



The response of periodontal cells to kaolinite

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Abstract

Objectives The impact of kaolinite on human periodontal cells is yet unknown. The aim of the study was to assess the response of human periodontal cells to kaolinite.

Methods Human periodontal cells were treated with kaolinite at reducing concentrations from 30 to 0.0015 mg/mL and with conditioned medium, which was depleted of kaolinite. Cell viability was evaluated with a resazurin-based toxicity assay, Live-Dead staining, and MTT assay and staining. The pro-angiogenic factors vascular endothelial growth factor (VEGF) and interleukin (IL)-6 and IL-8 were quantified via ELISA in periodontal fibroblasts. L-929, a standard cell-line used for cytotoxicity studies, served as control cell line. Composition of kaolinite was verified using energy-dispersive X-ray spectroscopy.

Results Kaolinite in suspension but not in conditioned medium impaired cell viability dose-dependently. VEGF, IL-6, and IL-8 production was not substantially modulated by kaolinite or the conditioned medium in periodontal cells.

Conclusion Overall, kaolinite can decrease cell viability dose-dependently while conditioned medium showed no toxic effect. No pronounced impact of kaolinite on VEGF, IL-6, and IL-8 production was observed. This study provided first insights into the impact of kaolinite on human periodontal cells thereby inferring to the basis for the evaluation of kaolinite as a carrier in regenerative dentistry.

Clinical relevance Kaolinite, a clay mineral, is successfully used in medicine due to its favorable properties. Also, applications in conservative dentistry are described. However, the response of oral cells to kaolinite is still unclear. Here, we assessed the impact of kaolinite on human periodontal cells.

Keywords Kaolinite · Clay minerals · Periodontal ligament fibroblasts · Gingival fibroblasts · L-929 cells · Cytotoxicity

Introduction

Tissue engineering is based on three main pillars: cells, scaffolds, and growth factors [1]. Several properties are to be considered before deciding for a specific scaffold. Among these, the most important are biocompatibility, biodegradability, mechanical properties, scaffold architecture, and manufacturing technology [2]. Scaffold properties impact the efficiency of a certain tissue engineering approach. Thus, the importance of choosing and

designing an optimal scaffold for a specific application is of high importance. Recently, clay-based materials have been advocated in regenerative medicine for tissue engineering as carrier materials for pharmaceuticals and biologicals [3–8].

Kaolinite is a clay mineral of the kaolin group with the structural formula $Al_2Si_2O_5(OH)_4$ [9]. Usually, it appears in stacks of pseudohexagonal platelets, which are less than 2 μm large [10], but the particle size can range from 0.3 to 100 μm [11]. Kaolinite is insoluble in water as well as in acids and alkali hydroxides [10]. Furthermore, it has a wide pH range [12], high lubricity [10], and is non-abrasive [12]. Kaolinite is a non-swelling clay [13], which makes it feasible for a variety of clinical applications compared to other clay minerals. Furthermore, it withstands temperatures up to 400 °C [14]; it is stable under acidic conditions and has a low equilibrium moisture content [15]. Due to these physical and chemical properties, kaolinite is used in the production of ceramics, paper, rubber, fertilizers, pesticides, and cosmetics. Applications in drug manufacturing and medical fields have been described [16].

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Kaolinite has been employed in medicine for a wide variety of applications. It is used as a pharmaceutical excipient due to its inertness [17, 18]. Its functions as an excipient are varied: as a diluent, binder, pelletizing agent, particle film coating, emulsifying, and suspending agent and many more [17, 18]. Its use as a diluent is based on the role of reducing dissolution or diminishing availability of drug molecules by adsorption [19]. Kaolinite as a drug carrier is also under use as the release kinetics are favorable at a broad pH span, thus, both under gastric (pH 1) and intestinal (pH 6.8) conditions [20].

The outstanding and interesting aspect of kaolinite is its application as active pharmaceutical ingredient in various medical fields [19]. Its use as a hemostatic wound dressing agent is based on its property of accelerating blood clot formation by activating the clotting pathway [21]. Due to the negative surface charge of kaolinite at a pH of around 7 (pH of blood), it starts to transform the factor XII of the coagulation cascade to its active form leading to accelerated coagulation [22]. Based on these findings, kaolinite is now commercially used in many wound dressings [23]. This aspect of kaolinite could be of special interest in oral surgery and periodontology, leading to a possible reduction of gum bleeding.

Kaolinite is also attributed to have antibacterial and antiviral effects. Antibacterial effects are explained by physical, such as electrostatic attraction to certain bacteria, and chemical effects of kaolinite. Chemical effects are due to the presence of Fe^{2+} in this special clay mineral. These are oxidized once they enter the bacterium and lead to the production of lethal hydroxyl radicals [24–26]. This antibacterial effect is also of special interest in dentistry as periodontitis is a bacterial disease with a high prevalence in central Europe, posing a problem to over 50% of young adults in Germany and nearly 2/3 of the elderly German population [27]. Thus, kaolinite could prove itself useful not only as a carrier but also as an active treatment ingredient in dentistry. Several studies have already been conducted on the effect of kaolinite on oral pathogens [28]. Holešová et al. prepared nanocomposites for oral application containing kaolinite with the intention of an antipathogenic effect and Jou et al. studied chlorhexidine mixed with silver-kaolinite, which leads to a stronger antibacterial effect than a combination of only two of the three components [29, 30].

Also, the anti-inflammatory properties of kaolinite may be of high relevance for dentistry. Its topical application in form of poultices has already been described as an anti-inflammatory treatment [31–33]. Gingivitis is characterized by the inflammation of oral gingiva and mucosa. Periodontitis is microbially associated and leads to periodontal attachment loss [34]. Thus, kaolinite may find its place in the treatment of these diseases. Other medical applications include the usage as a gastrointestinal protector, as anti-diarrheal substance and in pelotherapy [35,36].

