RESEARCH ARTICLE

The response of gingiva monolayer, spheroid, and ex vivo tissue cultures to collagen membranes and bone substitute

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Abstract

Collagen membranes and bone substitute are popular biomaterials in guided tissue regeneration for treatment of traumatized or diseased periodontal tissue. Development of these biomaterials starts in monolayer cell culture, failing to reflect in vivo tissue organization. Spheroid cultures potentially mimic in vivo tissues in structure and functionality. This study aims to compare gingiva cell (GC) monolayers and spheroids to ex vivo gingiva. Human GC monolayers, spheroids and gingiva ex vivo tissues were cultured on plastic surfaces, collagen membranes or bone substitute. Hematoxylin–eosin (HE) staining, immunohistochemistry for KI67 and caspase 3 (CASP3), resazurin-based toxicity assays, quantitative polymerase chain reaction for collagen I (*COL1A1*), vascular endothelial growth factor (*VEGF*), angiogenin (*ANG*), interleukin (*IL*)*6* and *IL8* and ELISA for COL1A1, VEGF, ANG, IL6 and IL8 were performed in all cultures. Morphology was different in all culture set-ups. Staining of KI67 was positive in monolayers and staining of CASP3 was positive in spheroids. All culture set-ups were viable. COL1A1 production was modulated in monolayers and ex vivo tissues at mRNA levels, VEGF in monolayers and ex vivo tissues at mRNA levels and in spheroids at protein levels, ANG in spheroids at mRNA levels and in monolayers and spheroids at protein levels, IL6 in monolayers and spheroids at mRNA levels and in spheroids and ex vivo tissues at protein levels and IL8 in monolayers and ex vivo tissues at mRNA levels. Modulations were surface-dependent. In conclusion, each culture model is structurally and functionally different. Neither GC monolayers nor spheroids mimicked gingiva ex vivo tissue in all measured aspects.

KEYWORDS
biocompatible materials, cell culture techniques, dentistry, gingiva, in vitro techniques, regeneration

1 | INTRODUCTION

Disease and trauma can lead to damage and loss of periodontal tissue. In order to overcome resulting physiological and psychological strain,

regenerative approaches have to provide functional and esthetic healing (Abou Neel, Chrzanowski, Salih, Kim, & Knowles, 2014). Tissue engineering strategies in dentistry are based on three main components, being cells (Tomokiyo, Wada, & Maeda, 2019), scaffolds (Talebi

Ardakani, Hajizadeh, & Yadegari, 2018) and signaling molecules (Kaigler, Cirelli, & Giannobile, 2006). Biomaterials are frequently used as scaffolds in approaches for guided tissue regeneration and guided bone regeneration.

In dentistry, collagen barrier membranes are widely applied biomaterials for guided tissue regeneration, as for example in bone augmentations before implant placement (Li, Chen, Zhu, & Zha, 2012). Currently, a wide range of differently manufactured barrier membranes is available for clinical applications and experimental studies. The ideal barrier membrane, which would have to provide systemic and tissue-specific compatibility, a stable barrier between periodontal soft tissue and bone, easy handling and facilitation of regenerative processes, has not been developed yet (Rakhmatia, Ayukawa, Furuhashi, & Koyano, 2013). During sinus augmentation, a barrier membrane should be placed between soft and hard tissues, such that periodontal soft tissue and bone regeneration can occur without disturbances. A limitation of such barrier membranes is that they are not always mechanically stable enough to reliably maintain the required space between soft tissue and bone (Caballé-Serrano et al., 2018). To study what consequences for the regenerative process it would have if a barrier membrane mechanically breaks down and periodontal soft tissue and bone tissue get in contact, an ideal in vitro model must imply the interactions of all components: soft and hard tissues.

