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Research Paper

Cytotoxicity of Bentonite, Zeolite, and Sepiolite Clay Minerals on Peripheral Blood Mononuclear Cells

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ABSTRACT

Background: Clays and clay minerals have great potential for exerting positive impacts on human health and implementation in medical applications. They are industrial minerals used in various medical applications, like drug delivery. Considering the abundance of clay resources in Iran, we decided to investigate the role of natural clays in peripheral blood mononuclear cells (PBMCs), as effective immune system cells that provide the health of the body in disease and standard times. Investigating the cytotoxicity of these minerals on PBMCs helps to understand their performance in medicine and the treatment of patients.

Materials and Methods: The studied clays, including bentonite, zeolite, and sepiolite, were prepared from Iran mines, and their characterizations were scanned by x-ray fluorescence (XRF), x-ray diffraction, and cation-exchange capacity (CEC) determination. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation, and 200000 PBMC cells were exposed to different concentrations of clays (1-1000 µg/mL) for 48 h in 96-well cell culture plates. Cell cytotoxicity response was determined using 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay.

Results: Bentonite inhibited cell proliferation after 48 h of incubation at a concentration above 0.05 mg/mL, whereas zeolite inhibited cell proliferation at 10 and 5 mg/mL. Sepiolite does not have any cytotoxic effect at all of the concentrations. CEC for bentonite, zeolite, and sepiolite were 86 cmol(+)/kg, 15.5 cmol(+)/kg, and 3.54 cmol(+)/kg, respectively, and showed a direct relationship with cell growth.

Conclusion: The cytotoxicity of the investigated clays is less than those reported in the literature review. This suggests that the studied clays with beneficial properties have great potential to be used in medicine, taking into account the size, type, and concentration of clays. In vivo and long-term studies on bio-culture and biodistribution are essential to understand better the role of the studied clays. Furthermore, our results could provide a new perspective on the safety of using cheap and naturally available clays in medical and industrial applications.

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1. Introduction

Chemically, minerals belong to two groups, including silicates and non-silicates. Silicates (aluminosilicates) are among the most plentiful minerals in the earth [1]. Silicates are divided into groups based on the amount of shared oxygen at the top of the tetrahedral. Phyllosilicates, also known as clay minerals, are one of the most important groups of silicates. This group is divided into 1:1 and 2:1 groups based on the number of octahedral and tetrahedral sheets. Each group is also divided into different types with entirely different features [2].

Some of the unique features of clay minerals are surface reactivity, high adsorption, cation exchange capacity, colloidal capacity, acid adsorption capacity, expansion in water and appropriate rheological behavior [2], diversity in reactivity [3], and variation in density, degree of hardness, morphology, and surface area [4].

The use of a particular clay for any specific application depends on (i) its mineralogical composition, i.e. the type of clay mineral, (ii) the structure of the clay mineral (1:1 or 2:1 layer type), and (iii) its chemical composition [5]. Variety in the properties of clay minerals has led to various applications, like paper making [4], rubber composites [6], ceramics industry [7], micro plastics [8], industrial wastewater treatment [9], food packaging [10], cosmetics [11], and using as an absorbent [12], polymers fillers [13], catalysts [14], bioelectrocatalysts [15], agricultural carriers [16], pharmaceutical carriers [17], food additives, carriers in insecticides and pesticides, soaps and detergents, washing powder, glue, crayons, deodorants, and water purifiers [4].

Although clay minerals have been applied in biomedical applications for many years [18-23], they have received global attention in recent years. Clays and clay minerals have been recognized due to their positive effects on human health and have great potential to be used in medicine. They can be used in a wide variety of medical applications, such as drug delivery [24, 25]. Applications of these materials in medicine and health care include (but are not limited to) the production of cosmetics, foam, film or implants, veterinary medicine, biomarkers, and parts of medical devices [20] as a lubricant in the manufacture of tablets, modifiers, flavorings, diluents, and carriers of biologically active molecules to improve the bioavailability of drugs [22], disinfectants, and alleviators of tissue damage and inflammation [26].