Thus, many different medical applications have been described in various fields and the effect of kaolinite on human

cells other than the dental field has been evaluated [37]. The dental and oral surgery field however is quite underrepresented. Kaolinite could serve as a medical application itself or act as a scaffold material for growth factors and other proteins in oral tissue regeneration. However, until now, there are no studies on the application of kaolinite in regenerative dentistry neither as scaffold material nor as carrier for signaling factors or pharmaceuticals. Thus, first steps have been taken here in evaluating the effect of kaolinite on cells of the L-929 line, a standard cell line for cytotoxicity testing and on primary human fibroblasts from the periodontal soft tissue and the gingiva with the intention to carve the way for kaolinite as a candidate biomaterial for oral tissue regeneration.

In regenerative dentistry, there is still a lack of studies on the feasibility of clay-based materials, including properties such as binding of diverse factors. Also, viability assays and biocompatibility testing are yet to be done. Thus, the aim was to assess for any toxic, pro-angiogenic, and anti-inflammatory effect of kaolinite on oral cells and cells from the L-929 line.

Materials and methods

Kaolinite

Kaolinite used in this study was obtained from Sigma-Aldrich (Kaolinite natural, $\text{Al}_2\text{O}_7\text{Si}_2$, faint beige powder, molecular weight 258.16 g/mol; PubChemSID: 329747737). Composition was validated with scanning electron microscopy and energy-dispersive X-ray spectroscopy.

Preparation of kaolinite suspension

Kaolinite powder was mixed with α -minimal essential medium (α -MEM) (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Linz, Upper Austria, Austria) and antibiotics (penicillin 100 U/mL, streptomycin 100 $\mu\text{g}/\text{mL}$, amphotericin B 2.5 $\mu\text{g}/\text{mL}$ solution, Invitrogen Corporation, Carlsbad, CA, USA) at a concentration of 30 mg/mL. A dilution series was performed at 1:3 (by volume) with the α -MEM leading to kaolinite in suspension of the following concentrations: 30 mg/mL, 10 mg/mL, 3.3333 mg/mL, 1.1111 mg/mL, 0.3704 mg/mL, 0.1234 mg/mL, 0.0412 mg/mL, 0.0137 mg/mL, 0.0046 mg/mL, and 0.0015 mg/mL.

Preparation of conditioned medium from kaolinite

Kaolinite at 30 mg/mL was suspended in α -MEM supplemented with 10% FCS and the antibiotics penicillin, streptomycin, and amphotericin B (Invitrogen) and incubated at 37 °C, 5% CO_2 , and 95% atmospheric moisture for 24 h. The suspension was then filtered with a sterile filter with a

cut off of 0.22 μm (TPP, Trasadingen, Switzerland). A dilution series of this conditioned medium was then performed at 1:3 with α -MEM supplemented with 10% FCS and the antibiotics penicillin, streptomycin, and amphotericin B (Invitrogen) for 10 consecutive concentrations.

Cell culture

Primary human fibroblasts from the periodontal soft tissue and the gingiva were isolated from extracted third molars based on a previously described protocol [38] after informed consent was given by the donors (Ethics Committee of the Medical University of Vienna, Vienna, Austria). The donors were included based on the absence of any inflammation in the region of the extracted tooth. In brief, the soft tissue was scraped off the root of the tooth to collect periodontal ligament fibroblasts (PDLF). Soft tissue from the neck of the tooth was extracted for the culturing of gingival fibroblasts (GF). Explant cultures were performed in α -MEM supplemented with 10% FCS and the antibiotics penicillin, streptomycin, and amphotericin B (Invitrogen) at 37 °C, 5% CO₂, and 95% atmospheric moisture. Cells from the L-929 cell line (ATCC, Rockville, MD, USA) were also cultured in α -MEM supplemented with 10% FCS and the antibiotics penicillin, streptomycin, and amphotericin B (Invitrogen). For the experiments, the cells were seeded at 30,000 cells/cm² in 96-well plates and incubated for 24 h.

MTT assay and staining

In order to assess the metabolic activity of the cells, PDLF, GF, and L-929 cells were incubated with 1 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 2 h. The medium with the MTT was discarded and 100 μL DMSO were added per well. Formazan formation was quantified using a Synergy HTX multi-mode reader (BioTek, Winooski, VT, USA) at a wavelength of 550 nm. Before discarding the medium, images were taken with a Nikon ECLIPSE TS 100 light microscope.

Resazurin-based toxicity assay

For the evaluation of the cell viability, the resazurin-based toxicity assay was conducted according to the protocol of the manufacturer. Resazurin dye solution (Sigma-Aldrich) in an amount equal to 10% of the culture medium was added to the cells. The cells were incubated at 37 °C for 4 h. Fluorescence was evaluated using a Synergy HTX multi-mode reader (BioTek) at a wavelength of 600 nm, using an excitation wavelength of 540 nm.

Live-Dead staining

In order to assess the integrity and status of the cellular plasma membrane as an indicator of vitality, cells were stained with the Live-Dead Cell Staining Kit (Enzo Life Sciences AG, Lausen, BL, Switzerland) according to the manufacturer's instructions. In short, to prepare the staining solution, 1 μL of solution A and 1 μL of solution B were added per 1 mL of the staining buffer. Then, the staining solution was added to the cells and incubated at 37 °C for 15 min. The cells were evaluated using fluorescence microscopy (Nikon Diaphot 300) for green and red, with a B-2A filter (excitation filter wavelengths: 450–490 nm), respectively. Vital cells appeared green while dead cells appeared red. Images were taken with the Nikon Diaphot 300. Staurosporine-treated cells were used as toxic control.

