

A clinoptilolite effect on cell media and the consequent effects on tumor cells *in vitro*

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1. ABSTRACT

Clinoptilolite is a nontoxic natural zeolite with properties of an ion-exchanger and adsorbent. Earlier studies showed that clinoptilolite could be an adjuvant in cancer therapy. The aim of this study was to define effects of clinoptilolite in cell media on cell viability and activity of key proteins regulating cell survival, cell division and stress response. The number of viable cells, DNA synthesis and activity of EGF-R, PKB/Akt and NFκB was reduced, while apoptosis was increased in cells that were cultured in medium supplemented with clinoptilolite. These results might be due to adsorption of some serum components such as EGF to clinoptilolite. In treated medium without serum the predominant role of clinoptilolite is that of cation exchange, likely affecting calcium levels and calcium-dependent signalling pathways. These results are in line with other data that confirm enhanced apoptosis in cells incubated in treated medium. Together, data presented here demonstrate that clinoptilolite affects cellular microenvironment through mechanisms that are dependent on adsorptive and ion-exchange characteristics of this material.

2. INTRODUCTION

Elongation of an average human life-span in 20th century, especially in industrialized Western countries (1), is accompanied with an increased incidence of elderly people diseases (2). Among them, malignant tumors take an important place (3, 4). Therefore, connection between aging and tumor appearance (5), as well as a possibility of avoiding tumor development by environmental control, specially pollution and nutrition, is investigated intensively (6).

Investigation of nutrition/tumor relationship has two major goals. First, discovering food ingredients which induce mutagenesis and cancerogenesis as well as mechanisms of their action (7, 8). Second, finding food ingredients and adjuvants which strengthen a mechanism of resistance to carcinogenesis (9-11). Examples of these substances are plant estrogens (12), flavonoids (12) and others (13, 14), called nutraceuticals (15).

Zeolites, special group of nutraceuticals, are hydrated natural and synthetic microporous crystals containing AlO₄ and SiO₄ tetrahedra linked through the

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common oxygen atoms (16). Zeolites have properties to act as catalysts, ion-exchangers, adsorbents and detergent builders (17-21). Except for being used in different industrial applications, it is known that silicates and aluminosilicates also possess a biological activity. Well defined structures and catalytic activity make aluminosilicates an attractive model system for protein and enzyme mimetics (22). The best known positive biological activity of natural clinoptilolite is its action as antidiarrheic drug (23). Recent results have demonstrated that it was very effective as glucose adsorbent (24) and immunostimulator (25-28). In addition, some results indicate that silicates and aluminosilicates induce changes of expression of genes whose products are involved in cell signaling (29). They activate MAPK, PKC and SAPK (30), transcription factors like AP-1 or NF κ B and proinflammatory cytokines IL-1 α , IL-6 and TNF- α (31). Very important results show antitumor effect of clinoptilolite and its potential role as adjuvant in anticancer therapy (27, 28, 32, 33).

The aim of this study was to determine more closely natural zeolite clinoptilolite action on cell medium and consequent effects on tumor cells *in vitro*. We wanted to determine whether clinoptilolite effect on tumor cells is a consequence of its ion-exchanger property or/and consequence of adsorption from cell microenvironment.

3. MATERIALS AND METHODS

3.1. Cell lines

Mouse fibrosarcoma (FSAR), small cell carcinoma (SCC VII) (present of Prof. Dr. Mladen Korbek, Vancouver, Canada), human pancreas carcinoma (MiaPaCa-2) cells and normal diploid fibroblasts (WI38) were maintained in Dulbecco's Modified Eagle medium DMEM (Gibco, SAD) with 1000 mg/mL glucose, 10 mM HEPES (Sigma, SAD), 2 mM L-glutamine, 500 U/mL penicillin (Pliva, Zagreb, Croatia) and 500 mg/mL streptomycin (Pliva), supplemented with 5% foetal bovine serum (FBS; Gibco) and 5% newborn bovine serum (NBS; Gibco).

3.2. Clinoptilolite

The clinoptilolite powder (Zeocem, Bystre, Slovakia), obtained by tribomechanical micronization (micronized zeolite; MZ), contained 85% of clinoptilolite and 15% silica, montmorillonite and zeolite mordenite. Chemical composition of MZ was determined by the atomic absorption spectroscopy: 50-55% SiO₂, 9.3-11.4% Al₂O₃, 2.2-2.8% Fe₂O₃, 0.8-1.1% Na₂O, 2.9-4.3% K₂O, 0.8-1.2% MgO, 13.7-17.2% CaO, 0.07-0.90% MnO, 0.14-1.22% TiO₂ and 14-16% H₂O (800°C). Seventy-five percent of particles had size of up to 3 μ m, average size of particles 2,5 μ m, and 25% of powder was consisted of particles which size was less than 1,5 μ m. Specific particle surface was 1,35 m²/g.