The clay minerals that are most commonly used in pharmaceutical formulations include bentonite, kaolinite, zeolite, and sepiolite. Montmorillonite (MMT) is a dioctahedral 2:1 phyllosilicate constituted. Bentonite is an absorbent swelling clay composed mostly of montmorillonite. Sepiolite is a hydrated magnesium silicate with a microfibrous morphology and a particular texture that provides a high specific surface area (about 200~300 m^2/g). It is built up of blocks structurally similar to montmorillonite. Zeolites generally have silicate or aluminosilicate crystalline materials with a highly regular and open microporous (<2 nm) structure. These minerals act as active acids or emulsions. In addition to their application in the medical field, they have also been studied as a carrier or active component in the study of biocompatibility and response to cell growth in bentonite [25], sepiolite [27] and zeolite [28].

A review of studies on clay minerals focusing on their use as carriers for delivery and sustained release of biologically active species showed that montmorillonite mineral has low cytotoxicity in both in vitro and in vivo [22]. According to these findings, montmorillonite may be effectively used as a carrier for several drug molecules [29].

Sepiolite is an alluring nano-material with diverse uses, including biomedicine. Interestingly, sepiolite does not exhibit significant toxicity and is not a health risk. Human cells are able to detect it, triggering a defense response, and may be able to expel the fibers [30]. Ragu et al. [30] presented that sepiolite may transfer DNA into mammalian cells, opening alluring avenues for biotechnological and biomedical applications. Strangely, sepiolite exposure did not alter the cell cycle distribution and triggers neither the DNA damage response program nor apoptosis, proposing that it does not significantly assault the genetic material in mammalian cells.

This paper studied the in vitro biocompatibility of different clays, including bentonite, zeolite, and sepiolite, collected from the Iranian mines for peripheral blood mononuclear cells (PBMCs) using the MTT method. The MTT assay has been widely used to assess cell viability and cytotoxicity [31].

Studying the role of natural clays in PBMCs is important because PBMCs are the effector of the immune system and provide the safety of the body in health and disease. Considering the numerous applications of clay minerals in medicine and health, it is essential to investigate their toxicity. Any side effects of drugs or pharmaceutical products can cause other adverse conse-

quences for the body by acting on them. As the existing natural clays are significantly different in mineralogical and chemical compositions, their effects on human health should be determined on a case basis. Therefore, it is necessary to ensure the safety of clay minerals at the doses used on PBMCs. Their cytotoxicity was also investigated for a better understanding of their function for use in biomedical, medical, and, ultimately, the treatment of patients.

2. Materials and Methods

Clay samples

Clay samples used in this study were bentonite, sepiolite, and zeolite from the mines in Iran. Natural bentonite from Arak mine was purchased from ZAMINKAV Engineering Company in 325 meshes. The natural sepiolite from the Fariman mine was 270 meshes. The ammonium acetate method determined the cation exchange capacity of bentonite and sepiolite at pH=8.2 [32]. Natural zeolite from Semnan mine was purchased from ZAMINKAV Engineering Company in 270 meshes. The internal and external (external) cation exchange capacity of zeolite was determined using the sodium acetate method [32] and replacing sodium with tert-butyl ammonium ion [33].

Characterization of clay samples

To examine the purity of clays, x-ray diffraction patterns of the studied minerals were analyzed by the Bruker D8-Advance XRD. X-ray fluorescence (XRF) was used to check the clay compositions and the concentrations of the heavy metals. A gamma-ray was used to sterilize the clays. The sterilization process was performed by Gamma Cell 220. For this purpose, the samples were cultured in the laboratory, and after determining the type and the amount of bacterial and other contaminants, the appropriate intensity of gamma rays was determined at 30 kg to eliminate the existing contaminants.

Cation-exchange capacity (CEC) for zeolite was different because of its particular structure and screening. The Ming and Dixon method determined both external (non-zeolite) and internal zeolite CEC (1987). Briefly, 30 mL of 1 M sodium acetate (pH=5) was shaken with 2 g of zeolite and then, the sample was washed four times with water and alcohol and then exposed to 60˚C for 24 hours with 30 mL of semi-normal tetra-butyl ammonium chloride. The surface solution was collected in a 100 mL flask. A flame photometer read the amount of sodium to calculate the non-zeolite CEC. Then, the zeolite was washed with 95% alcohol to remove the excessive tetra-butyl ammonium chloride and washed three times with 1 M ammonium acetate. Finally, the supernatant was collected to determine the sodium content and calculate the internal CEC of the zeolite [33].