Immunoassays

Culture supernatants were subjected to the measurement of vascular endothelial growth factor (VEGF), interleukin (IL)-6, and IL-8 levels by ELISA (Standard ABTS ELISA Development Kits, Peprotech, Rocky Hill, NJ, USA). In short, the appropriate capture antibody in PBS was used to coat 96-well ELISA plates. The plates were sealed and left at room temperature for incubation overnight. Then, plates were washed with wash buffer and blocked using the blocking buffer. After washing, the samples and the standard were added and plates were incubated at room temperature for 2 h followed by a washing step. Then, the detection antibody was added and the plates were incubated for 2 h at room temperature followed by a washing. Avidin-HRP conjugate was applied to each well and the plates were incubated for 30 min at room temperature followed by a washing. Finally, ABTS was added. The optical density obtained from the samples was measured using a Synergy HTX multi-mode reader (BioTek) at the recommended wavelength of the ELISA kit manufacturer. The concentration of total VEGF, IL-6, and IL-8 was calculated with the standard curve method as described by the manufacturer.

Scanning electron microscopy

Kaolinite was evaluated with the Quanta 200 system (FEI Company, USA). The powder samples were located in a sufficient quantity on an aluminum sample holder for scanning electron microscopy (SEM) analysis. EDS was used to analyze the powder particle composition.

For cell images, cells cultured on coverslips with and without kaolinite at 0.123 mg/mL and the respective conditioned medium were fixed with 4% neutral buffered formalin and dried before they were sputtered. Images were taken after sputtering with gold for 30 s (approximately 100 nm Au) using the EM ACE200 sputtering device (Leica, Germany). An accelerating voltage of 12.5 and 25 kV in SE mode at 1000-fold magnification was used.

Bicinchoninic acid protein assay

Bicinchoninic acid protein assay (BCA) (Thermo Scientific, Rockford, IL, USA) was used to determine the protein concentration cell culture medium (α -MEM with 10% FCS) and the kaolinite conditioned medium according to manufacturer's guidelines [39]. BCA A and B were mixed at 1:50 ratio. One hundred microliters of this solution were added per 10 μ L of the 1:100 diluted sample. The samples were incubated for 30 min at 37 °C. Evaluation was carried out using the Synergy HTX multi-mode reader (BioTek, Winooski, VT, USA) at a wavelength of 550 nm.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics Version 23 (IBM Corporation, Armonk, NY, USA), using the ANOVA and *post hoc* Dunnett test. The level of significance was set at $p < 0.05$.

Fig. 1 Kaolinite powder evaluated by scanning electron microscope (a). Images were taken using the Quanta 200 system (FEI Company, USA) and an accelerating voltage of 25 kV in SE mode at 1000-fold. The white bar represents 50 μ m. A Energy-dispersive X-ray spectroscopy (EDS) was used to analyze the powder particle composition (b)

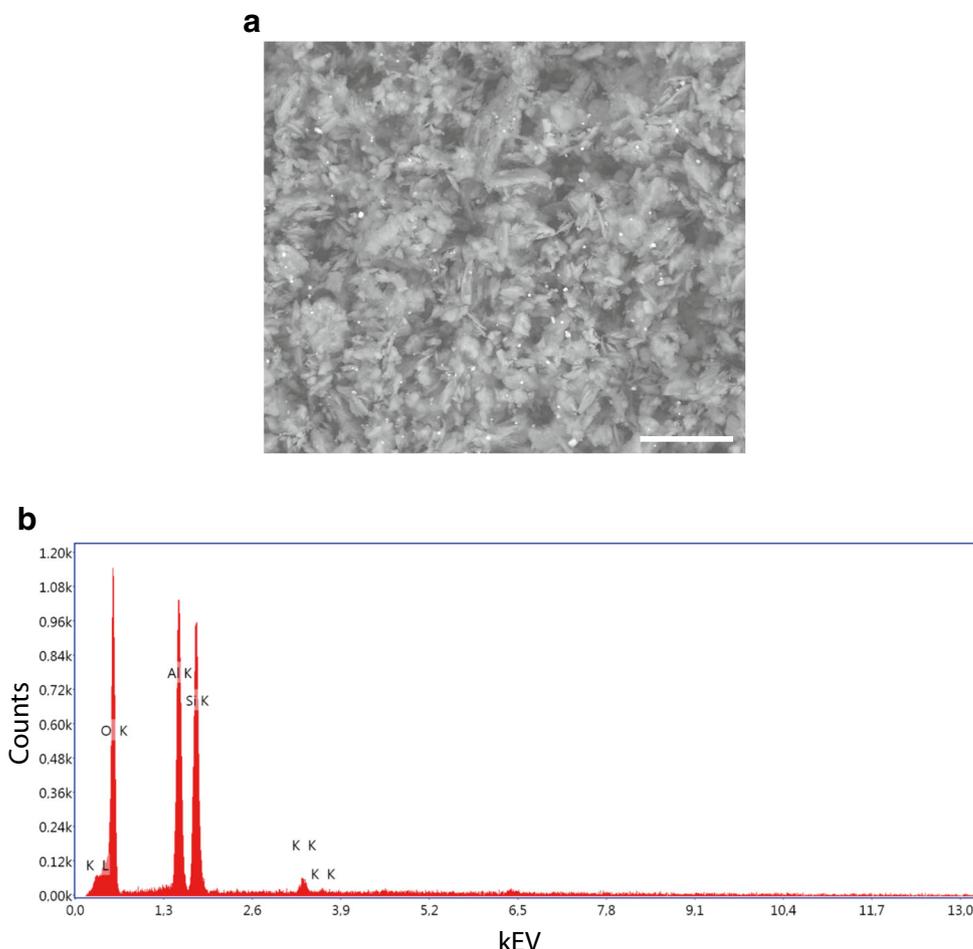


Table 1 Evaluation of kaolinite powder by scattered electron microscopy

Element	Weight %	Atomic %
O	50.81	64.29
Al	22.38	16.79
Si	24.83	17.90
K	1.98	1.03

An EDS-system (EDAX Company, Germany) installed in a Quanta 200 system (FEI Company, USA) was used for evaluation of energy-dispersive X-Ray spectroscopy to analyze the powder particle composition.

