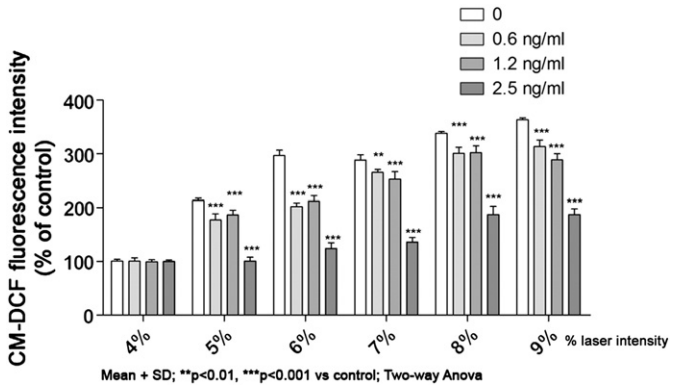

Fig. 2. Detection of mitochondrial ROS generation. Retinoic acid differentiated SH-SY5Y cells were preincubated with different concentrations of MZ (0.6, 1.25, 2.5 ng/ml) for 24 h before being exposed to increasing percentage of laser intensity. Fluorescence emission intensity was calculated as average green level value per pixel and corrected for background. Data are expressed as mean ± SD of at least three different cell preparations and showed the percentage of fluorescence intensity compared to the baseline emission (4% of laser intensity). $^{**}p < 0.01$, $^{***}p < 0.001$ vs the corresponding control values; two-way Anova, followed by Bonferroni post-hoc test.

In vivo evaluation of antioxidant properties of MZ

Due to the previously described properties of MZ to reduce ROS levels in our in vitro experiments, our next goal was to investigate whether MZ treatment was also able to reduce oxidative stress in vivo. For this purpose we planned to use an in vivo mouse model of aging-related neurodegeneration, the APPswePS1dE9 mice (tg mice), in which there is an early onset (6–7 months of age) of the pathological hallmark of neurodegeneration, that means plaque formation and accumulation, a process that increases with animal aging leading both to the chronic progression of neurodegeneration and to an increase in oxidative damage (Hamilton and Holscher, 2012). Animals have been randomly divided into two experimental groups: (1) tg mice that have been watered with MZ supplemented water and (2) tg mice watered with only water (control tg mice). Animals started the treatment at the age of 7 months, a time in which it has been reported that Aβ begins to accumulate in the brain of this model of aging-related neurodegeneration (Jankowsky et al., 2004). Brain cerebral areas were taken from 12 month old mice and the antioxidant capacity was measured in both the experimental groups. Among the enzymatic antioxidant defenses that mammalian cells possess to cope with oxygen free radicals, there are the cytosolic copper–zinc superoxide dismutase (CuZnSOD), also named SOD1, and the mitochondrial manganese superoxide dismutase (MnSOD or SOD2), which catalyze the dismutation of superoxide anions to hydrogen peroxide. In our experimental groups SOD1 and SOD2 expression levels in cerebral cortex and hippocampus of MZ tg mice have been assessed and compared with those of age-matched control tg mice. Our experiments demonstrated that there was not any significant difference in the expression levels of both SOD1 and SOD2 protein levels both in the cortex and the hippocampus of MZ-treated tg mice compared to control tg mice (data not shown).

Since enzymatic functionality is determined either by protein expression level and enzymatic activity we decided to investigate whether SOD antioxidant activity was changed in tg mice. In our experimental groups the total SOD activity (SOD1 and SOD2) showed a brain area selective activation. Notably, SOD activity was significantly increased up to about 112% in the hippocampus of MZ treated tg mice compared to age-matched control tg mice (mean ± SD: 0.068 ±