PBMC isolation

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. Briefly, phosphate-buffered saline (PBS) solution diluted all fresh peripheral blood samples with heparin coagulant in a one-to-one ratio. Then, the diluted blood sample was gently laid on the Ficoll-Hypaque layer (Sigma, St Louis, MO, USA). Finally, PBMCs were separated from this solution via density centrifugation at 400 g for 25 min. The viability of isolated cells was more than 95%, as assessed by the Trypan blue exclusion test of cell viability.

Biological tests

Clay minerals were screened in vitro against PBMCs. PBMCs were seeded at 200000/well onto 96-well culture plates and then treated with different concentrations of clays (10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 mg/mL) in 96-well culture plates for 48 h in an incubator at 37˚C with 5% CO_2. The control sample (PBMCs without clay) was also examined. All experiments were conducted in three replications. Cell cytotoxicity response was determined according to the 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay.

MTT assay

MTT assay is an indirect method to assess cell viability and proliferation since the absorbance at 570 nm can be correlated to the number of cells [31]. Briefly, MTT was dissolved in PBS at 5 mg/mL and after 48 h, stock MTT solution (10 µL per 100 µL medium) was added to all wells, and plates were incubated at 37˚C for 4 h. After the incubation period, the suspension was gently removed and the formazan crystals were resolved in 100 mL acidic isopropanol (0.04 M HCl in isopropanol), and absorbance was read at 570 nm with a plate reader. Data were presented as the proliferation inhibitory rate of the cell, and evaluated by the MTT assay according to the following formula (Equation 1):

$$1.\quad \text{Cytotoxicity (\%)} = 100 - \frac{\text{Cell OD}}{\text{Control OD}} \times 100$$

Where:

Cytotoxicity=cell proliferation inhibition rate

Cell OD=OD value of a cell by a clay treatment

Blank OD=OD value of clay without cells

Control OD=OD value of a cell without the treatment clay

Statistical analysis

Data analyses were performed by GraphPad Prism software, version 9. Data were expressed as Mean±SE of the mean (SEM) of the three replications. Statistical analysis was carried out by one-way ANOVA after the Shapiro-Wilk and Anderson-Darling tests. Differences were considered significant at $P<0.05$, $P<0.01$, and $P<0.001$.

3. Results

Structural characterization

Diffractograms presented typical patterns for the studied clays. Sepiolite with no evidence of significant accumulation of secondary phases was detected (Figure 1). Only a few amounts of quartz and dolomite were observed. A sharp peak at 12.1 Å and moderate reflections at 3.7, 3.3, and 3.2 Å show the high purity of the sepiolite sample.

The XRD pattern of bentonite is given in Figure 2. A peak at 14 Å in $MgCl_2$ treatment increased to about 17 Å in treatment with ethylene glycol. This increase and the sharpness of the peak indicate high purity and good crystallinity of bentonite. Also, in the KCl treatment, the peak at 12 Å collapsed to 10 Å after heating to 550°C.

The zeolite pattern is shown in Figure 3. Peaks at 4.2, 4.6, 5, 5.9, 7.9, 3, 3.16, 3.3, 3.5, 3.71, 3.91, 3.98 Å belong to clinoptilolite mineral, which is one of the types of zeolites [2]. The predominant zeolite peaks are shown by c-spacing at 9.07, 7.98, and 3.98 Å. Weak peaks at 4.2 and 3.3 Å indicate only the presence of a few amounts of quartz and feldspar in the sample.

XRF data in Table 1 confirm the absence of heavy metals in the clay samples.

Table 1. XRF analysis of the studied clays

Clay Minerals	(%)									
	SiO_2	Al_2O_3	Fe_2O_3	CaO	MgO	Na_2O	K_2O	TiO_2	MnO	P_2O_5
Sepiolite	55.4	0.6	2.04	2.56	38.9	0.01	0.09	0.06	0.02	0.01
Zeolite	55.7	13	4.7	1.5	3.9	2.9	0.3	-	-	-
Bentonite	47	15.5	3.2	2.6	2.7	3.6	-	-	-	-