Results

Scanning electron microscopic evaluation of kaolinite and protein absorption analysis

First, kaolinite powder in terms of its composition regarding molecular weight and atom distribution was evaluated. The image shows kaolinite particles below 50 μ m (Fig. 1a). Using EDS O at 50.81 wt% and 64.29 atomic %, Al at 22.38 wt% and 16.79

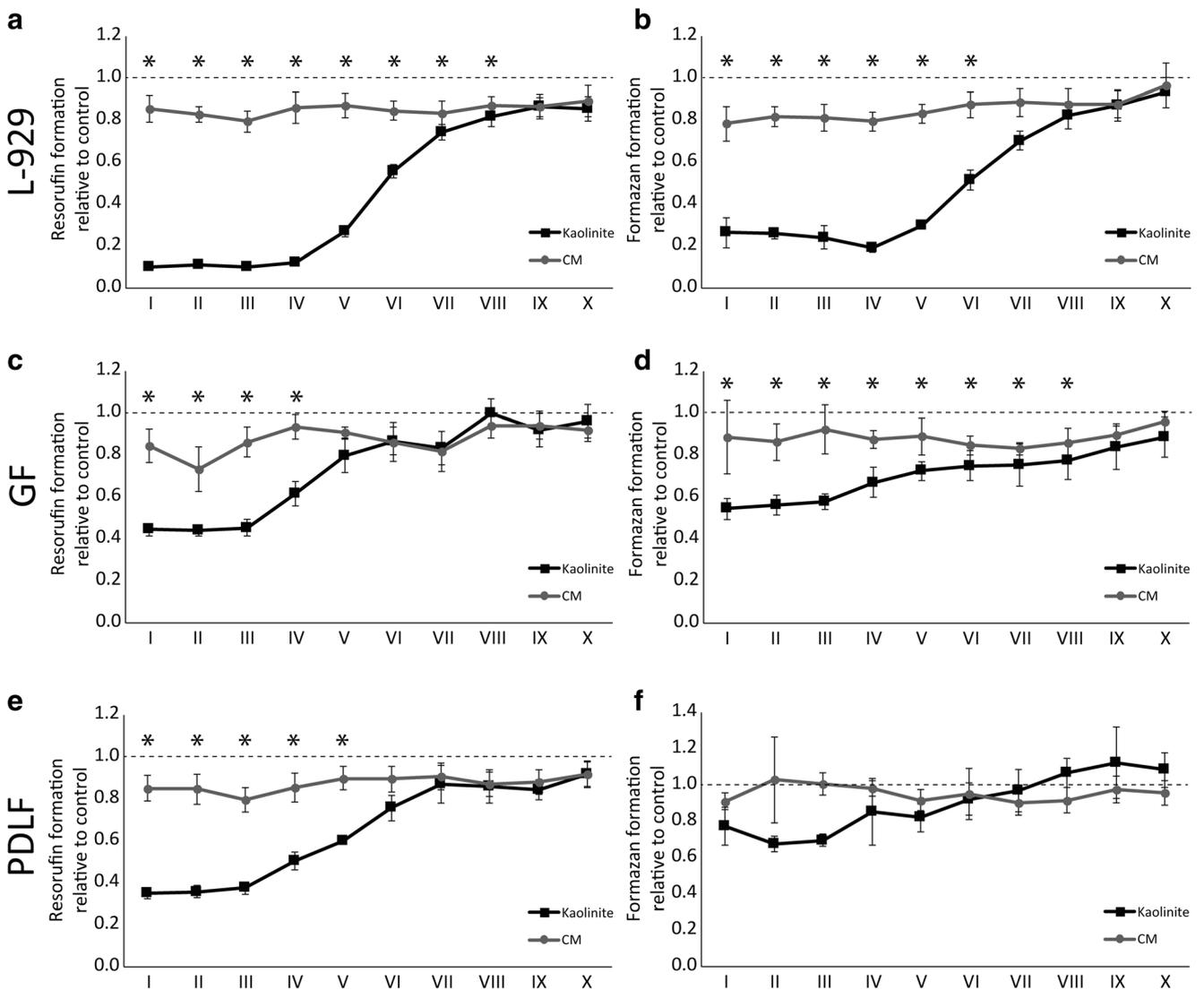


Fig. 2 The effect of kaolinite on the activity of L-929 cells and human periodontal cells. L-929 cells, human gingival fibroblasts (GF), and periodontal ligament fibroblasts (PDLF) were exposed to kaolinite in suspension and with kaolinite-conditioned medium from 30 mg/mL (I)–0.0015 mg/mL (X). Stimulants were prepared as a dilution series of 1:3. Viability was assessed based on resorufin formation (a, c, e). b, d, f

Furthermore, formazan formation was assessed via the MTT assay (b, d, f). Data are given relative to untreated cells. The black lines represent the cells incubated with kaolinite, while the gray lines represent the cells incubated with conditioned medium (CM). Data points represent mean ± standard deviation. Four repetitions were performed with four donors. **p* < 0.05 vs. control (dashed line)

atomic %, Si at 24.83 wt% and 17.90 atomic %, and K at 1.98 wt% and 1.03 atomic % was found (Fig. 1b; Table 1).

