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Original article

Clinoptilolite induces cell death in THP-1 cells in oxidative stress conditions

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Summary. *Aim:* Zeolites are tectosilicates which appear as minerals in nature and can also be synthesized in the laboratory conditions. Clinoptilolite is a natural zeolite which has ion exchanging and adsorbent characteristics. We have aimed to display the effect of clinoptilolite to apoptotic, autophagic and antioxidant characteristics of cancer cell lines under oxidative stress conditions. *Material and Methods:* The cytotoxicity of clinoptilolite was evaluated using the MTT assay. The total antioxidant status was measured by the total antioxidant status (TAS) detection kit. The western blotting analysis was performed in order to assess the protein expression levels of the apoptosis and to autophagy markers like Beclin1, Bcl-2 and LC3B. *Results:* 24 hours incubation of cancer cells with clinoptilolite reduced cell proliferation in a dose dependent manner. Clinoptilolite lowered the autophagy in cancer cells, in particular, directing the cells towards apoptosis and leading to a fall in the TAS levels. Clinoptilolite to cause a decrease in antioxidant defence. *Discussion:* Parallel to these findings, it has been seen that there was an increase in apoptosis and a decrease in autophagy in cells induced with hydrogen peroxide effect. In this study, clinoptilolite was shown to cause a decrease in antioxidant defense.

Key words: clinoptilolite, cancer, autophagy, apoptosis, antioxidant

Introduction

Zeolites are tectosilicates which have been hydrated, and which appear as minerals in nature and can also be synthesized as artificial materials, in the laboratory (1-2). It is known that there are more than 40 zeolite structures in nature, while there are a further 229 types of zeolites which have been synthesized (3). Zeolitization is the process of zeolite formation from feldspathic rocks (4). This zeolitic transformation cause their chemical stability in solutions at different pH values which is essential for human applications (5).

Zeolites have a big potential for biomedical applications. It has been shown that microporous Faujasite Zeolite could be used as a drug delivery system to facilitate the oral delivery of poorly water soluble compound (6). Current needs for the synthesis and characterization of novel mesoporous and microporous materials, which would be better suited for biomedical applications (7).

Zeolites have been used as hemostatic components, gastro-protective drugs and antioxidative agents. Clinoptilolite, is a natural zeolite, which has ion exchanging and adsorbent characteristics and is not toxic. It has been shown in previous studies that clinoptilolite can be used as an auxiliary product in the treatment of cancer (8,9). Pavelic et all. Also shown that antiproliferative and proapoptotic effects of zeolites can be used for tumor treatment (7).

Zeolites can be used as antibacterial agents, especially when Ag is incorporated into these materials by ion exchange. (10,11). Zeolites, are used for as biosensor in some applications (12). Intestinal cell mediated antiinflammatuar effects of clinoplitolite has been shown in some studies (13, 14) Recent studies have estabileshed a possible link "gut brain axis" between intestinal microbiome and neurological disorders (15).

Based on the antioxidant effects of zeolites, clinoptilolite's antioxidant effects on hepatocytes, following partial hepatectomy in rats has been shown. It is found that, the levels of malondialdehyde, has decreased which is an indicator of oxidative stress on the liver tissue, following the oral application of clinoptilolite (16).

Programmed cell death is defined as regulated cell death mediated by an intracellular program (17). Defects in the system of apoptosis play a very important role in cancer. New cancer treatment strategies target to reduce apoptotic mechanisms. There are pro-anti apoptotic proteins like Bcl-2 in the process of apoptosis in the cell (18). Studies have shown that clinoptilolite reduces cell viability, DNA synthesis and increases apoptosis (16).

