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Natural Micronised Clinoptilolite and Clinoptilolite Mixtures with *Urtica dioica* L. Extract as Possible Antioxidants

Višnja Šverko[1], Sandra Sobočanec[1], Tihomir Balog[1], Miroslav Colić[2] and Tatjana Marotti[1]*

[1]Rudjer Bošković Institute, Division of Molecular Medicine, Bijenička 54, HR-10000 Zagreb, Croatia

[2]Molecular Technologies Inc., 6512 Segovia, Goleta, CA 93117, USA

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Summary

In this study the *in vivo* effect of natural micronised clinoptilolite and clinoptilolite *forte* (clinoptilolite + 40 % extract of pulverised dried leaves of *Urtica dioica* L.) on the lipid peroxidation and antioxidative capacity in mice was examined. This was done by measuring the concentration of thiobarbituric acid reactive substances and total superoxide dismutase content in the liver homogenate, and total antioxidant status in the plasma. The results obtained showed that the 12.5 % of tribomechanically micronised mineral zeolite *forte* supplemented to food during 3 weeks significantly reduced lipid peroxidation process in the liver. This was paralleled with a significant increase of total superoxide dismutase content, which was observed with both clinoptilolite and clinoptilolite *forte*. This study demonstrates that clinoptilolite *forte* might be a novel class of lipid peroxidation inhibitors and potent antioxidant.

Key words: clinoptilolite, *Urtica dioica* L., antioxidant capacity

Introduction

Zeolites are natural or synthetic mesoporous or microporous silicate and alumosilicate crystals. They act as catalysts, ion-exchangers, non-specific immunostimulators or adjuvants in anticancer therapy (*1*). Zeolites possess the ability to bind oxygen, nitric oxide and 4-hydroxy-nonenal and may also have antioxidative or prooxidative effect. Because of the need for novel compound that will interact with free radicals, and which would enable significant advances in the treatment of diseases, these properties make it interesting for pharmaceutical industry and biomedical application in recent years. Lipid peroxidation processes (LPO) generate highly toxic molecules, which play substantial role in the organism during aging and in several diseases/disorders (*2*). Free radicals are eliminated from the body by their interaction with antioxidants. Measurements of the capacity of all the antioxidants present in plasma (TAS) establish the links between antioxidant capacity and the risk of disease. They also provide a tool for monitoring antioxidant therapy. At present, the assessment of the antioxidative status of the body, as a clinical marker of oxidative stress, is established using three approaches: (*i*) determination of TAS, which provides information about overall antioxidants and antioxidant enzyme concentrations without the exact qualitative differentiation, (*ii*) determination of activity of selected antioxidant enzymes such

* Corresponding author; Phone: ++385 1 456 11 72; Fax: ++385 1 456 10 10; E-mail: asverko@irb.hr

as total superoxide dismutase content (tSOD), (*iii*) monitoring the marker of oxidative stress, *e.g.* the concentration of thiobarbituric acid reactive substances (TBARS).

As shown in our previous results natural micronised clinoptilolites (TMAZ) could have an important influence on the immunological parameters and inflammatory processes (*1*), through the action on the superoxide anions and nitric oxide. The purpose of our current investigation was to determine whether the TMAZ containing plant extract (TMAZ *forte)* would have increased, decreased or unchanged antioxidant properties. Recently, compounds with antioxidative properties have been found within many natural substances isolated from plant extracts (*Rosmarinus officinalis*, *Polygonum hydropiper*, *etc.*) (*3*).

It has recently been shown that the strongest anticancer action has been observed by natural compounds with multifunctional activity. Plant extracts from *Urtica dioica* L. have pharmaceutical activities and clinical assessment (*4*), they inhibit the synthesis of interleukin 1, tumor necrosis factor and the proinflamatory transcription factor NF-κ B (*5*). The elements important for free radical reactions like Zn, Cu, Mg and Se were observed in the leaf of *Urtica dioica* L. (*6*). Reduced serum levels of Se may correlate with an increased incidence of cancer. Thus, the characterisation of novel natural solids such as natural micronised clinoptilolites (TMAZ) and TMAZ *forte* (clinoptilolite + 40 % extract of *Urtica dioica* L.), which would be more suitable for biomedical application, are investigated. This article focuses on the oxidative/antioxidative effect of TMAZ and TMAZ *forte.*

Materials and Methods

Four-month-old female mice of the CBA strain were fed either with standard food or with standard food supplemented with 12.5 % of TMAZ or TMAZ *forte* during 3 weeks. TMAZ *forte* contained the extract of pulverised dried leaves of *Urtica dioica* L. (Urticaceae) (40 % referred to the raw materials). TMAZ and TMAZ *forte* were prepared freshly with water from pulverised standard food supplemented with pulverised TMAZ or TMAZ *forte*, respectively. Each group of mice consisted of 7 animals. Experiments were repeated 3 times.