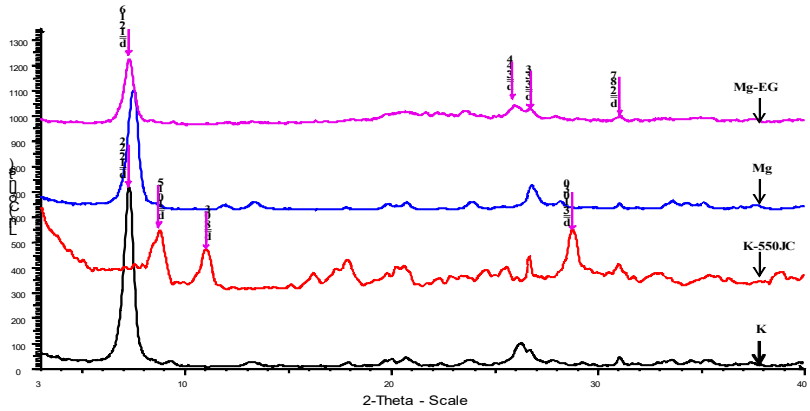

Figure 1. X-ray diffractogram of the sepiolite

Mg: The sample saturated with $MgCl_2$; K: The sample saturated with KCl; Mg-Eg: The sample saturated with $MgCl_2$ and ethylene glycol; K-550: The sample saturated with KCl after heating to 550°C; d: D-spacing based on angstrom (Å).

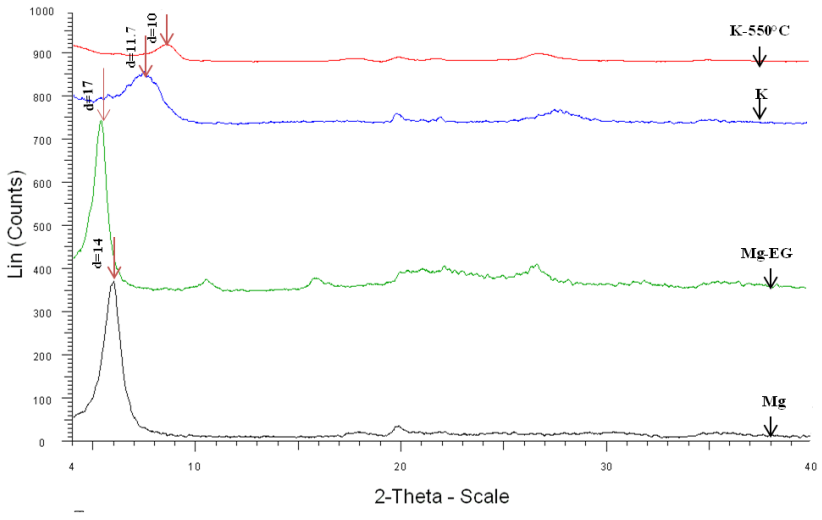

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Figure 2. X-ray diffractogram of bentonite clay

Mg: The sample saturated with $MgCl_2$; K: The sample saturated with KCl; Mg-Eg: The sample saturated with $MgCl_2$ and ethylene glycol; K-550: The sample saturated with KCl after heating to 550°C; d: D-spacing based on Angstrom (Å).

Cell proliferation

Figure 4 displays data pertinent to the effects of bentonite, zeolite, and sepiolite on cellular cytotoxicity determined using the MTT assay.

The results suggest that clays had different toxic effects on the PBMCs, depending on the type of clay minerals. Bentonite inhibited cell proliferation after 48 h of incubation at concentrations above 0.05 mg/mL, whereas zeolite inhibited at 10 and 5 mg/mL. Sepiolite had no effect at any concentrations on cell proliferation. Cells treated with 10-0.5 mg/mL of bentonite had more than 50% cytotoxicity, and cytotoxicity immediately reduced at the next concentration (0.1 mg/mL). Other minerals did not have 50% cytotoxicity at any of the studied concentrations (Figure 4). As the performance of each clay depends on its characteristics, the effect of different clays with different physical and chemical properties on cells would be different [34, 27].