Autophagy is a cellular mechanism by which cellular materials are delivered to the lysosome for degradation. With this mechanism, cellular components are recycled and cellular energy and precursors of macromolecules are obtained (19). Some studies reveal that autophagy is associated with oncogenes and tumor suppressor genes. The mechanism of the autophagic process is controlled by a series proteins such as Beclin-1, ATG5, LC3B, ULK (20)

Extensive research over the last two decades has shown that ongoing oxidative stress also can cause chronic inflammation, which can mediate cancer (21). Thus extanuate oxidative stress is a potential strategy for therapeutic prevention of cancer. In this study we evaluated the effect of clinoptilolite on cell death mechanisms on the leukemia of the human peripheral blood monocyte cell line (THP-1) under oxidative stress conditions. High levels of oxidative stress exhibit cytotoxicity, inhibiting cell proliferation and leading to apoptotic/necrotic cell death.

Material and Methods

Clinoptilolite (MEGADETOX® TMAZ® Tribomechanisch Mikronisierter Aktivierter clinoptilolite-Zeolith % 100), and the human peripheral blood monocyte cell line (THP-1, ATCC®-TIB202TM-THP-1 Manassas, VA, USA) were used in this study. Cells were cultured with the Roswell Park Memorial Institute1640 medium (RPMI 1640), containing 10 % FBS, 0.2 mM glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin at 37°C, under 5 % CO2 and 1 atm pressure. THP-1 cell line without clinoptilolite (control) was used as negative control. Only 200 mM H_2O_2 treated group without clinoptilolite administration was used as positive control. Cells were incubated with increase concentrations of clinoptilolite ($5x10^{-5}$, 10^{-5}, $5x10^{-4}$, 10^{-4}, and 10^{-3} M) for 24 hour. After incubation with clinoptilolite, H_2O_2 added each treated groups and only H_2O_2 treated group for 1hour.

The Measurement of Cytotoxicity

Clinoptilolite's effect on cell viability was evaluated using the MTT [3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit (Promega-The CellTiter 96® Non-radioactive Cell Proliferation Assay) This is a colorimetric assay for measuring cell metabolic activity. It is based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye MTT to its insoluble formazan, which has a purple color.

THP-1 cells placed on 96-well plates with 2.5 × 10^4 cells in each well and incubated for 24 h, with serum-medium. Then, the medium was replaced with serum-free medium containing the clinoplitolite at increasing concentrations ($5x10^{-5}$, 10^{-5}, $5x10^{-4}$, 10^{-4}, and 10^{-3} M) concentrations, based on MTT assay, followed by incubation for 24 h. The absorbance was recorded at 570 nm using 96-well plate reader.

Total Antioxidant Status (TAS)

After incubation with serum-free medium containing clinoptilolite for 24 h and H_2O_2 for 1 hour, cell lysates were prepared with lysis buffer. (Thermo Fisher Scientific, Cat No: FNN0021). The total antioxidant status (TAS) was measured using the TAS detection kit

(Rel Assay Diagnostic) in cell lysates. The absorbance was recorded at 660 nm using 96-well plate reader.

Western blot analysis

After incubation with clinoplitolite cells were washed with PBS and centrifugated, the supernatant fluid was discarded and the cellular pellet was lysate in 1 ml RIPA buffer (Mybiosource, Cat No: MBS169028) containing protease inhibitor cocktail. After 20 min incubation with RIPA, the cell lysate was centrifuged at 16.250 g, 20 min, 4°C. The supernatant was collected and the amount of protein was calculated using the Bradford reagent (Sigma Aldrich, USA). Western blotting was performed with the following antibodies Rabbit anti-beclin-1 (Santa Cruz, USA) and mouse anti-Bcl-2 and anti-LCB (Santa Cruz, USA) were used to determine autophagy and apoptosis related protein expression levels.

Signals were detected with an imaging system (Bio-Rad ChemiDoc MP Imaging System, Singapore). The density was analyzed using Image J software (W. Ras Band, Research Service Brunch, NIMH, NIH, Bethesta, MD) and normalized with the signal of actin for equal protein loading control of each sample and each experiment (20)

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using non-parametric Mann–Whitney U test. P value < 0.05 (*) and < 0.01(**) was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5 software.

Results

Cell Viability

Cell viability is a parameter for the proliferation index of the cells.