Distilled water and/or media were pretreated with clinoptilolite (MZ) by overnight rotation at room temperature, centrifuged 5 minutes at 1500 g (Eppendorf

centrifuge, 5810R, Eppendorf, Germany) and supernatants were filtered through 0,2 μ m filter (Sartorius, Germany).

3.3. Antibodies

Rabbit anti-pAkt and anti-pJNK1/2 (New England Biolabs) and mouse anti-pERK1/2, anti-PKB/Akt, anti-ERK1/2 and anti-JNK1/2 (Santa Cruz, USA) were used. NF κ B subunits were detected using monoclonal anti-p65 and polyclonal anti-p50 antibodies (Transduction Laboratories, USA). Phosphorylated tyrosine was detected using 4G10 antibody (Upstate Biotechnology, USA) and EGF-R with polyclonal anti-RK2 antibody (from Dr. Ivan Dikic Laboratory). Horseradish peroxidase labeled Protein A (Amersham, USA) and anti-IgM (Amersham) in 5% milk were used for detection and Protein A Sepharose 4B (Zymed, USA) for immunoprecipitation.

3.4. MTT assay

Medium with serum was pretreated with 20 mg/mL and 50 mg/mL clinoptilolite. Distilled water was pretreated the same way and next day it was used for preparation of a fresh medium with serum.

FSAR and SCC VII, MiaPaCa-2 and WI38 cells (5x10³, 6x10³ and 1.4x10⁴ cells/well, respectively) were plated onto 96-microwell plates (Greiner, Germany) in quadruplicates. After an overnight incubation, standard medium was replaced with either MZ-pretreated medium or medium prepared from MZ-pretreated water. Both untreated media were used as controls. After incubation of 24, 48 and 72 hours cell viability was measured using MTT assay as previously described (27). The cell viability is expressed as a percentage of absorbance of MZ-pretreated, in relation to the absorbance of control cells. Experiments were repeated three times and results were statistically analysed with ANOVA, Tuckey test. Statistical significance was p \leq 0,05.

3.5. Timidine incorporation assay

To determine DNA synthesis, 10⁴/well FSAR cells were plated onto 96-microwell plates. After overnight starvation cells were incubated in serum-free medium and MZ-pretreated and untreated medium with serum for 5 minutes, 10 minutes and 24 hours. Cells were then incubated with radioactive thymidine (1 μ Ci/mL) ([methyl-³H]-thymidine of 1 mCi/mL concentration, specific activity of 82,04 Ci/mmol) NEN/DuPont, USA) for 18 hours and washed over filter (Glas Fiber Filter Strips 240-1, Cambridge Technology, Inc., USA) on PHD Cell Harvester (Cambridge Technology, Inc.) apparatus. Amount of radioactivity was measured on scintillator with scintillator liquid 0,1 mM 1,4-Bis[2-[2-Methyl-5-phenyloxazolyl]benzene] (Fisher Scientific, USA) and 18,1 mM 2,5-diphenyl-1,3-oxazol (Beckman, USA) dissolved in the mixture of toluene (EM Science, USA) and Triton X-100 (Sigma, USA) (75:25 v/v).

3.6. Isolation of apoptotic DNA fragments

FSAR and SCC VII cells were grown in a 10-cm dish for 24 hours, medium was discarded and replaced with MZ-pretreated or control medium with serum. After 24

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hours the cells were trypsinized and apoptotic fragments were isolated (34) and visualized on 1 % agarose gel.

3.7. Cell stimulation and lysis

After overnight starvation cells were stimulated with growth factors or MZ-pretreated media, washed twice with cold PBS, lysed 10 minutes with buffer (50 mM Hepes pH 7.5; 150 mM NaCl; 1.0 mM EDTA; 0.2 mM EGTA; 1% glycerol; 1% Triton X-100; 1.0 mM Na-orthovanadate; 1.0 mM PMSF; 1.0 µg/mL aprotinin; 2 µg/mL leupeptin) and centrifuged 15 minutes at 14000 g. Concentration of proteins was determined with Bradford method and total cell lysates were prepared.

For NFκB activity determination, nuclear and cytoplasmic proteins were isolated. The cells were incubated for 5, 30 and 60 minutes in MZ-pretreated and untreated DMEM with and without serum in 10-cm dish, washed with PBS, lysed on ice 5 minutes with 300 µL of buffer (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF; 1 mM Na-orthovanadate; 1.0 µg/mL aprotinin; 2.0 µg/mL leupeptin) and 9.375 µL of 10% NP-40 was added. Lysates were vortexed and centrifuged 3 minutes at 3300 g (Microfuge R, Beckman, Germany). Supernatants (cytoplasmic fractions) were frozen at -20°C before further analysis. After pellets (nuclear fractions) were washed with lysis buffer and centrifuged as before, 150 µL of Leamli buffer was added to each sample.