Although the traditional monolayer cell culture has provided fundamental knowledge in basic science for large part, it fails to mimic complex processes of the in vivo situation in a living organism, such as tissue regeneration. Cells in an artificial environment like a petri dish or plastic culture plates look and behave differently in terms of, for example, proliferation and gene expression, compared with in vivo tissues and three-dimensional (3D) cell cultures (Edmondson, Broglie, Adcock, & Yang, 2014). Spheroid cell cultures are a promising and feasible 3D model where cells form a 3D sphere and therefore closer resemble an in vivo microenvironment (Laschke & Menger, 2017). Spheroids made of gingiva cells (GCs) were already evaluated for different fields of regeneration, including basic research (Janjić, Kurzmann, Moritz, & Agis, 2017), research in host-microbial interaction (Bugueno et al., 2018) as well as substance and material testing (Tigani, Skrtic, Valerio, & Kaufman, 2019), (Hsu et al., 2012).

Extensive research has been performed to compare monolayer and spheroid cultures, demonstrating differences in cell signaling (Schyschka et al., 2013), differentiation behavior of stem cells (Baharvand, Hashemi, Kazemi Ashtiani, & Farrokhi, 2006), metabolic activity and general response to cell treatments (Breslin & O'Driscoll, 2016; Sun, Jackson, Haycock, & MacNeil, 2006; Pickl & Ries, 2009). Considering the differences of measured effects, these findings suggest that biological processes in cell culture depend on the culture model. Further, it would be of interest to determine which model is the most suitable to represent a situation in the patient. Although 3D cultures are proposed to be closer to the in vivo situation by adding a third dimension, in fact, we only know that 2D and 3D models differ in substantial aspects of cell morphology and behavior, but we do not know which of these models provides more similarity to in vivo tissue.

As an example for an application where an in vivo-like model would be desirable to solve a research question, we aimed to test our in vitro models on different biomaterials that are used in guided tissue regeneration. During guided tissue regeneration, a collagen barrier membrane is inserted between gingiva and bone. If the barrier membrane is not stable enough and collapses, gingiva gets in contact with bone tissue. To assess the response of soft tissue to such a situation in an in vitro model, we cultured gingiva monolayers, spheroids and ex vivo tissues on collagen membranes as well as bone substitute material.

Therefore, the objective of this study was to compare gingiva monolayers and gingiva spheroids toward the similarities and differences to gingiva ex vivo tissue, in terms of morphology, proliferation and apoptosis, metabolic activity as well as the production of collagen type I (*COL1A1*/COL1A1), vascular endothelial growth factor (*VEGF*/VEGF), angiogenin (*ANG*/ANG), interleukin 6 (*IL6*/IL6) and interleukin 8 (*IL8*/IL8). These components cover key elements of tissue regeneration, including production of extracellular matrix and pro-angiogenic and pro-inflammatory capacities.

2 | MATERIALS AND METHODS

2.1 | Isolation and culture of cells and tissue

Human wisdom teeth were extracted by oral surgeons at the University Clinic of Dentistry, Medical University of Vienna, Vienna, Austria. Inflammatory or diseased teeth were excluded from this study. Gingiva tissue that remained at the tooth neck after extraction was removed from the tooth with a scalpel. Tissue pieces were placed in α-minimal essential medium (Gibco, Invitrogen Corporation, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Linz, Austria) and 1% antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin [Gibco, Invitrogen Corporation]). The heterogeneous cell population that grew out from the tissue pieces was used for experiments as human GC or kept in liquid nitrogen for long-term storage. GC fulfilled the minimal criteria for mesenchymal stromal cells (Oberoi et al., 2018), (Dominici et al., 2006). Tissue pieces for gingiva ex vivo tissue culture were used directly for experiments. Cells and tissues in experiments were cultured at 37°C, 5% CO_2 and 95% humidity and processed under sterile conditions. GC were used until passage 7 for experiments.

The Ethics Committee of the Medical University of Vienna, Vienna, Austria (631/2007) has approved the procedure to isolate and use patient material from the gingiva upon written informed consent of the donor.

2.2 | Gingiva monolayer culture

Gingiva monolayer culture was performed to represent the traditional in vitro 2D cell culture.

A cell suspension of 50,000 human GC in α-minimal essential medium with FCS and antibiotics was seeded onto 1 cm^2 of a collagen

membrane (Geistlich Bio-Gide®; Geistlich Pharma AG, Wolhusen, Switzerland) or bone substitute (Geistlich Bio-Oss®; Geistlich Pharma AG) covering 1 cm² of plastic. The same amount of GC was seeded onto the plastic surface of a cell culture plate as control. GC monolayers were cultured for 24 h on each surface.