24 hours incubation with clinoptilolite reduced cell proliferation in a dose-dependent manner, in the THP-1 cells. The non-toxic concentrations of $5x10^{-5}$,10^{-5}, $5x10^{-4}$, 10^{-4}, and 10^{-3} M clinoptilolite were determined according to the MTT results (Fig. 1)

The Total Antioxidant Status in the THP-1 Cells Treated with Clinoptilolite

A statistically significant decrease was observed in positive control (H_2O_2 treated) group in TAS level, (*p<0.05). TAS levels were intent to increase with clinoptilolite +H_2O_2 treated groups but these increases were not significantly important (Fig. 2)

The Autophagy Markers

The protein expressions of Beclin-1 and LC3-B, the early and late markers of autophagy respectively

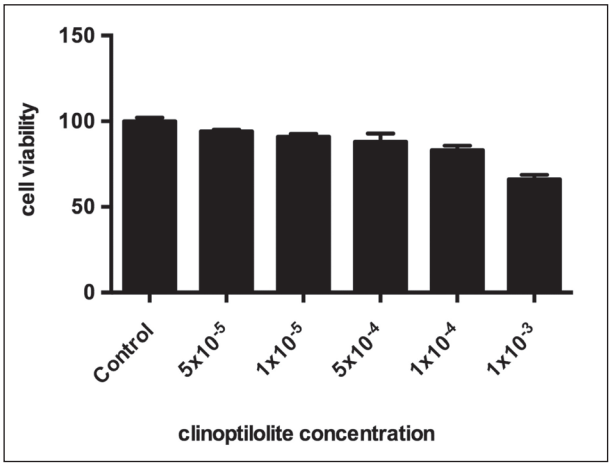

Figure 1: Effect of different clinoptilolite doses on the viability of THP-1cells measured by the MTT assay.

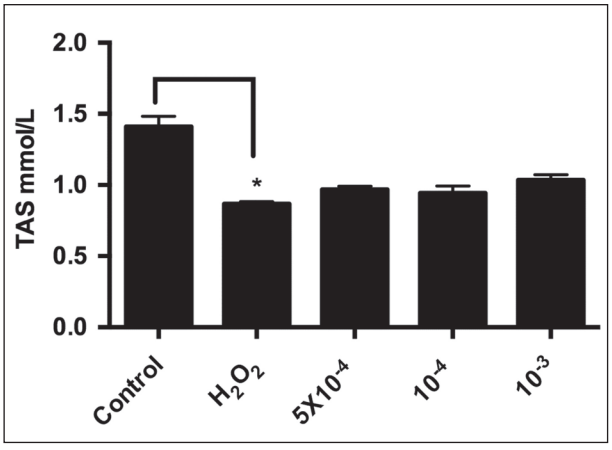

Figure 2: Graphical comparison of Total Antioxidant Status (TAS) mmol/L values for the control, H_2O_2, and different doses of clinoptilolite in the THP-1cells. Mean±SD, *p<0.05 for control group vs H_2O_2.

and the expressions in the THP-1 cells incubated with clinoptilolite for 24 hours and H_2O_2 for 1 hour were determined using the western blot method.

Beclin-1 protein levels were decreased in H_2O_2 and clinoptilolite incubated groups in a statistically significant manner (*p<0.05, **p<0.001 respectively (Fig 3).

LC-3B protein levels were also decreased in H_2O_2 and clinoptilolite incubated groups in a statistically significant manner (*p<0.05, **p<0.005 respectively) There was no significant differences only in $5X10^{-4}$ concentration of clinoplitolite incubated group (Fig 4).

The Apoptosis Markers

BCL-2 is an anti-apoptotic marker. It is known that some tumor containing cells inhibit apoptosis to escape death.

We found significantly increased BCL2 protein level in H_2O_2 treated group compared with control. Clinoplitolite was decreased this protein levels in all concentrations. These decreases were statistically different from H_2O_2 treated group.