The BCA assay showed that the protein concentration was not significantly different in the cell culture medium compared to the conditioned medium. The relative protein content of the conditioned medium amounted to 112.5% (± 43.6%) relative to the medium.

The effect of kaolinite on the metabolic activity of L-929, GF, and PDLF

The effect of kaolinite in suspension and medium conditioned with kaolinite on cells of the line L-929, human GF, and human

PDLF was evaluated using the MTT assay and a resazurin-based toxicity assay. Both the kaolinite in suspension and the conditioned medium were applied in a dilution series. L-929 cells maintained their ability of resorufin and formazan formation above 70% compared to the control upon treatment with kaolinite in suspension at concentrations of 0.041 mg/mL and below. Resorufin and formazan formation was decreased below 30% of the control in L-929 cells for kaolinite concentrations between 1.111 and 30 mg/mL (Fig. 2).

In human GF and PDLF, resorufin and formazan formation was above 70% compared to the control at concentrations 0.370 mg/mL and below. Only the MTT assay in PDLF exhibited reduced formazan formation at just below 70%.

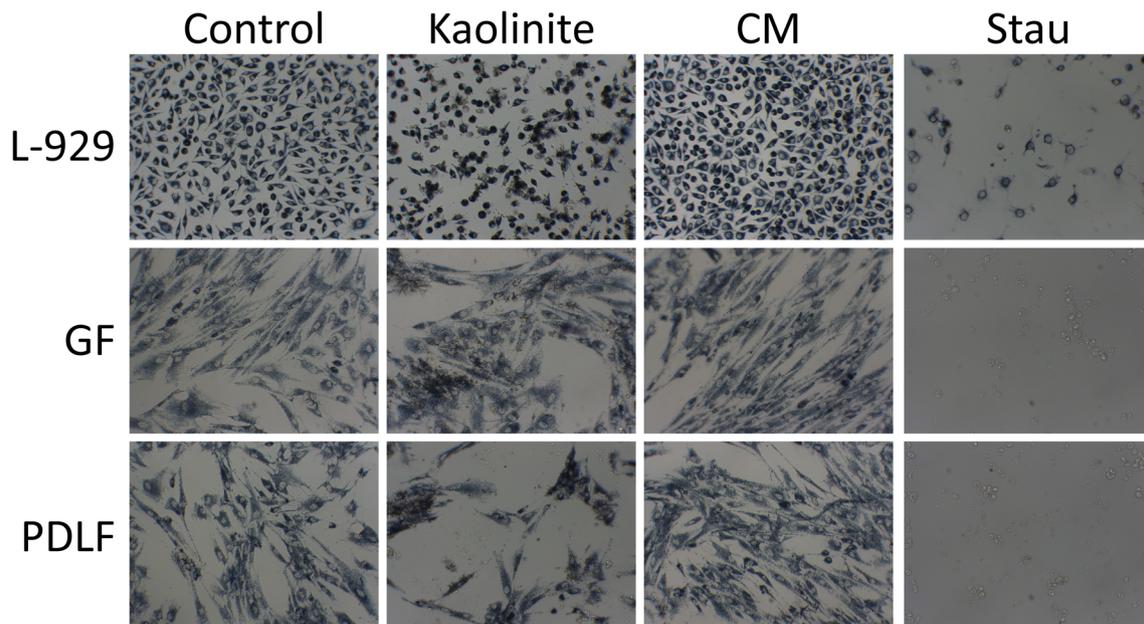


Fig. 3 Evaluation of the cellular response to kaolinite using the MTT staining. L-929 cells, human gingival fibroblasts (GF), and periodontal ligament fibroblasts (PDLF) were exposed to a kaolinite suspension at

0.123 mg/mL and to medium depleted from kaolinite at this concentration (Conditioned medium, CM). Staurosporine (Stau) acted as the negative control. MTT staining was performed

Furthermore, resorufin and formazan formation is reduced to 30–60% in GF and PDLF at the highest kaolinite concentrations of 30 mg/mL (Fig. 2).

When exposed to conditioned medium, resorufin and formazan formation was not majorly affected in all three cell types. Cell viability stayed above 70% independent of the

kaolinite concentration used for conditioning the medium (Fig. 2).

Formazan formation is not impaired in GF and only little in PDLF when exposed to kaolinite at a concentration of 0.123 mg/mL. L-929 cells contract and detach from the surface. All three cell types do not appear to be influenced in their formazan for-