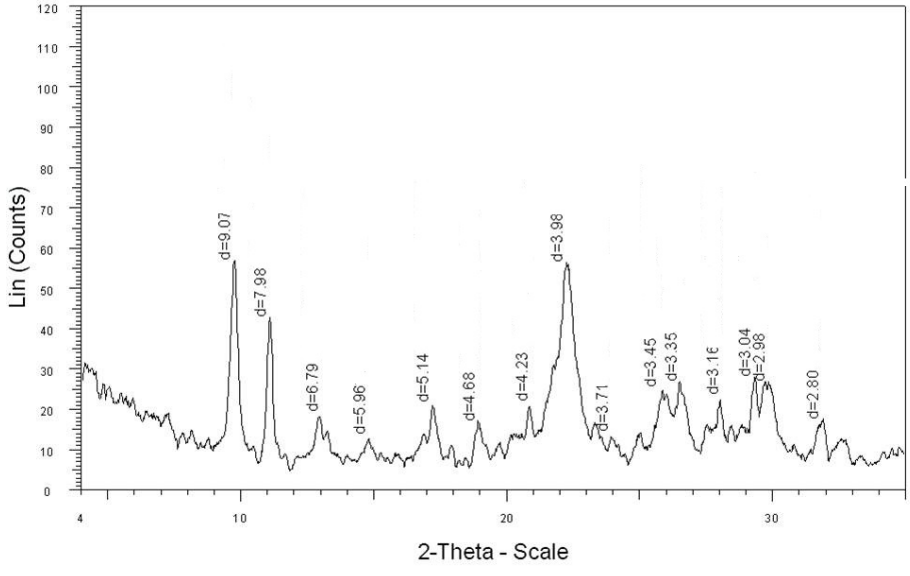

Figure 3. X-ray diffractogram of zeolite clay

d: D-spacing based on angstrom (Å).

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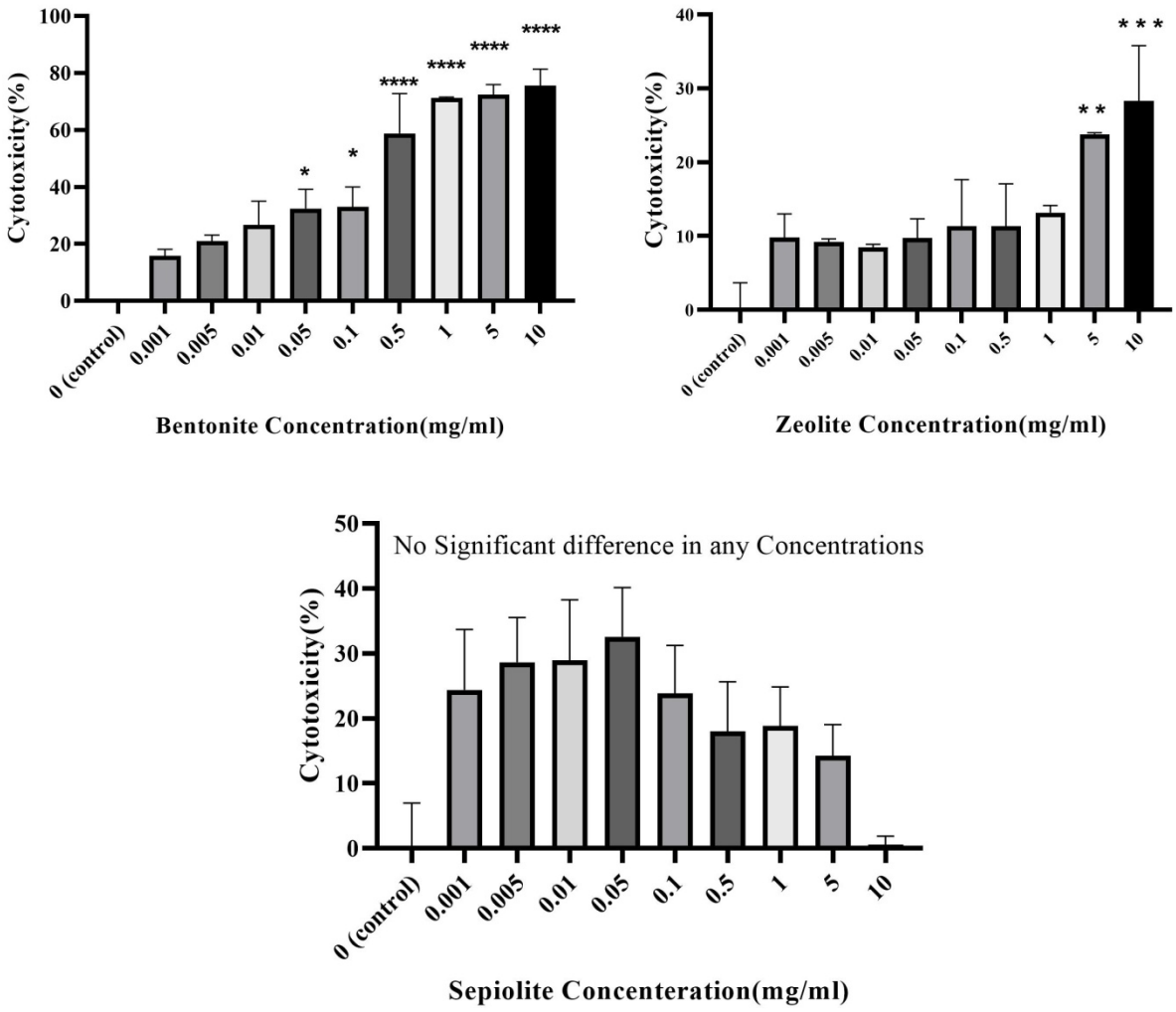