Discussion

In the last twenty years, the routes followed in the formation and treatment of cancer have changed to a great extent, at molecular levels. Thus, today, targets have been revealed at numerous molecular levels for the development of alternative cancer treatments, which have started to be clinically implemented (22). Strategically targeted alternative cancer treatments focus on fundamental signal mechanisms, along the lines of cell growth and cell death (23).

Apoptosis, which is also known as programmed cell death, is a physiological cellular process, which is seen in organisms during their normal development (24). Many anti-cancer drugs impact the DNA synthesis and separation of cells in the tumor cell, causing apoptosis inductions (25).

Autophagic cell death is characterized by the appearance of cytoplasmic organelles, such as mitochondria and endoplasmic reticulum, or vesicles with two or multiple membranes, which swallow the cytoplasm mass. Autophagy can be a protective mechanism against apoptosis. Damage to the autophagy process contributes to the development of cancer (26).

At the same time, autophagy may also be a tumor suppressant in the early stages of tumor formation. Reduced autophagy is found in tumor cells and may be associated with malignant transformation. Under these circumstances, the induction of autophagy is seen to be beneficial for the prevention of cancer. Together with this, autophagy could also support the

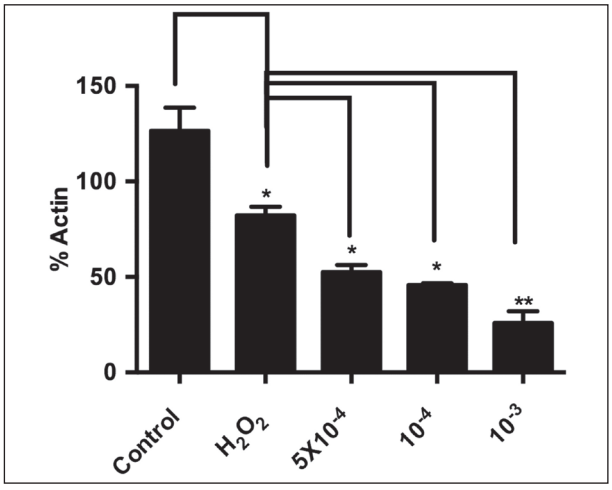

Figure 3: Graphical comparison of protein levels of Beclin-1 using Western Blot for the control, H_2O_2, and drug groups at different doses of clinoptilolite in the THP-1 cells. Mean±SD, *p<0.05 for control group vs H_2O_2 group, H_2O_2 group vs $5X10^{-4}$ M and 10^{-4} M clinoptilolite. **p<0.01 for H_2O_2 vs 10^{-3} M clinoptilolite.

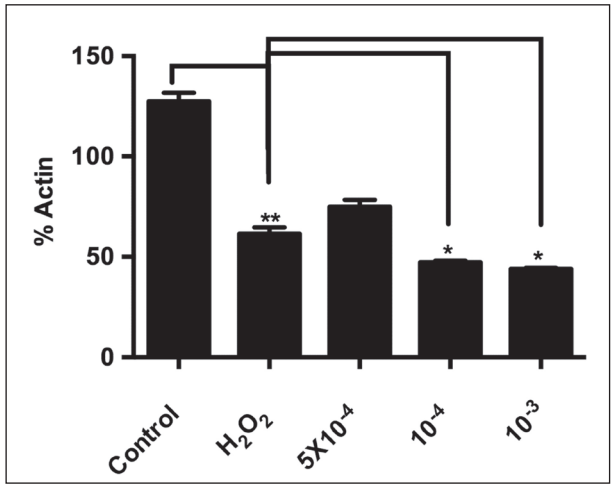

Figure 4: Graphical comparison of protein levels of LC-3B using Western Blot for the control, H_2O_2, and drug groups at different doses clinoptilolite in the THP-1 cells. Mean±SD, **p<0.01 for control group vs H_2O_2 group, H_2O_2 group vs 10^{-4} M and 10^{-3}M clinoptilolite **p<0.01

tumor, when the presence of a tumor has been shown (that is to say, it is a tumor inhibiting mechanism) and cancer cells can use developed autophagy in order to survive under metabolic and therapeutic stress (27).