The fine powder of natural clinoptilolites from the eastern Balkan region was obtained by tribomechanical micronisation. As it was described earlier (*7*), tribomechanically treated natural clinoptilolite contained mass fraction of approximately 80 % clinoptilolite. The remaining 20 % consisted of silica, mordenite and montmorillonite zeolite. Chemical composition of clinoptilolite was: SiO_2 70.06 %, Al_2O_3 12.32 %, Fe_2O_3 1.48 %, CaO 3.42 %, MgO 0.96 %, TiO_2 0.71 %, P_2O_5 0.05 %, MnO 0.02 %, Na_2O 0.68 %, K_2O 2.38 %, SO_3 0.17 % and H_2O 7.3 %. Humidity at 105 °C was max. 6 %, pH=6.9–7.1, density 2.39 g/cm^3, specific area 360–390 m^2/g and NH_4^+ substitution capacity 8 500 mg/kg. Maximum frequency of clinoptilolite particle size appeared at 1 μm. Particle size distribution curves of clinoptilolites were taken by a Mastersize XLB (Malvern) laser light-scattering particle size analyser.

TBARS in the liver was estimated according to the method of Okhawa *et al.* (*8*). TAS was determined by commercially available kit (TAS Cat. No. NX 2332, RANDOX Lab. Ardmore, UK). tSOD was determined by the method of Cord and Fridovich (*9*). Protein concentration was measured by the method of Lowry *et al.* (*10*).

The results are presented as mean values ± SEM. Statistical analysis of the data was done according to the Student's t-test for independent samples or the Mann Whitney test. Values < 0.05 were considered significant.

Results

The effect of TMAZ or TMAZ forte on TAS, tSOD and TBARS

As seen from Fig. 1A neither TMAZ ((0.45±0.04) mmol/L) nor TMAZ *forte* ((0.52±0.04) mmol/L) changed the TAS significantly as compared to TAS in the control group ((0.48±0.04) mmol/L), fed with standard food. The increase of TAS after the supplementation with TMAZ *forte*, as compared to the control group, was not significant. On the contrary, if standard food was supplemented with 12.5 % of TMAZ (Fig. 1B), tSOD content significantly increased (control: (1.41±0.18) μg/mg protein; TMAZ: (1.84±0.17) μg/mg protein; $p<0.049$). The increase of tSOD content was much more pronounced when TMAZ *forte* ((1.94±0.11) μg/mg protein; $p<0.011$) was added, as compared to control animals. The effect of TMAZ or TMAZ *forte* on the liver TBARS concentration is shown in Fig. 1C. The concentration of TBARS was unaffected if mice were fed with TMAZ ((1.13±0.03) nmol/mg protein), as compared to the control mice ((1.14±0.02) nmol/mg protein), fed with standard food. However, TMAZ *forte* ((1.01±0.03) nmol/mg protein) significantly ($p<0.034$) reduced LPO processes, as seen from TBARS concentration determined in the control.

Discussion

In our previous study (*7*) 12.5 % of TMAZ provided beneficial effect on LPO process. In this study, we observed that 12.5 % of TMAZ does not have significant effect neither on LPO process nor on TAS capacity. This was probably due to the form of feeding (*per os*). Therefore, the mice were fed *ad libitum* with TMAZ or TMAZ *forte*. Unlike TMAZ, TMAZ *forte* is able to inhibit LPO. Although without the increase of TAS capacity, both TMAZ and TMAZ *forte* have an impact on antioxidant function through significant increase of tSOD content ($p<0.049$ and $p<0.011$, respectively) in mice.

Because of the increased tSOD content, TMAZ and TMAZ *forte* may have potentially important and beneficial effect against cytotoxicity, chronic intoxication with ethanol, mutagenicity and/or carcinogenicity, stress, prevention of aging and the decline of immune system or hazardous effects from environmental factors (*11*). TMAZ and especially TMAZ *forte* contain Cu, Mg or Zn, which are necessary for the production of tSOD. Through these positive electrons TMAZ may neutralise free radicals. TMAZ *forte*, because of the supplementation with *Urtica dioica* L., in relation to TMAZ, contains additionally Se, Zn, phytosterole, glucoquinone, phenolic compounds,