3.8. Immunoprecipitation

Total proteins (300 µg) were mixed with 5 microliters of anti-RK2 antibody 90 minutes at 4°C. Samples were mixed for 30 minutes with 37 µL of Protein A/Sepharose, spun down at 4°C and supernatants were aspirated with vacuum. Beads were washed twice with lysis buffer and once with TBS. Twenty microliters of Leamli buffer were added to each sample, vortexed shortly, incubated for 3 minutes at 98°C and spun down at room temperature.

3.9. Western blot

An equal amounts of cell proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Immunoblots were blocked with TBS/5% BSA (10 mM Tris-HCl, pH 7.4; 150 mM NaCl) overnight at 4°C, incubated for 90 minutes with primary antibodies in TBS, washed thoroughly in TBS/0.05% Triton X-100 and incubated for 1 hour with appropriate secondary antibody. Following further washes, immunoblots were visualized using enhanced chemiluminescence reagent (Boehringer-Manheim, Germany). Blots were reprobed by incubation in stripping buffer (62.5 mM Tris-HCl, pH 6.7; 2% SDS; 100 mM 2-mercaptoethanol) at 58°C for 25 minutes, washing with TBS, reblocking and reblotting with the appropriate antibodies. Blots were quantified and analysed by Imagemaster® VDS Software Version 2.0 programme (Amersham).

4. RESULTS

4.1. Clinoptilolite-pretreated cell medium decreases cell viability and DNA synthesis and increases apoptosis

To determine whether clinoptilolite effect is consequence of its dissolving properties and/or desorption

or adsorption of molecules/proteins from cell medium, distilled water and medium were pretreated with clinoptilolite. While clinoptilolite overnight treatment did not affect medium pH, it significantly increased pH of distilled water. Overnight incubation itself did not affect pH of water or medium. Although buffered system of medium is sufficient to maintain medium pH, the change of water pH indicates that clinoptilolite affects ions in solution. Additionally, MTT assay (figure 1) showed that DMEM prepared from pretreated water did not affect viability of cells, which indicates that it is not dissolved or not dissolved sufficiently to affect cells. However, MZ-pretreated medium significantly decreased viability of FSAR and MiaPaCa-2 cells and slightly decreased viability of SCC VII and WI38 cells.

After decreased viability was observed, DNA synthesis and apoptosis were measured. While the presence of serum increased DNA synthesis in FSAR cells, 5-minute incubation in MZ-pretreated medium with serum decreased DNA synthesis compared to the medium with serum. After 12 hours it was decreased about 30% (data not shown). After 24-hour incubation of FSAR and SCC VII cells in MZ-pretreated and untreated medium with serum, apoptosis was shown in both cell lines (data not shown). However, it was more prominent in FSAR cells (where unfragmented DNA was still present).

These results indicate that MZ-pretreated medium causes inhibition of proliferation and activation of apoptosis at the same time, and are in agreement with results of MTT assay.

4.2. Cell signalling

Since FSAR and SCC VII cells showed different response, they were chosen for intracellular signalling pathways analysis. We analysed the effect of MZ-pretreated media on survival (PKB/Akt1/2), mitogenic (ERK1/2) and stress (JNK1/2) signalling pathways as well as EGF-R activity in these cells.

First, FSAR and SCC VII cells were incubated in untreated and MZ-pretreated medium with serum (figure 2). PKB/Akt was slightly decreased after 1 minute and was additionally decreased in both cell lines after 30 and 60 minutes of incubation. The effect was more prominent in FSAR cells. Similar results were obtained with ERK1/2 activity. PKB/Akt activity was increased in response to EGF, insulin and serum (data not shown). In another experiment, after 30 minutes of incubation in MZ-pretreated medium with serum, 5 minutes incubation with growth factors EGF and PDGF restored PKB/Akt activity in FSAR, but not SCC VII cells. ERK1/2 and JNK1/2 activities were also different in FSAR and SCC VII cells. While ERK1/2 activity in FSAR cells was decreased, it was slightly increased in SCC VII cells after 30-minutes incubation in MZ-pretreated medium. However, JNK1/2 activity was significantly decreased in SCC VII cells, while it remained unchanged in FSAR cells (data not shown). Growth factors restored its activity in both cell lines. These results suggest that pretreated medium does not activate stress pathway in neither cell line. However, different results obtained with