In many studies have been shown that reactive oxygen species (ROS) could be effective in the formation of autophagosomes, as well as having regulatory effects on autophagy for cell death or survival. At the same time, high levels of ROS within the cell, promotes apoptosis (28).

Autophagy is an alternative mechanism, which is used especially by tumor cells to survive (29). According to our findings, it was seen that clinoptilolite suppresses autophagy in THP-1 cells (Fig 3 and 4). Thus, there was a negative effect on the survival rate of this cancer cells. The inhibition of autophagy will result in an increase in the inflammation within the cell, and this will induce the cell towards apoptosis. In one study, stated that inflammation in the cells would increase during the process of getting away from autophagy (30), authors also stated that apoptosis increases during the process of inflammation (31). Similarly, in our study, it was observed that autophagy had been inhibited as a result of the application of clinoplilolite, and that, in parallel with the information contained in the literature, this inhibition had increased the level of apoptosis, but also led to a relative fall in the TAS level.

The effects of clinoptilolite on Bcl-2, which is an anti-apoptotic marker, were also examined. The Bcl-2 protein levels only showed an increase, when compared to the control group, in the cells incubated with H_2O_2. Accordingly, incubation with H_2O_2 has directed THP-1 cell lines away from apoptosis, in order to protect them from oxidative stress. In one study it has been observed that hydrogen peroxide induced cytotoxicity increased the levels of the Bcl-2 protein (32).

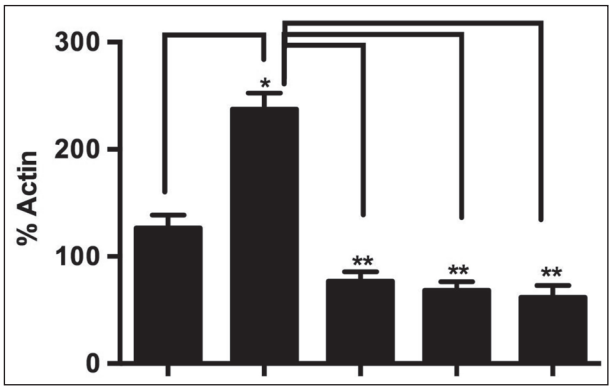

Figure 5: Graphical comparison of protein levels of Bcl-2 using Western Blot for the control, H_2O_2, and drug groups at different doses clinoptilolite in the THP-1 cells. Mean±SD, *p<0.05 for control group vs H_2O_2 group, H_2O_2 group vs $5X10^{-4}$ M, 10^{-4} M and 10^{-3} clinoptilolite, **p<0.01 for H_2O_2 vs 10^{-3} M clinoptilolite.

On the other hand, clinoptilolite incubation, was significantly decreased the Bcl-2 levels, when compared to the H_2O_2 group (Fig. 5). Based on these findings, it can be said that clinoptilolite suppresses the anti-apoptotic routes and directs the cells towards apoptosis, in cells which have suffered oxidative damage.

Even if the previous studies in the literature have yet to fully explain the relationship between clinoptilolite and apoptosis, in their study using macrophages derived from human peripheral blood monocytes and we can also say that clinoptilolite was increased apoptosis in dose dependent manner in THP-1 cells (Fig. 5).

As a summary, in concordance with the literature, there was an increase in apoptosis and a decrease in autophagy in cells induced with hydrogen peroxide effect. In this study, clinoptilolite was shown to cause a decrease in antioxidant defense. Parallel to these findings, it was seen that while clinoptilolite inhibits autophagy, it directs cells to apoptosis. This apoptotic effects of clinoptilolite may related to the suppression of oxidative stress. There is still a need for more extensive and more descriptive research to promote the clinical use of clinoptilolite in cancer. Further human studies are necessary to understand clinoptilolite contributions on anticancer therapy.

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