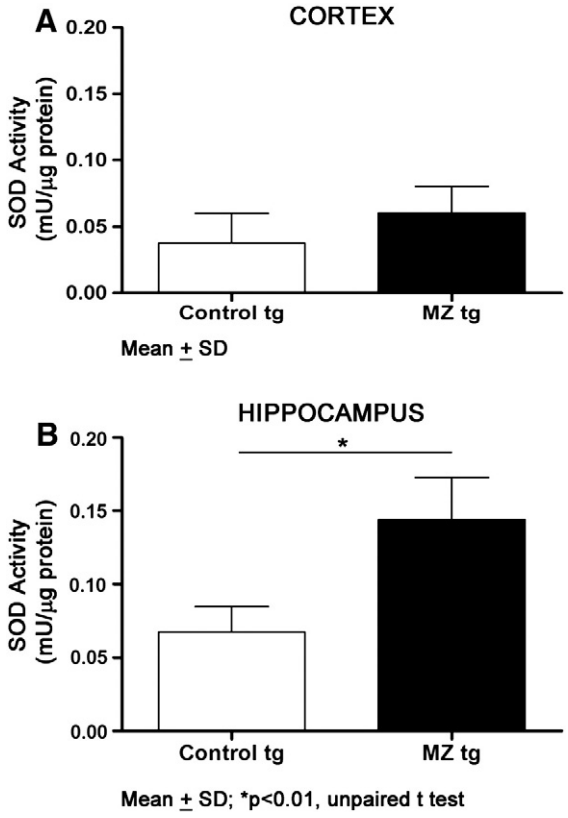

Fig. 3. Endogenous antioxidant activity in the brain of tg mice. A: SOD activity in the cortex of 12 month old tg mice after a five month long period of MZ treatment as water supplementation. B: SOD activity in the hippocampus of tg mice. Data are expressed as mean ± SD, $^{*}p < 0.01$, unpaired *t* test.

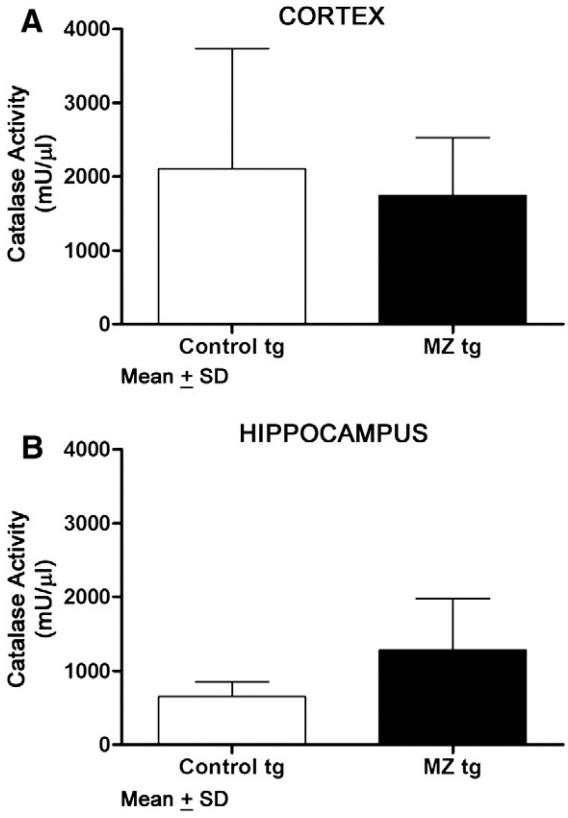

Fig. 4. Catalase activity in the brain of tg mice. A: Catalase activity in the cortex of 12 month old tg mice after a five month long period of treatment with water (white bar) or MZ (black bar). B: Catalase activity in the hippocampus of water treated tg mice (white bar) or MZ treated tg mice (black bar). Data are expressed as mean ± SD.

0.017 milliUnits for μg of proteins and 0.144 ± 0.029 milliUnits for μg of proteins, $p < 0.01$, in control tg and MZ-treated tg mice respectively, Fig. 3B). In the cerebral cortex instead, there was only a trend toward an increase (60% increase, $p > 0.05$) in the SOD activity in MZ-treated tg mice but such trend did not reach significant differences (mean ± SD: 0.038 ± 0.022 milliUnits for μg of proteins and 0.060 ± 0.020 milliUnits for μg of proteins, in control tg and MZ-treated tg mice respectively, Fig. 3A).

Catalase (CAT) is an antioxidant enzyme whose role is to cope with H_2O_2 formation, thus limiting superoxide production. In our experimental groups CAT activity showed a trend toward an increase in the hippocampus (+96%, $p > 0.05$; Fig. 4B) of MZ-treated tg mice compared to age-matched control tg mice, but such increase did not reach statistical difference. In the cortex of tg mice we did not observe any difference between control tg and MZ-treated tg mice (Fig. 4A).