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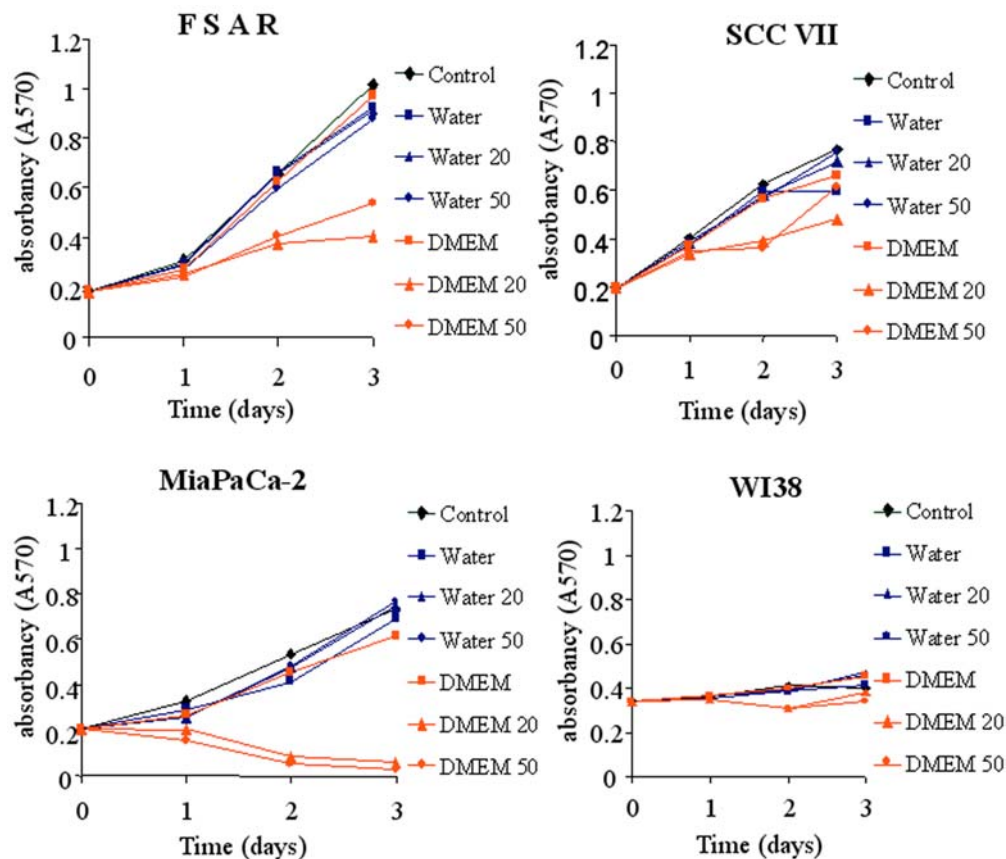


Figure 1. Cell growth (viability) of FSAR, SCC VII, MiaPaCa-2 and WI38 cells. The viability is expressed as absorbance at 570 nm, as function of incubation in different media; control medium and those prepared from non-treated or clinoptilolite-pretreated water.

ERK1/2 and JNK1/2 activity in FSAR and SCC VII cells suggest that clinoptilolite might act on different MKKs in these cells (i.e. MKK4 and MKK7 in SCC VII cells and MKK1 and MKK2 in FSAR cells).

EGF ability to reverse MZ effects suggested that the effect could be associated with EGF-signalling pathway. However, activity of EGF-R in both cell lines incubated in the pretreated medium with serum was not significantly changed. To further investigate the hypothesis that decreased PKB/Akt activity was a consequence of clinoptilolite interaction with serum components, we used EGF as a model system. First, EGF was incubated overnight in the medium without serum. After 5-minutes incubation of cells in that medium, EGF was able to activate PKB/Akt and ERK1/2 (figure 3). However, when EGF was incubated overnight in the medium together with clinoptilolite, previously observed effect of EGF on PKB/Akt and ERK1/2 activity was diminished. These results implicated that the mechanism of clinoptilolite action was growth factors adsorption. That was further confirmed by measurement of EGF-R activity under the same conditions (figure 4). While EGF activated EGF-R, overnight incubation with clinoptilolite EGF did not activate EGF-R. These results indirectly confirm that the clinoptilolite particles adsorb growth factor(s).

Additionally, PKB/Akt and ERK activity was determined in FSAR and SCC VII cells incubated in untreated and MZ-pretreated medium without serum. There was difference in control FSAR compared to control SCC VII cells, because PKB/Akt activity was decreasing in FSAR cells in time (opposite of its activity in FSAR cells incubated in medium with serum), while it was increasing in control SCC VII cells (the same as in SCC VII cells incubated in medium with serum). PKB/Akt activity was even more decreased in FSAR cells incubated in MZ-pretreated medium than in control cells. However, in SCC VII cells PKB/Akt activity was increased compared to control SCC VII cells after 1 and 5 minutes of incubation, started to decrease after 30 minutes and was significantly decreased after 60 minutes of incubation (data not shown).

In FSAR cells ERK1/2 activity is transiently increased after 30 minutes of incubation in MZ-pretreated medium without serum. While ERK1/2 activity in control SCC VII cells is decreasing in time, its activity in SCC VII cells incubated in MZ-pretreated medium is already slightly increased after 5 minutes of incubation and it is getting more increased in time (data not shown). These results were opposite from the results obtained with MZ-pretreated medium with serum. The difference was more prominent in SCC VII cells.