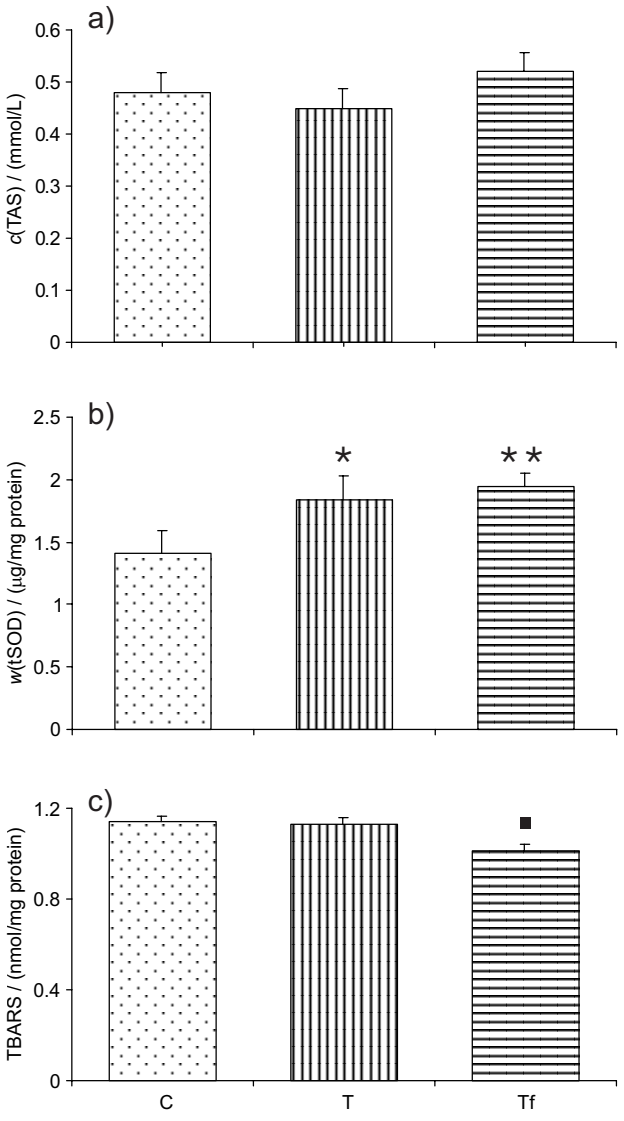

Fig. 1. a) total antioxidant status (TAS), b) total superoxide dismutase content (tSOD) and c) thiobarbituric acid reactive substances concentration (TBARS) in control (C), mice fed with standard food supplemented with 12.5 % of TMAZ (T) or 12.5 % of TMAZ *forte* (Tf) during 3 weeks. Each experimental group consisted of 7 animals. Data are presented as mean value ± SEM of 3 repeated experiments

*$p<0.049$; **$p<0.011$; ■$p<0.034$; compared to control

vitamins C, A and E, which are essential for powerful antioxidant activity (*3*). Our study demonstrates that the effect of TMAZ *forte*, as an antioxidant and LPO inhibitor, is stronger than that of TMAZ. These effects are stronger probably due to tribomechanical micronisation of the extract of *Urtica dioica* L. containing Se, flavonoids and vitamin E. Vitamin E is relevant as LPO scavenger. The extract of *Urtica dioica* L. decreased LPO level, as observed by Kanter *et al.* (*12*) and Gulcin *et al.* (*13*). It has recently been shown that extracts from *Urtica dioica* L. have modulatory effects on phase II detoxification enzymes, including biotransformation enzymes, antioxidant enzymes as well as lactate dehydrogenase and LPO in mice (*14*). Many other natural products, the so-called xenobiotics, induce phase II drug metabolising-detoxification enzymes (*15*). Our results about the increased tSOD activity along with the results of Ozen *et al.* (*14*) suggest that TMAZ *forte* might be used as primary antioxidant or inducer of phase II detoxification enzymes. As such, TMAZ *forte* may prevent formation of new free radical species. We could say that TMAZ *forte* might be considered as novel class of LPO inhibitors.

Other possible mechanisms of synergistic action between TMAZ and *Urtica dioica* L. extract also exist. It has recently been shown that anticancer activity of TMAZ is due to its immunomodulatory effects (*7*). Recently strong immunomodulatory effects have also been observed for *Urtica dioica* L. extracts. Phenolic compounds such as kaempherol or quercetin 3-O-ruthenoside present in *Urtica dioica* L. extracts are responsible for immunostimulatory effects (*16*). Synergistic activity of TMAZ *forte* components may indeed have a complex mechanism. Future studies should be performed to identify how much the micronised zeolites can potentiate the effects of *Urtica dioica* L. extract.

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Antioksidativno djelovanje prirodnog mikroniziranog klinoptilolita i smjese klinoptilolita s ekstraktom *Urtica dioica* L.

Sažetak

U radu je istraživan *in vivo* učinak mikroniziranog prirodnog klinoptilolita i klinoptilolita *forte* (klinoptilolit + 40 %-tni ekstrakt suhoga lišća u prahu *Urtica dioica* L.) na peroksidaciju lipida i na antioksidativni kapacitet miševa. To je postignuto mjerenjem koncentracije spojeva koji reagiraju s tiobarbiturnom kiselinom i određivanjem udjela superoksid dismutaze u homogenatu jetre te utvrđivanjem ukupnog antioksidativnog statusa u plazmi. Rezultati su pokazali da 12,5 %-tni tribomehanički mikronizirani mineral zeolit *forte*, dodavan hrani tijekom tri tjedna, bitno smanjuje proces lipidne peroksidacije u jetri. Istodobno je jako povećan udjel superoksid dismutaze, što je opaženo pod utjecajem klinoptilolita i klinoptilolita *forte.* Istraživanja su pokazala da se klinoptilolit *forte* može smatrati novom vrstom inhibitora lipidne peroksidacije i snažnim antioksidansom.