To confirm that MZ treatment in tg mice induced an antioxidant response in the mouse brains we measured the relative levels of 3-nitrotyrosine (3-NT), a post-translational nitration of protein tyrosine residues related to the oxidative status of the environment. In the hippocampus of MZ-treated tg mice there was a statistically significant 30% reduction of 3-NT expression levels compared to age-matched control tg mice suggesting that the increased SOD activity induced an effective antioxidant response in MZ-treated tg mice (mean ± SD: 100.00 ± 11.15 percentage of expression level and 70.27 ± 18.55 percentage of expression level, $p < 0.05$, in control tg and MZ-treated tg mice respectively, Fig. 5B). The expression levels of 3-NT in the cortex of tg mice were not modified by MZ treatment (mean ± SD: 100.00 ± 16.68 percentage of expression level and 86.96 ± 13.90 percentage of expression level in control tg and MZ-treated tg mice respectively, Fig. 5A).

Amyloid beta levels and plaque load in the tg mouse brain

Several reports proposed the existence of a causal relationship between oxidative damage and Aβ accumulation since it has been demonstrated that pro-oxidants increase Aβ production (Tamagno et al., 2005) and conversely, Aβ promotes molecular oxidation (Butterfield et al., 2002; Li et al., 2004). Therefore we wondered whether also in APPswePS1dE9 mice a 5 month-long period of intake of MZ might affect the amyloidogenic processing of Aβ in the mouse brain. Several works demonstrated that APPswePS1dE9 mice begin to accumulate plaques at 6–7 months of age (Jankowsky et al., 2004) and that plaque load increases over time. So we planned to investigate whether a long-term MZ treatment, started at the beginning of Aβ accumulation, might maintain the brain balance of neurochemical factors needed to counteract age-dependent amyloid fibrillogenesis. First we measured the levels of Aβ (x-42) by an ELISA assay. In the cortex of MZ-treated tg mice we observed a trend toward a decrease (60% decrease, $p > 0.05$) of Aβ (x-42) levels compared to control tg mice (mean ± SD: 81.85 ± 68.00 pg/ml and 33.05 ± 18.53 pg/ml, in control tg mice and MZ-treated tg mice respectively, Fig. 6A), but it did not reach statistical difference. On the contrary, in the hippocampus of tg mice we found a statistically significant 54% decrease of Aβ (x-42) levels in MZ-treated tg mice compared to age-matched control tg mice (mean ± SD: 323.1 ± 85.35 pg/ml and 147.4 ± 101.6 pg/ml, $p < 0.05$, in control tg mice and MZ-treated tg mice respectively, Fig. 6B).

The amyloid load in the whole brain of tg mice was evaluated by performing the Congo red staining. Representative images of amyloid plaque deposition in the brain of tg mice are shown in Fig. 7. Few plaques were observed in 12 month old MZ tg mice (Fig. 7B)

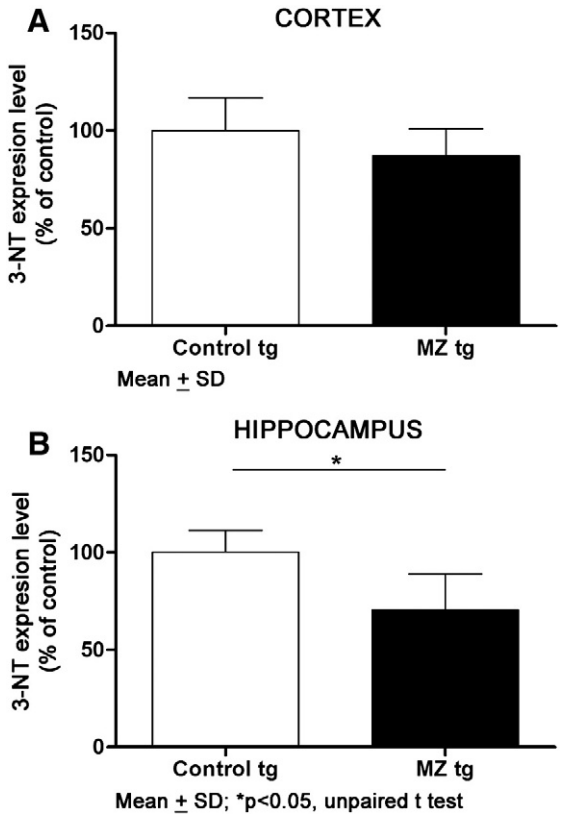

Fig. 5. 3-NT expression level in the brain of tg mice. 3-NT expression level in the cortex (A) and in the hippocampus (B) of 12 month old tg mice that received only water (white bar) or MZ supplemented water (black bar) for five months. Data are expressed as mean ± SD.