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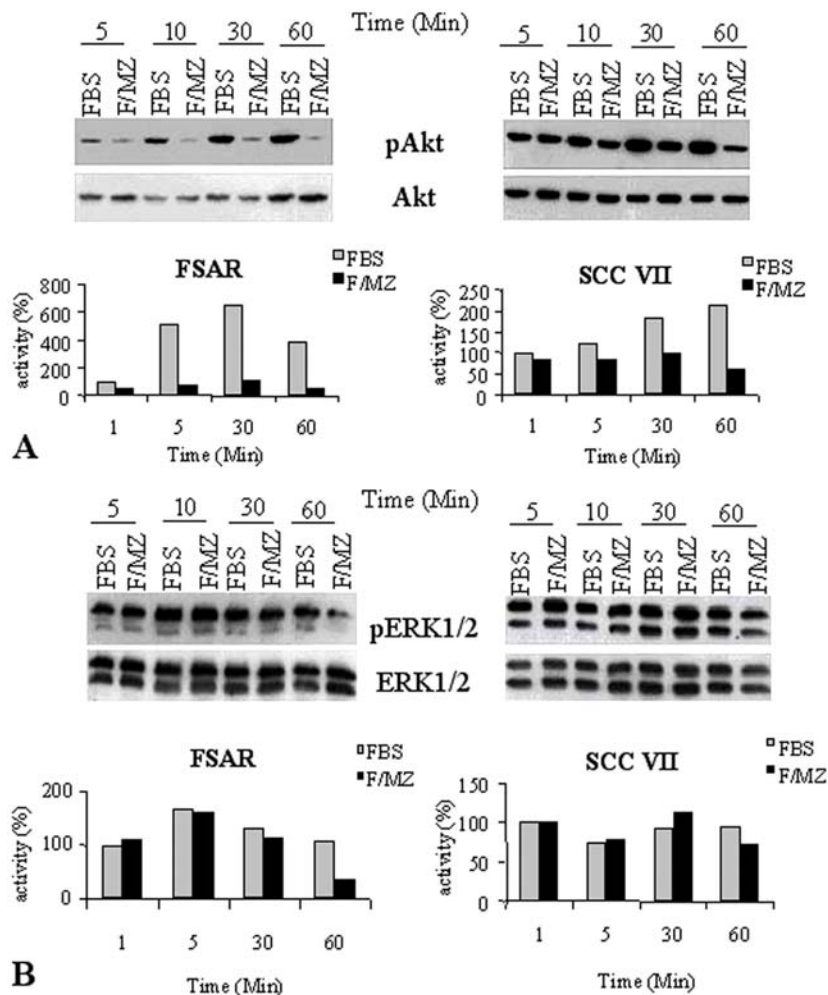


Figure 2. PKB/Akt and ERK1/2 activity in FSAR and SCC VII cells. Western blot analysis of A. PKB/Akt and B. ERK1/2 activity in FSAR and SCC VII cells incubated in untreated (FBS) or MZ-pretreated (F/MZ) medium with serum for 1, 5, 30 and 60 minutes. Three experiments were done and representative result is shown.

Transcription factor NF κ B is placed in cytoplasm in its inactive form, bound to its inhibitor I κ B. It can be activated with PKB/Akt, which promotes survival of the cells. To investigate possible downstream targets of PKB/Akt, involved in antiapoptotic pathway, we analyzed NF κ B activity in SCC VII cells incubated for 5, 30 and 60 minutes in MZ-pretreated medium with and without serum. Results have shown that NF κ B is activated after 30 minutes in medium without serum, although its activation is even higher in the medium with serum. However, both MZ-pretreated media decreased NF κ B activity after 30 minutes of incubation (figure 5).

5. DISCUSSION

Clinoptilolite is a nontoxic natural zeolite with properties of an cation exchanger and adsorbent of proteins and small molecules. It possesses a biological activity; it can adsorb glucose (24), improve diarrheic syndrome (23),

and act as an immunostimulator (25-28). Our previous studies showed that clinoptilolite could be an adjuvant in cancer therapy because of its anticancer, antimetastatic and immunostimulating properties (27, 28, 32, 33). We assume that these effects are consequences of changes in cell signalling influenced by clinoptilolite. The aim of this study was to define the mechanism underlying the effects of clinoptilolite on cell media and the consequent effects on cell viability and underlying activity of key proteins involved in signalling pathways regulating cell division (ERK1/2), survival/apoptosis (PKB/Akt) and stress response (JNK1/2).

Our results indicate that clinoptilolite changes ion composition. However, since incubation of tumor cells in the medium prepared from clinoptilolite-pretreated water did not affect cell viability we assumed that changed ion composition was not the main cause of cell death. Clinoptilolite-pretreated medium with serum, on the other