GC from 6 different donors were used for histological and immunohistochemical analysis (n = 6), and GC from 6 different donors were used for metabolic activity, mRNA level and protein analysis (n = 6).

2.3 | Gingiva spheroid culture

Gingiva spheroid culture was performed to represent an in vivo-like 3D cell culture.

GC spheroids were produced using 3D Petri Dishes® (Microtissues, Inc., Providence, RI, USA), following the instructions of the manufacturer (Janjić, Lilaj, Moritz, & Agis, 2018). Three GC spheroids with a total cell number of 50,000 and an average diameter of 356 μm/spheroid were seeded onto 1 cm² of a collagen membrane (Geistlich Bio-Gide®; Geistlich Pharma AG) or bone substitute (Geistlich Bio-Oss®; Geistlich Pharma AG) covering 1 cm² of plastic. Three GC spheroids with the same amount of cells were seeded onto the plastic surface of a cell culture plate as control. The three GC spheroids together approximately equaled the total diameter of one piece of gingiva ex vivo tissue. GC spheroids were cultured for 24 h on each surface.

GC from 6 donors were used for histological and immunohistochemical analysis (n = 6) as well as metabolic activity, mRNA level and protein analysis (n = 6).

2.4 | Gingiva ex vivo tissue culture

Gingiva tissue culture was performed to represent an ex vivo situation.

Gingiva tissue from extracted teeth was cut into pieces of approximately 1 mm³ diameter to match the diameter of three GC spheroids. Tissue pieces were seeded onto 1 cm² of a collagen membrane (Geistlich Bio-Gide®; Geistlich Pharma AG) or bone substitute (Geistlich Bio-Oss®; Geistlich Pharma AG) covering 1 cm² of plastic. Gingiva ex vivo tissues were cultured for 24 h on each surface.

Tissue pieces of gingiva from four different donors were used for histological and immunohistochemical analysis (n = 4), and tissue pieces of gingiva from six different donors were used for metabolic activity, mRNA level and protein analysis (n = 6).

2.5 | Morphology

GC, GC spheroids and gingiva ex vivo tissues were stained with hematoxylin–eosin to visualize the morphology of the cells and tissues.

Fixation with phosphate-buffered 4% formaldehyde was performed for 24 h after seeding onto collagen membranes, bone substitute or plastic. After several rinses with distilled water, staining with Mayer's hematoxylin was performed for 5 min, followed by rinsing with distilled water and addition of 0.1% HClOH. Afterwards, samples were rinsed several times with tap water and stained with 0.5% Eosin G for 3 min with subsequent rinsing with tap water and a stepwise dehydration with 90% EtOH for 3 min and 100% EtOH for 5 min, followed by two repetitions of xylene for 5 min each. Formaldehyde-fixed GC monolayers were directly stained in the cell culture plates. GC spheroids and gingiva ex vivo tissues were placed into a small block of HistoGel™ (Thermo Fisher Scientific, Waltham, MA, USA) before dehydration. GC spheroids and gingiva ex vivo tissues were permanently embedded in paraffin and finally cut into 4 μm thick sections. Images were taken at 200-fold magnification under a light microscope.

2.6 | Proliferation and apoptosis

Immunohistochemical staining for KI67 and caspase 3 (CASP3) was performed in GC monolayers, spheroids and gingiva ex vivo tissues to visualize proliferating and apoptotic cells, respectively.