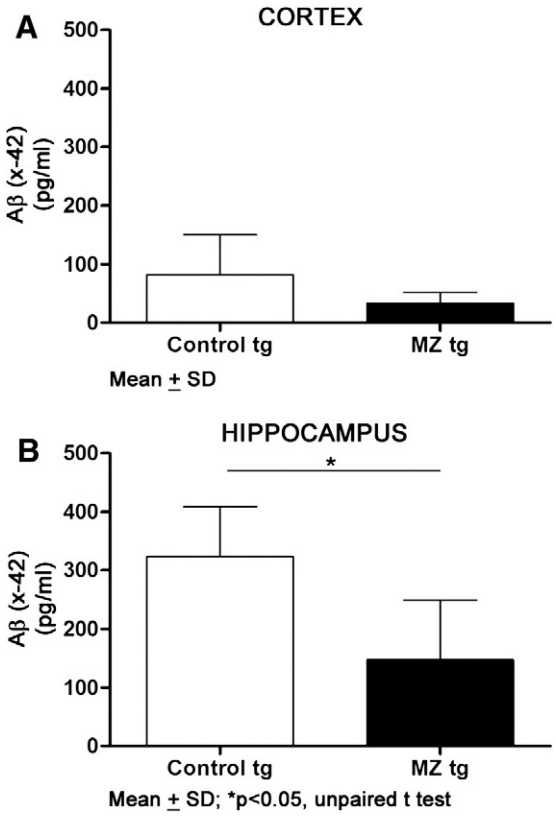

Fig. 6. Detection of Aβ (x-42) levels in the brain of tg mice. A: Aβ (x-42) total levels in the cortex of 12 month old tg mice watered with only water (white bar) or with MZ treated water (black bar). B: Aβ (x-42) total levels in the hippocampus of water treated tg mice (white bar) or MZ treated tg mice (black bar). Data are expressed as mean ± SD, $p < 0.05$, unpaired *t* test.

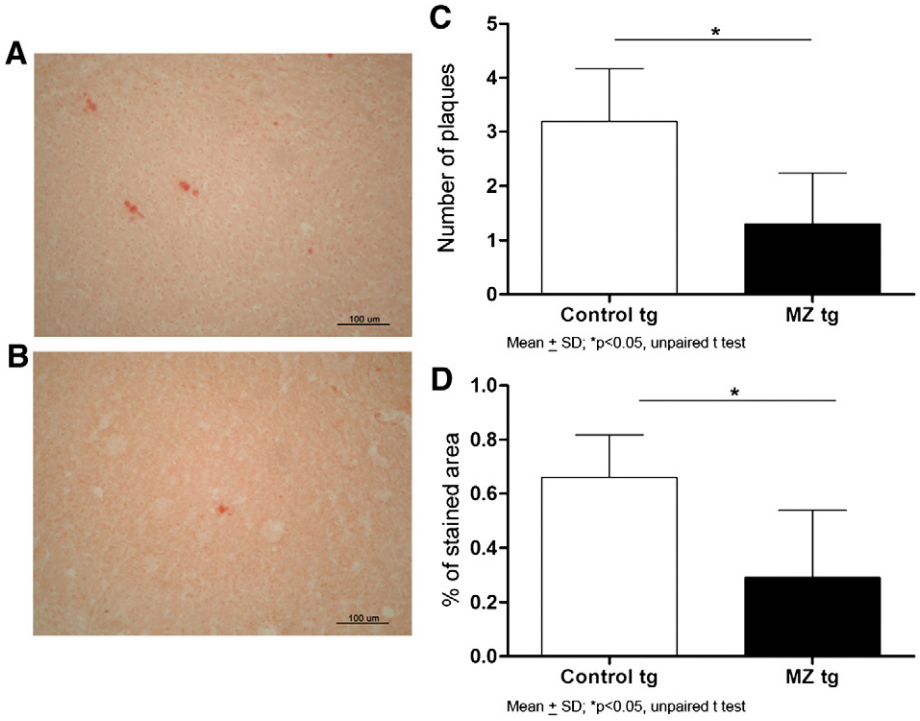

Fig. 7. Amyloid plaque burden in the brain of 12 month old tg mice. Representative images of Congo red stained plaques in control tg (A) and MZ-treated tg mice (B). Six 20× magnification field were acquired on five previously selected coronal brain sections and a total of 30 field per mouse were used to quantify (C) plaque number and (D) percentage of Congo red stained area in water treated tg mice (white bar) and MZ treated tg mice (black bar). Data are expressed as mean ± SD, $p < 0.05$, unpaired *t* test. Scale bar: A, B 100 μm.