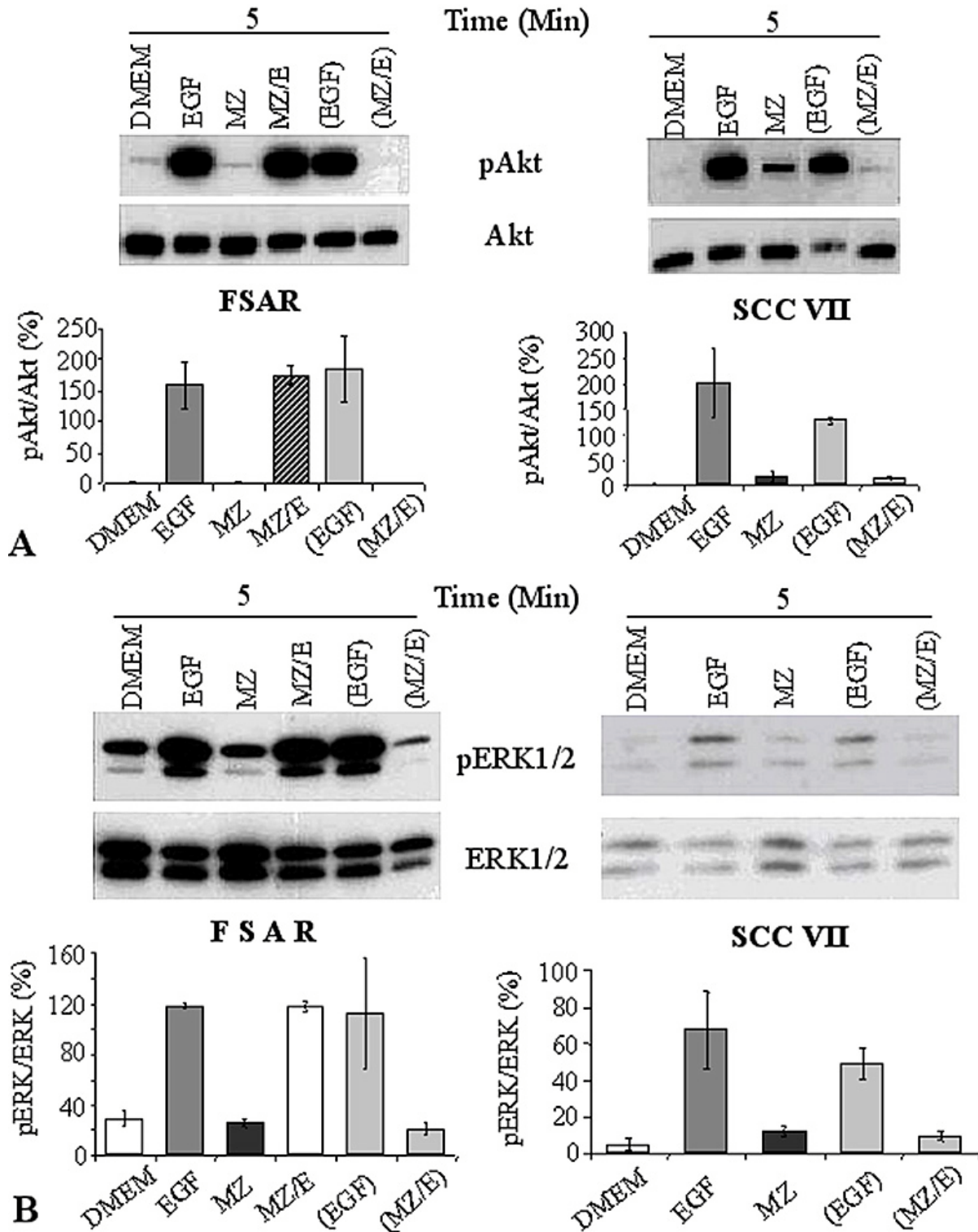


Figure 3. PKB/Akt and ERK1/2 activity in FSAR and SCC VII cells. Western blot analysis of A. PKB/Akt, and B. ERK1/2 activity in FSAR and SCC VII cells after 5-minutes incubation in untreated medium without serum (DMEM), medium without serum with addition of EGF (EGF), MZ-pretreated medium without serum (MZ), MZ-pretreated medium without serum and addition of EGF (MZ/E), medium without serum incubated with EGF overnight (EGF) and medium without serum incubated with MZ and EGF overnight (MZ/E).