Figure 4. Comparison of cell toxicity rate (%) of bentonite, zeolite, and sepiolite onPBMCs with different concentrations for 48 h
*P≤0.05, **P≤0.01, ***P≤0.001 compared to the control.

4. Discussion

CEC is a unique property of clays, allowing for interactions with cell surfaces and intracellular signaling pathways [35]. It has two origins: One origin can be isomorphic substitution in the tetrahedral- and/or octahedral sheet of the clay mineral layer. For example, substituting aluminum for magnesium or silicon with aluminum leads to a negative net charge. This part of the CEC is considered to be constant as it is almost not sensitive to the system's pH. The second origin is the dissociation of luminol groups on the edges. Since the acidity of these groups is weak, the edge charges are pH-dependent, and the CEC depends on the pH [36]. Interaction of the cationic edge charges of the clay with cell membrane molecules facilitates cellular transport and uptake [37, 38]. Among the studied clays, the highest CEC belongs to bentonite [2]. At pH 7, about 20% of the CEC of bentonite is located at the edges [36]. Our results show that CEC for bentonite, zeolite, and sepiolite are 86 cmol(+)/kg, 15.5 cmol(+)/kg, and 3.54 cmol(+)/kg, respectively, directly related to cell growth.

Particle size and structure may also facilitate cellular transport and uptake [38-40]. Their uptake directly and strongly affects cellular physiological responses [41]. Because the particle size of bentonite clay is smaller than zeolite and sepiolite fibers [2], the cytotoxic effects of bentonite can be due to the increase in the entrance of clay sheets to the cells. Sabzevari et al. [42] also reported that increasing cell death could be due to the increase in the internalized sheets of montmorillonite. Transport into the cells is through endocytosis or by cell receptors [43]; it seems that bentonite can enter the cells and in-

crease cell death due to its smaller size and higher CEC (higher activity). As a result, it can cause cell dysfunction, apoptosis, and as a result, cellular death.

Related studies also reported that cytotoxic concentrations vary for different clays [44-46, 40]. Our study showed that bentonite decreased cell viability at 10, 5, 1, 0.5, 0.1, and 0.05 mg/mL (Figure 4). This range is more expansive than similar concentrations of bentonite investigated in other research. For example, in vitro, the cytotoxicity of montmorillonite on Chinese hamster ovary (CHO) cells was only evident at >1 mg/mL concentrations [39]. Another study on proliferation and colony formation of human normal intestinal cells publicized inhibition at 5 mg/mL of montmorillonite within 24 h [47]. A significant loss of viability was reported in the human hepatic cell line HepG2 in response to low concentrations of both unmodified and organically modified montmorillonite nanoclays [48]. MMT significantly inhibited cell proliferation after 24-72 h of incubation in a concentration-dependent manner at concentration levels above 100 µg/mL [49]. Laponite (a type of smectite clay) at 100 µg/mL for seven days showed no effect on morphology, viability, or cell proliferation of bone marrow cells and fat-derived stroma. However, a decrease in metabolic activity was observed at high doses (>1 mg/mL) of clay [40, 50]. No significant decrease in human hepatoma cell line HepG2 viability was observed after montmorillonite (6.25 and 62.5 µg/mL) treatment for 4 and 24 h [51].