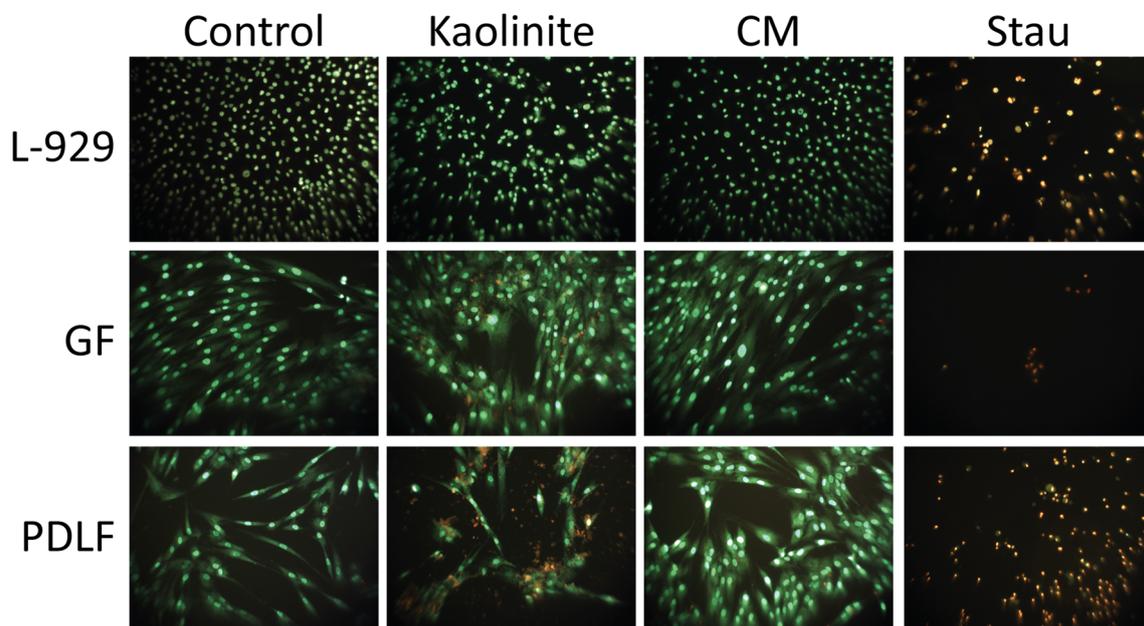
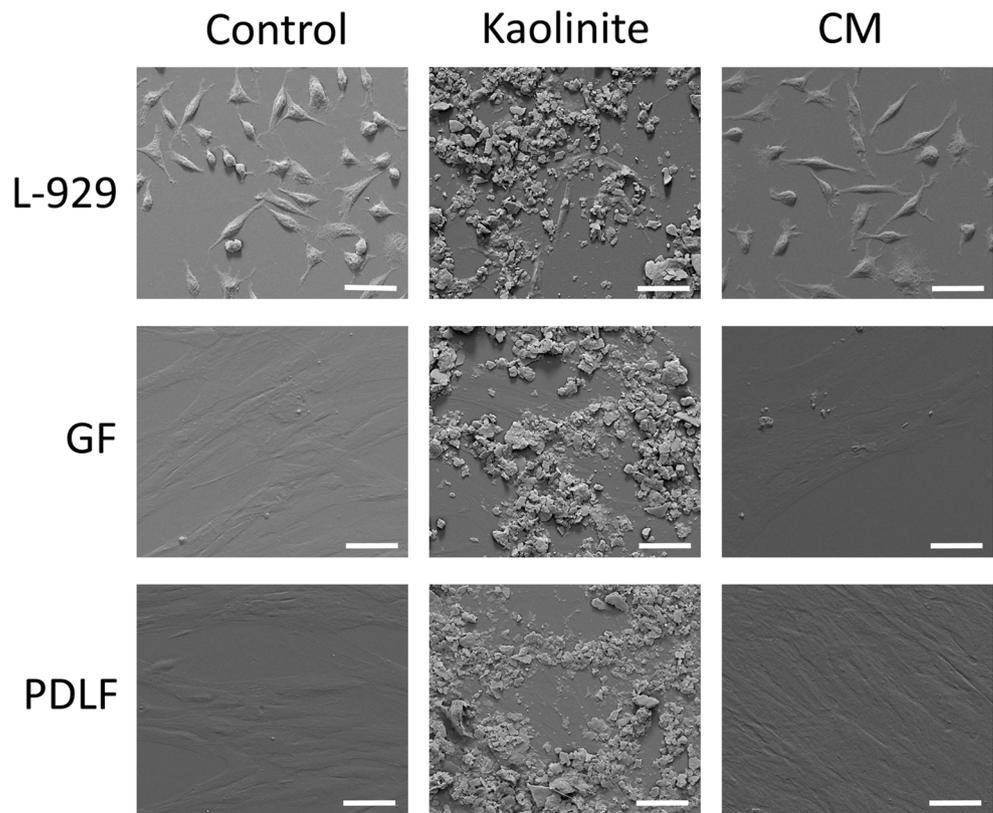


Fig. 4 Evaluation of the cellular response to kaolinite using the Live-Dead staining. L-929 cells, human gingival fibroblasts (GFs), and periodontal ligament fibroblasts (PDLFs) were exposed to a kaolinite suspension at 0.123 mg/mL and to medium depleted from kaolinite at

this concentration (Conditioned medium, CM). Staurosporine (Stau) acted as the negative control. Live-Dead staining was performed. Green represents vital cells, red shows impaired viability

Fig. 5 Evaluation of the cellular response to kaolinite using scanning electron microscope. Cells were cultured with and without (Control) kaolinite at 0.123 mg/mL or the respective conditioned medium (CM). Images were taken using the Quanta 200 system (FEI Company, USA) and an accelerating voltage of 12.5 kV in SE mode at 1000-fold. The white bar represents 50 μm



mation when exposed to the conditioned medium (Fig. 2).

Viability of L-929 cells, GF, and PDLF exposed to kaolinite could also be validated via MTT staining and Live-Dead staining. The MTT staining showed blue stained cells, which indicates vitality (Fig. 3). Vital cells appeared green while dead cells appeared red under the fluorescence microscope when applying the Live-Dead staining. The L-929 cell number appeared to be reduced when exposed to kaolinite at a concentration of 0.123 mg/mL. When exposed to conditioned medium, cells of all three types appear green, thus vital (Fig. 4). Also, SEM showed vital cells with normal fibroblastic morphology when cultured with

kaolinite at 0.123 mg/mL or the respective conditioned medium (Fig. 5). In the groups with kaolinite, the cells were covered by the powder.

Taken together, the data suggest that kaolinite can dose dependently impair viability of murine L-929 cells while human GF and PDLF are more resistant. Conditioned medium does not affect viability in any of the cell types.

VEGF, and interleukin production in L-929, GF, and PDLF upon kaolinite exposure

To evaluate VEGF and IL-6 production, L-929 cells, GF, and PDLF were exposed to kaolinite at a concentration of 0.123 mg/mL and to medium conditioned with the same kaolinite concentration for 24 h and the supernatant was harvested. VEGF and IL-6 levels in the supernatant was assessed via ELISA.