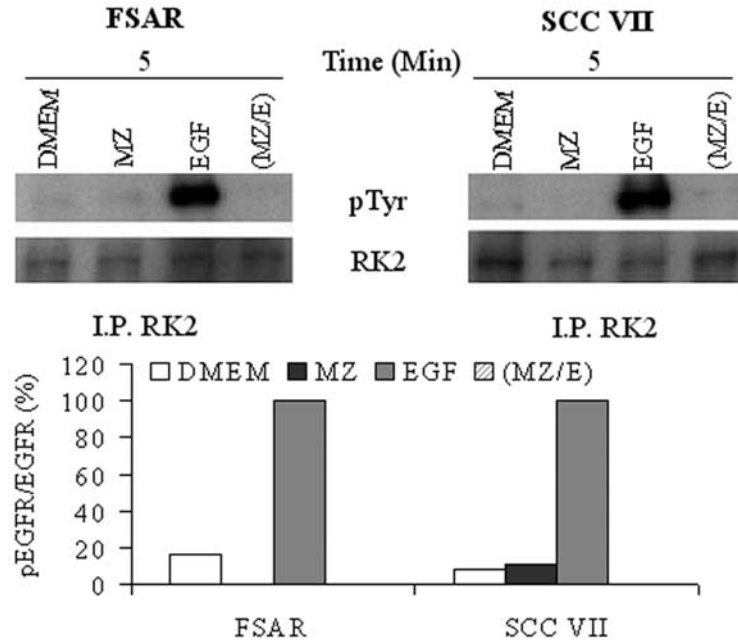


Figure 4. EGF-R activity in FSAR and SCC VII cells. Western blot analysis of EGF-R activity in FSAR and SCC VII cells after 5-minutes incubation in untreated medium without serum (DMEM), medium without serum with addition of EGF (EGF), MZ-pretreated medium without serum (MZ), MZ-pretreated medium without serum and addition of EGF (MZ/E), medium without serum incubated with EGF overnight (EGF) and medium without serum incubated with MZ and EGF overnight ((MZ/E)).

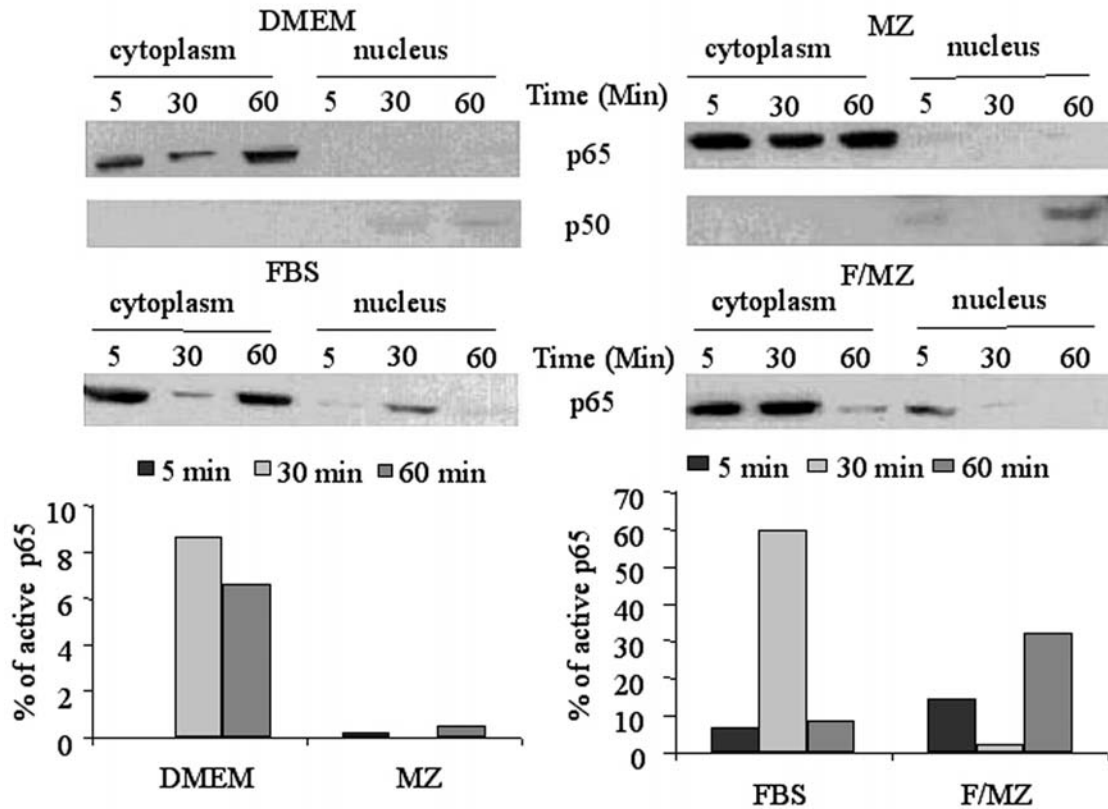


Figure 5. NFκB activity in SCC VII cells a) NFκB p65 i p50 subunits activity in SCC VII cells measured by Western blot; b) p65 activity calculated as an amount of p65 in nuclear fraction compared to total amount of p65 in the cell. One of three similar results is shown.

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hand, significantly decreased the cell viability by decreasing DNA synthesis with concomitant increase in apoptosis. That is in agreement with previous results obtained with human cell lines HeLa, CaCo2, HT-29 and MCF-7 (27). This effect is not influenced by the origin of cells (mouse, human) or by type of malignancy (fibrosarcoma, carcinoma, normal cells) but rather by the rate of cell proliferation. The best effect was obtained with the cells that proliferate the most intensively (FSAR and MiaPaCa-2).