As shown in Figure 4, there were no significant differences in all concentrations of sepiolite (0.0010-10 mg/ml). Castro-Smirnov et al. [52] also reported that sepiolite did not affect cell viability up to 50 ng/µL after 24 h. They mentioned that 100 ng/µL of sepiolite received for 48 h could slightly increase toxicity (80% of survival cells). In vitro, the toxicity of sepiolite nano clays (NC) showed that macrophages derived from human peripheral blood monocytes were less affected in viability (25% decrease at 48 h) [53]. Indeed, human cells are able to detect sepiolite, triggering a defense response, and might be able to expel the fibers. Nevertheless, the choice of the sepiolite source for current and future applications should be based on crucial parameters, like fiber size and chemical composition, which in turn might be directly related to the geological origin [30]. Our results also showed that the zeolite's cytotoxic concentrations are higher than those reported by Demircan et al. [54]. They mentioned cell death at 5×10^{-5}, 10^{-5}, 5×10^{-4}, 10^{-4}, and 10^{-3} M of clinoptilolite in the human peripheral blood monocyte cell line (THP-1) was non-toxic.

PBMCs showed different responses after bentonite, zeolite, and sepiolite exposure. Cells showed the highest inhibition toward bentonite. The mortality rate was more than 50% at high concentrations of bentonite. Zeolite showed growth inhibition only at high concentrations (10 and 5); immediately after that, the inhibition reached a constant level in others. Indeed, toxic concentration is unique for each type of clay, and it is necessary to check the toxic concentrations before using the target clays. Although the results showed that the toxicity of the investigated clays was less than those observed in the literature review, sufficient care should be taken in using these particles in healthcare and medicine according to the mechanisms of entering these particles into the cell and their intracellular effects.

Our finding showed that the effect of clays on cell growth was concentration-dependent. Sabzevari et al. [42] reported that the antiproliferative effect of montmorillonite was concentration-dependent. Also, in oxidative stress conditions after 24 hours of incubation, clinoptilolite reduced cell proliferation in a dose-dependent manner in THP-1 cells [54]. Natural clinoptilolite treatment inhibited the proliferation and survival of diploid fibroblasts (Hef522), cervical carcinoma (HeLa), colon carcinomas (CaCo-2, HT-29, and SW 620), mammary carcinomas (MCF-7 and SkBr-3), and also mouse fibrosarcoma cell line in a concentration-dependent manner [44]. One of the factors in the occurrence of this behavior might be the flocculation of clay colloids at increasing concentrations and in the high salt concentrations of the cell culture media. Because of surface charge, clays electrostatically repel each other upon dispersion in cell culture media, avoiding aggregation. In dilute suspension, the clays are well dispersed and negatively charged. However, at increased concentrations, strong van der Waals forces make the clays adhere to each other, such as in the mechanism of flocculation or aggregation [40]. This mechanism especially occurs for bentonite because of its small particle size. Many studies [24, 40, 50, 55] also point out that for this type, clay particles will typically aggregate into micro-sized clusters/agglomerates with a tendency to accumulate around cells. Accumulation can block membrane channels and damage cellular metabolism and cytoskeleton organization.

The results indicated that bentonite, sepiolite, and zeolite clays had favorable behaviors in terms of cytotoxic in vitro. Many toxicology studies have verified negligible effects of clay nanoparticles on human or animal cells at related physiological concentrations [35]. These findings will provide fundamental information about the potential toxicity of bentonite, sepiolite, and zeolite

clays in Iran mines and their application at safe levels as well. Results could also provide a new perspective on the safety of using these inexpensive and naturally available clays in medical and industrial applications.

5. Conclusion

Bentonite was internalized more than other clay minerals due to its high CEC and fine-grained plates and caused PBMC death. Due to the special sieve structure and having internal and external cation exchange capacity, zeolite destroyed cells. Interestingly, sepiolite does not exhibit significant toxicity and has no health risks. Therefore, sepiolite is non-toxic, zeolite has low toxicity only at high concentrations, and bentonite shows more toxicity. The cytotoxicity of the investigated clays is less than those reported in the literature. Since the leukocytes are the main effector cells in immune response and this study was done on PBMCs, the results of this paper may suggest that the studied clays have great potential and can be used for immune system responses. Nevertheless, in vivo and long-term studies on bio-culture and biodistribution are essential to understand better the role of the studied clays. It is also important to evaluate the toxicity in animal models as the in vitro cell culture system does not complexity of the in vivo system.

Ethical Considerations

Compliance with ethical guidelines

There is no ethical principle to be considered in doing this research.

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Authors' contributions

Conceptualization, project administration, funding acquisition and supervision: Mohammad Hassan Salehi and Tooba Ghazanfari; Investigation, methodology and writing–original draf: Fariba Nemati Shamsabad; Advising Jalaleddin Sham: advising.

Conflicts of interest

The authors declared no conflict of interest.

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