VEGF production was significantly decreased to 80% in GF and PDLF compared to the control when exposed to kaolinite at a concentration of 0.123 mg/mL. Conditioned medium did not affect the VEGF production in human cells. In L-929 cells, the VEGF production seems to be rather increased but due to the high standard deviation, results are not significant (Fig. 6).

Table 2 Evaluation of IL-8 levels in the supernatant of human periodontal cells exposed to kaolinite in suspension and conditioned medium depleted of kaolinite

	Kaolinite	CM
GF	1.140 ± 0.115*	1.017 ± 0.104
PDLF	0.974 ± 0.116	0.988 ± 0.069

The pro-angiogenic response to kaolinite in suspension (0.123 mg/mL) and conditioned medium was measured based on IL-8 levels in the culture media. Data are given relative to untreated cells (1.000). Data are given as mean +/- standard deviation. Experiments were performed twice with three donors

**p* < 0.05 vs. control

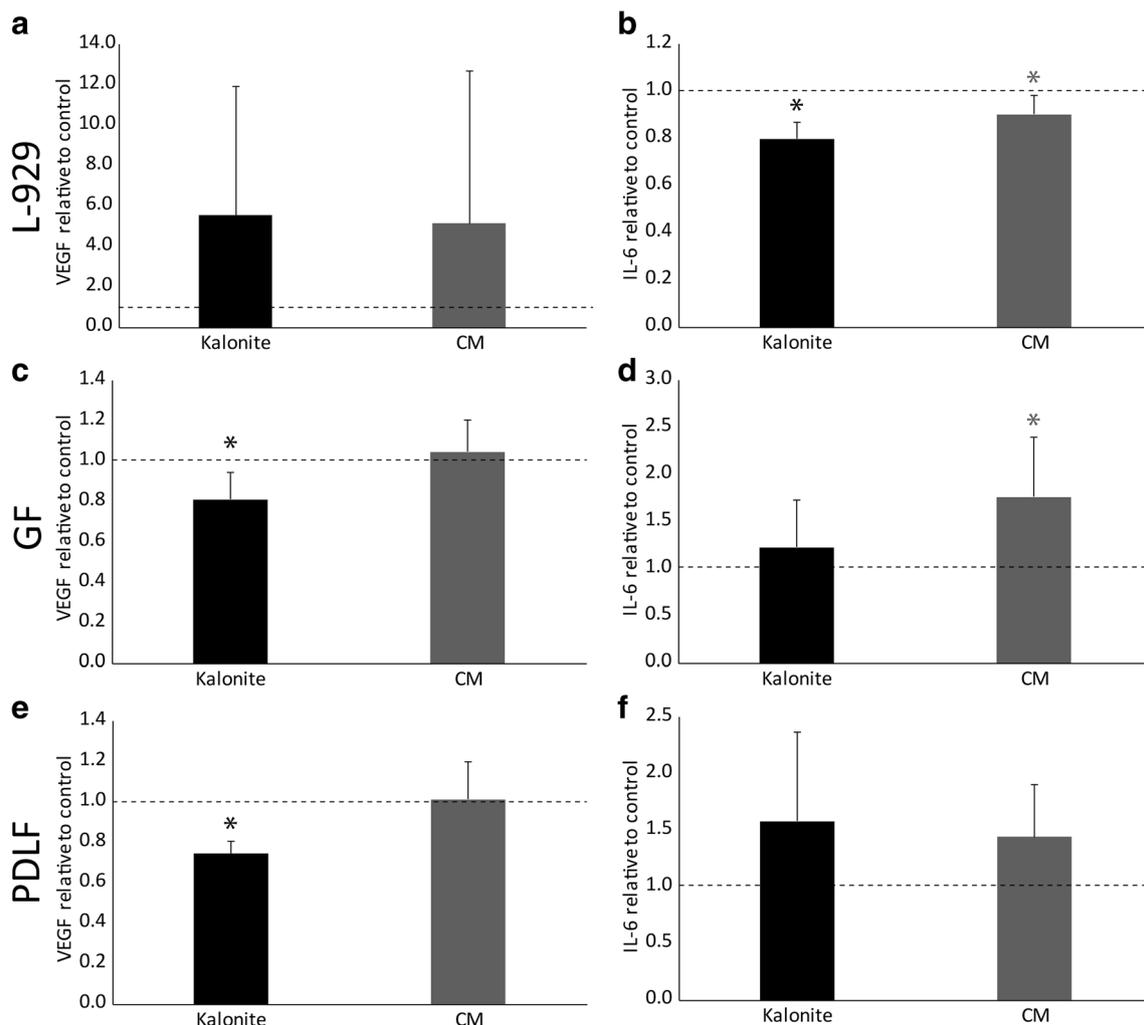


Fig. 6 Evaluation of VEGF and IL-6 protein levels in the supernatant of L-929 cells and human periodontal cells exposed to kaolinite in suspension and conditioned medium depleted of kaolinite. The pro-angiogenic response to kaolinite in suspension (0.123 mg/mL) and

conditioned medium was measured based on VEGF levels (a, c, e) and IL-6 levels (b, d, f) in the culture media. Data is given relative to untreated cells. Data points represent mean + standard deviation. Two repetitions were performed with three donors. * $p < 0.05$ vs. control (dashed line)

When L-929 cells were exposed to the kaolinite suspension (0.123 mg/mL) and conditioned medium, IL-6 production was decreased to 80% and 90% respectively. In GF and PDLF on the other hand, IL-6 was increased compared to untreated cells when incubated with kaolinite suspension and with conditioned medium. The increase was only significant in GF when exposed to the conditioned medium (Fig. 6).