To investigate that effect in more detail, we analysed activity of key proteins involved in signalling pathways regulating survival/apoptosis (PKB/Akt), cell division (ERK1/2) and stress response (JNK1/2) as well as NFκB activity. The most prominent change was shown with PKB/Akt and ERK1/2 activity that was decreased in both cell lines compared to controls. Since ERK1/2 activity is necessary for cell proliferation (35, 36), this result is in agreement with the result of decreased viability of the cells. Although it is not clear whether decreased ERK1/2 activity is a cause or a consequence of decreased DNA synthesis, this result shows that pretreated medium is cytostatic and not cytotoxic to the cells. PKB/Akt is antiapoptotic kinase that inactivates proapoptotic BAD (37, 38), caspase 9 (39) and AFX (40) proteins, avoiding apoptosis. The other mechanism of PKB/Akt antiapoptotic effect is activation of NFκB transcription factor by NFκB (41, 42, 43) and/or its inhibitor IκB (44) phosphorylation. Simultaneous decrease in PKB/Akt and NFκB activity in SCC VII cells is in agreement with the result of decreased viability of the cells and confirm the apoptosis of these cells at the signalling level.

While PKB/Akt activity was decreased in clinoptilolite-pretreated medium with serum, addition of growth factors reactivated PKB/Akt. Since PKB/Akt is activated by EGF, PDGF and insulin it is likely that clinoptilolite-pretreated medium affects the cells due to clinoptilolite adsorption of these growth factors. We confirmed that by incubation of the cells in the medium that was incubated overnight with EGF/clinoptilolite. PKB/Akt, ERK1/2 and EGF-R were activated by EGF-pretreated, but inactivated by EGF/clinoptilolite-pretreated medium. Clinoptilolite might also have adsorbed IGF1 that is very important growth factor in serum (45, 46, 47), also responsible for oxidative stress resistance of cells (48).

Pretreated medium had different effects on ERK1/2 and JNK1/2 activity in different cell types. ERK1/2 activity in SCC VII cells was less sensitive to the incubation in pretreated medium than in FSAR cells. JNK1/2 activity was not increased in either cell line incubated in pretreated medium with serum; it was even decreased in SCC VII cells. Thus, stress-response pathways are not triggered by pretreated medium with serum. Clinoptilolite-pretreated serum-free medium induced PKB/Akt and ERK1/2 activity in SCC VII cells. Increased EGF-R, and consequently ERK1/2, activity can be explained by increased calcium concentration in the cells (49). These results are in agreement with the results obtained by microarray analysis (not shown) of SCC VII

cells grown in clinoptilolite-pretreated and control serum-free medium for 24 hours, which showed increased transcription of *CalM* and *NHE1* genes in the cells grown in pretreated medium. CalM, Ca²⁺ receptor, activates many phosphatases and kinases and, among others, activates proapoptotic BAD proteins. On the other hand, NHE1 is Na⁺/H⁺ ion-exchanger, activated by increased intracellular Na⁺ concentration. The results indicate changed concentration of ions (most probably Ca²⁺ and Na⁺) in both the medium and the cells.

However, different effects of pretreated media on different cell lines could be explained by different nature of these cells. FSAR cells are used for immunotherapy model since they are very immunogenic (50) in contrast to SCC VII cells (51). Both cell lines are used as a model for examination of nitric oxide role for therapy response (52), because SCC VII cells generate relatively large and FSAR cells relatively small amounts of endogenous NO. In addition, SCC VII cells do not express manose-6-phosphate/insulin-like growth factor II receptor (M6P/IGFII-R) which could be cause of their metastatic potential. SCC VII cells are also resistant to radiation, because of highly active mitochondrial superoxide-dismutase (53, 54). Some of these properties could also underlay a different response of SCC VII compared to FSAR cells to MZ-pretreated media.

Tumor-suppressor protein P53 is responsible for expression of many proteins (55), among which *GADD45* (56) and *CIP1/WAF1/p21* (57) expression. Thus, increased *p53* transcription obtained by microarray analysis (not shown) is in agreement with our previous result of increased *CIP1/WAF1/p21* expression in cells incubated in clinoptilolite-pretreated medium (27). Interestingly, increased *p53* expression was found in irradiated and paclitaxel treated SCC VII tumors *in vivo* (58), although these cells did not undergo apoptosis. However, all together, our results suggest that SCC VII underwent apoptosis.

In conclusion, we have shown that clinoptilolite effect is, at least partially, due to adsorption of growth factors from serum in the medium. It also changes calcium concentration in the medium. All these changes decrease PKB/Akt, ERK1/2 and NFκB activity. It has been shown previously that mice and cows fed with zeolite-supplemented food had changed serum ion composition (59, 60). Our results partially explain clinoptilolite effect on serum, extracellular liquid and liquid in gastrointestinal tract. Adsorption of active substances from serum or intestine is important mechanism of clinoptilolite action. In addition, we have shown relative insensitivity of normal cells to clinoptilolite effect compared to tumor cells. Also, changed ion concentrations in cellular microenvironment, and indirectly in the cells, changes activity of signalling pathways. Properties of clinoptilolite to influence signalling pathways and induces immunity can also explain the clinoptilolite effect on wound healing. We have shown that clinoptilolite has antitumor effect *in vitro* due to its indirect action on the cell media.

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Clinoptilolite effect on media and cells *in vitro*

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