compared to the numerous plaques seen in age-matched control tg mice (Fig. 7A). Quantitative analysis revealed that MZ-treated tg mice had a significant reduction of about 60% in the total Aβ plaque number compared to age-matched control tg mice (mean ± SD: 3.190 ± 0.975 and 1.306 ± 0.932, $p < 0.05$, in control tg and MZ-treated tg mice respectively, Fig. 7C). Notably, MZ protection in the accumulation of the pathological hallmarks of AD was also confirmed when we investigated whether plaque number reduction was due to an increase in the plaque size. Also these data demonstrated that MZ treatment was able to significantly reduce about 56% of the brain total Aβ plaque load (percentage of pixel area occupied by Congo red staining) in MZ-treated tg mice compared to control tg mice (mean ± SD: 0.660 ± 0.158 percentage of positive stained area and 0.292 ± 0.247 percentage of positive stained area, $p < 0.05$, in tg control and MZ tg mice respectively, Fig. 7D).

Discussion

This study demonstrated that pretreatment of SH-SY5Y neuronal-like cells with zeolite significantly reduced ROS-induced cell death and that such effect was linked to zeolite activation. Moreover, our experiments highlight that the observed decrease in ROS-induced cell death was due to the ability of MZ to counteract endogenous mitochondrial ROS production and accumulation. In order to investigate whether the MZ antioxidant effect was also present, other than in neuronal-like cultures, in a more complex biological system like the central nervous system, we translated our experiments in an in vivo model of aging-related neurodegeneration. To this aim we used APPswePS1dE9 transgenic mice. In these animals an early (6–7 months of age) production of plaques in the brain has been described and this effect has been associated with hyperproduction of ROS (Hamilton and Holscher, 2012). In our experimental groups, mice exposed to a long-term intake of MZ which started at the beginning of plaque accumulation showed a significant increase in SOD activity and a parallel statistically significant reduction in post-translational oxidative protein nitration in the hippocampus of MZ-treated tg mice compared to age-matched control tg mice. Moreover, in MZ treated tg mice we observed a significant 54% reduction of $A\beta_{42}$ total levels in the hippocampus of tg mice compared to age-matched control tg mice and about a 60% and 56% reduction of both plaque number and amyloid load respectively in the mouse brains.

The results reported in this paper, according to our opinion, are extremely relevant since we described for the first time an antioxidant effect of MZ in the brain of a mouse model of aging-related neurodegeneration. These results agree with recent studies in vivo which demonstrated the ability of zeolite to reduce ROS levels and to increase the total antioxidant status in the liver and in the plasma of rats exposed to partial hepatectomy (Saribeyoglu et al., 2011) and in the serum of healthy males that assumed zeolite for 4 weeks (Dogliotti et al., 2012). In line with these findings our experiments demonstrated that long-term MZ treatment, besides not affecting mice survival (data not shown), induced a significant 112% increase of SOD activity in the hippocampus of tg mice, while in the cortex of MZ-treated tg mice there was only a trend to increase (+60%) that did not reach statistical difference. In addition, we observed a trend toward an increase in the hippocampal CAT antioxidant activity (+96%) in MZ-treated tg mice compared to control tg mice. The reduction of the extent of the oxidative stress in MZ-treated tg mice was further confirmed when measuring the expression level of 3-NT, a marker related to the oxidative status of the environment. Evidence in literature suggests the involvement of nitrosative stress in modulating the activity and function of key enzymes involved in vital cell function and whose inactivation is a salient event leading to the etiopathogenesis of AD and other neurodegenerative diseases (Smith et al., 1997; Castegna et al., 2003). In the hippocampus of MZ-treated tg mice there was a statistically significant 30% reduction of the expression level of 3-NT indicative of an overall reduction of the oxidative stress following MZ treatment.