IL-8 was measured in the supernatants of GF and PDLF (Table 2). No substantial modulation of IL-8 in the culture medium upon treatment with kaolinite or the conditioned medium was found.

These data suggest that VEGF production is increased in murine L-929 cells but decreased in human GF and PDLF when exposed to kaolinite suspension and conditioned medium and vice versa for IL-6 production.

Discussion

Kaolinite is a clay mineral with widespread medical applications. Especially its hemostatic and antimicrobial properties may be of high interest when considering it as a scaffold material in regenerative dentistry [21–23, 29, 30, 40]. Kaolinite can readily adsorb proteins [41], which makes it a promising candidate as a scaffold. Depending on the accessibility of the application site and the surgical procedure, there might be a few obstacles.

Kaolinite is not soluble in aqueous solutions and therefore becomes a suspension when stirred into, e.g., growth medium. Thus, for easier handling, the powder could either be applied as powder or mixed with other clay materials, which can form hydrogels. Mixing kaolinite with other substances may also overcome a second hurdle. Although human GF and PDLF

appear to be far more robust than L-929, they still only tolerate kaolinite at concentrations below 0.370 mg/mL when suspended in medium. Also, no pronounced impact on cell morphology was found at non-toxic concentrations. Thus, diluting kaolinite with other clay minerals may be a practical approach. L-929 cells are derived from murine subcutaneous areolar and adipose tissue. They are used as a standard cell line for cytotoxicity testing according to ISO 10993-5:2009 and were thus also utilized in this study. The dilutions were chosen based on previous studies with TiO₂ powder [42].

We have studied other clay materials as scaffolds in regenerative dentistry, and laponite® seems to be a promising candidate, as it readily forms injectable hydrogels, making it easy to handle, especially when the accessibility of the application site is limited. Thus, combining the favorable properties of kaolinite with those of laponite® may be an auspicious strategy and is worth looking into as a next step [43].

In order to exclude the possibility that kaolinite releases factors into the cell culture medium or that components of the culture medium bind to certain structures of kaolinite and thereby influencing the impact of kaolinite on L-929 cells, human GF, and PDLF, conditioned medium was prepared by incubating medium with kaolinite for 24 h at 37 °C, 5% CO₂, and 95% atmospheric moisture and then filtrated the medium under sterile conditions. As seen in the results, the conditioned medium does not show any dominant effect on the three cell types when compared to the untreated control. Thus, the effect shown can be led back to kaolinite as a substance and not any reactions with the culture medium.

Furthermore, analysis of total protein content of the conditioned medium was performed and compared to untreated medium. This was based on a previous study [42]. The results showed no pronounced difference in protein content between the conditioned medium and the untreated culture medium. Thus, the proteins in the medium were not substantially depleted by the kaolinite.

In tissue regeneration, new vessel formation is essential. Among others, markers representing the potential of vessel formation are VEGF and IL-6 [44]. In L-929 cells, VEGF levels in the supernatant did not reach the level of significance when treated with kaolinite suspension or the conditioned medium compared to untreated cells. In human GF and PDLF cells, VEGF production is slightly decreased in cells treated with the kaolinite suspension and unchanged when treated with conditioned medium. IL-6 remains unchanged in human GF and PDLF when incubated with kaolinite but slightly decreased in L-929 cells. Interestingly, an increase of IL-6 was observed in GF when treated with the conditioned medium. IL-6 is not only pro-angiogenic but also is involved in the creation of a pro-inflammatory microenvironment, as is IL-8. IL-8 was not modulated by kaolinite or the conditioned medium. Increased IL-6 levels and a decreasing trend in IL-

8 levels were also found in human GF and PDLF when exposed to TiO₂ powder [42].

Cytotoxicity testing of substances on L-929 cells and human cells frequently show a higher viability impairment in L-929 cells [45]. The reason for L-929 cells reacting differently compared to human PDLF and GF concerning viability as well as IL-6, IL-8, and VEGF production cannot be fully explained. The fact that the cells are obtained from different organisms could have an influence. Furthermore, PDLF and GF are derived by explant culture whereas L-929 are cells from a cell line.

In previous studies, it was shown that hypoxia mimetic agents increase the expression of pro-angiogenic markers in human oral tissue cells. Furthermore, laponite® can act as a carrier for hypoxia mimetic agents as it can bind these agents and release them in a time-dependent manner [43]. Thus, it would be of great value to test whether a combination of laponite® with kaolinite or kaolinite by itself is feasible in terms of a function as a carrier for these hypoxia mimetic agents and other growth factors. Therefore, the release kinetics of various factors should be assessed and thereby evaluate kaolinite further as a scaffold material.

Owing to the fact that studies on the application in nanocomposites and mouth rinses have already shown kaolinite to be a potential dental material based on its promising applications [28–30], it is of high importance to assure the first steps in biocompatibility testing.

A limitation of our study is that kaolinite samples of one source were used. It is possible that when using other sources, impurities of the kaolinite and heavy metals in the kaolinite may have an additional impact. Thus, it is important to validate the material before clinical application.

Conclusion

In conclusion, cell viability was decreased by kaolinite dose-dependently. GF and PDLF were more resistant to the impact of kaolinite than L-929. The kaolinite conditioned medium had no toxic effect. VEGF production was slightly decreased in human GF and PDLF.

Overall, this study provides first insights into the impact of kaolinite on cells of the line L-929 and on human GF and PDLF. Kaolinite represents a potential carrier for pro-regenerative factors in regenerative dentistry. These results indicate that kaolinite might require modifications to be applied successfully in regenerative dentistry.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The protocol for tooth preparation and cell isolation was accepted by the ethics committee of the Medical University of Vienna (631/2007 and 1065/2013).

Informed consent Informed consent was obtained from all individual participants included in the study.

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