Recent studies in literature demonstrated a relationship between SOD activation and plaque accumulation in several experimental

models of aging-related neurodegeneration (Li et al., 2004; Massaad et al., 2009; Lee et al., 2012). This led us to wonder whether also in our tg mice there was a causal relationship between the antioxidant activity and the amyloidogenic processing of Aβ. Thus we measured the $A\beta_{42}$ levels and the plaque load in tg mice demonstrating that MZ treatment was effective in reducing $A\beta_{42}$ total levels in tg mouse hippocampus and in counteracting plaque load even if the treatment was started at the age in which tg animals began to accumulate Aβ. Up to now, we are not aware of which mechanisms lead to MZ-driven reduction in oxidative stress and plaque accumulation and whether there is a link between both these events. MZ is not adsorbed from the gastrointestinal tissue, therefore its observed in vivo effect in reducing oxidative stress and plaque burden cannot be due to a direct biochemical interaction. One explanation for the mechanisms by which MZ affected oxidative damage in the tg mouse brain might be sought in the ability of MZ to modulate the electrolyte homeostasis between the gastrointestinal lumen and the systemic system. The preserved electrolyte homeostasis in the blood leads to an overall reduced oxidative stress in the entire organism, brain included. Moreover immunomodulatory effect of MZ has been recently reported in various experimental models of cancer, where the authors demonstrated an anticancer and antimetastatic effect of MZ (Pavelic et al., 2002). The relationship between oxidative damage and immune system modulation might be related to the fact that ROS have been implicated as key mediators of cell signaling pathways able to activate redox-sensitive transcription factors, which in turn upregulate inflammatory gene expression (Cuzzocrea et al., 2001). Therefore, a reduction in the oxidative damage might be a key player in the modulation of the immune system and both these events act simultaneously to counteract Aβ accumulation and deposition (Dudal et al., 2004; Meyer-Luehmann et al., 2008; Zhang et al., 2012).

Antioxidant compounds, like vitamins, have been tested in epidemiological prospective cohort studies as a diet supplement in order to investigate whether they are able to slow down the risk to develop AD. Up to now, the results obtained by these studies remain rather controversial, with no clear results of whether they are able to counteract AD progression over a long-term period of diet intake (Engelhart et al., 2002; Morris et al., 2002; Luchsinger et al., 2003; Laurin et al., 2004; Miller et al., 2005). Other compounds with antioxidant effect were reported to be able to reduce plaque accumulation and Aβ levels in several transgenic mouse models of AD (Bareggi and Cornelli, 2012). These antioxidant agents are clioquinol and its derivative PBT2 that acting as metal chelators are able to reduce redox-active metal ion availability at synaptic levels, that represents an important source of oxidant species and whose presence is considered to be the triggering event for Aβ fibrillation. However it has been reported that metal chelation therapeutical approach, being able to bind the redox-active ions, chelates metals like Zn^{2+} and Cu^{2+} that are essential co-factors at synaptic levels of signaling transduction, thus impairing both synaptic transmission and brain plasticity. In addition, a number of toxicity studies (Tateishi, 2000; Tsubaki et al., 1971) reported that clioquinol exerted severe toxicity in the central nervous system after a chronic treatment, with relevant neurological side effects. The antioxidant effect of MZ that we reported in this paper highlights, according to our opinion, a novel compound in the field of the neurodegenerative diseases, with no toxicity or side effects even if it was administered for a long-term period. Moreover MZ treatment, differently than metal chelators that act by counteracting Aβ deposition and accumulation, seems to act by preventing amyloidogenic processing of Aβ, since we reported a significant reduction in Aβ42 levels in MZ-treated tg mice.

Conclusions

The results reported in this work highlight the importance of counteracting oxidative stress in in vivo experimental models of aging-related neurodegeneration, demonstrating that this early event might represent some important item in the cascade triggering the pathological progression of neurodegeneration. Importantly, the goal of these results is that we demonstrate that MZ treatment, even if started at the beginning of plaque accumulation, is still able to counteract ROS production and accumulation as well as amyloid plaque deposition. Although more targeted experiments are needed to further investigate the mechanisms through which MZ acts to impair the progression of neurodegeneration, our results indicate that MZ long-term treatment in an experimental model of aging-related neurodegeneration has the potential of being efficacious in slowing down oxidative damage and inflammation, hallmarks of several neurodegenerative diseases.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was in part supported by Panaceo International Active, Goedersdorf, Austria and by grants from Regione Lombardia (Network-Enabled Drug Design). We would like to thank Dott. Chiara Prandelli for her expert technical assistance in the enzymatic assay assessment.

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