Sections obtained from samples prepared for histology were deparaffinized. For KI67 and CASP3 staining, sections were steamed with EDTA at pH 9, followed by a wash with TBS. Subsequent blocking was performed with BloxAll (Vector, Burlingame, CA, USA). Afterward, sections were washed with TBS and incubated for 1 h with primary antibodies for KI67 (RM-9106-S1, rabbit monoclonal SP6, 1:200; Thermo Fisher Scientific) or CASP3 (9,661, rabbit polyclonal, 1:100; Cell Signaling, Danvers, MA, USA). Afterward, sections were washed with TBS, incubated with secondary antibodies (VWRKDPVR110HRP, antirabbit; Immunologic, Amsterdam, Netherlands) and washed again with TBS. For detection, NovaRED peroxidase substrate (SK-4805; Vector, Burlingame, CA, USA) was used. The staining process was performed directly in cell culture plates for GC monolayers and automatically by a Lab Vision Autostainer 360 (Thermo Fisher Scientific) for sections of GC monolayers and gingiva ex vivo tissues. Sections without incubation with primary antibodies served as negative controls. Images were taken in 200-fold magnification under a light microscope.

2.7 | Metabolic activity analysis

A resazurin-based toxicity assay (TOX8; Sigma-Aldrich GmbH, Vienna, Austria) was performed to measure metabolic activity of GC monolayers, GC spheroids and gingiva ex vivo tissue cultures.

The assay was conducted 24 h after seeding onto collagen membranes, bone substitute or plastic, following the instructions of the manufacturer. Resazurin-based solution was incubated for 8 h, and resorufin conversion was analyzed in a photometric fluorescence measurement at 540/34 nm excitation and 600/40 nm emission wavelength. Cell culture medium only on collagen membranes, bone substitute or plastic was used as blank and subtracted from results. Results were further normalized to the respective plastic control with GC or tissue.

2.8 | Relative mRNA expression analysis

RNA was isolated from GC monolayers, GC spheroids and gingiva ex vivo tissue 24-hr after seeding onto biomaterials or plastic using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Control samples without cells were included. RNA quality was verified photometrically by the 260/280 ratio.

Undiluted RNA was transformed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol.

Quantitative polymerase chain reaction (qPCR) was performed to analyze changes in *COL1A1* (Hs00164004_m1; Thermo Fisher Scientific), *VEGF* (Hs00900055_m1; Thermo Fisher Scientific), *ANG* (Hs04195574_sH; Thermo Fisher Scientific), *IL6* (Hs00985639_m1; Thermo Fisher Scientific) and *IL8* (Hs00174103_m1; Thermo Fisher Scientific) at mRNA levels. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs02758991_g1; Thermo Fisher Scientific) was used as reference gene. Results of qPCR were evaluated by the $\Delta\Delta$CT method and normalized to the respective plastic control.

2.9 | Proteinlevel analysis

ELISA was performed to analyze changes in COL1A1 (R&D Systems, Inc., Minneapolis, MN, USA), VEGF (Peprotech, Vienna, Austria), ANG (Peprotech), IL6 (Peprotech) and IL8 (Peprotech) at protein levels. For this, GC monolayers, GC spheroids and gingiva ex vivo tissue culture supernatant was collected 24 h after seeding onto collagen membranes, bone substitute or plastic. Control samples with cell culture medium on plastic, collagen membranes or bone substitute, but without cells, were also included. ELISA was performed following the protocol of the manufacturer. Results were evaluated using a standard curve as recommended by the manufacturer and normalized to the respective plastic control, produced separately for collagen membrane and bone substitute samples.

2.10 | Statistics

Differences between groups were tested for significance with the Kruskal-Wallis-test, followed by the Mann–Whitney test for pairwise comparison using a statistical software (IBM SPSS Statistics Version 24; IBM, Armonk, NY, USA). The level of significance was set at $p < 0.05$. Data are displayed as mean + standard deviation.

3 | RESULTS

3.1 | Morphology

GC monolayers showed even distribution and elongated nuclei in HE staining (Figure 1a). On collagen membranes, a monolayer of GC was visible on top of the smooth side of the membrane (Figure 1a).

GC spheroids showed oval-shaped nuclei in close contact to each other and even distribution in HE staining (Figure 1b). On collagen membranes, GC cell spheroids appeared on top of the smooth side of the collagen membrane, but without forming any visible connection to it (Figure 1b).

Gingiva ex vivo tissues on plastic and collagen membranes contained cells, fibers and small vessels in HE staining (Figure 1c).

GC monolayers, spheroids and gingiva ex vivo tissues on bone substitute could not be visualized in HE staining.
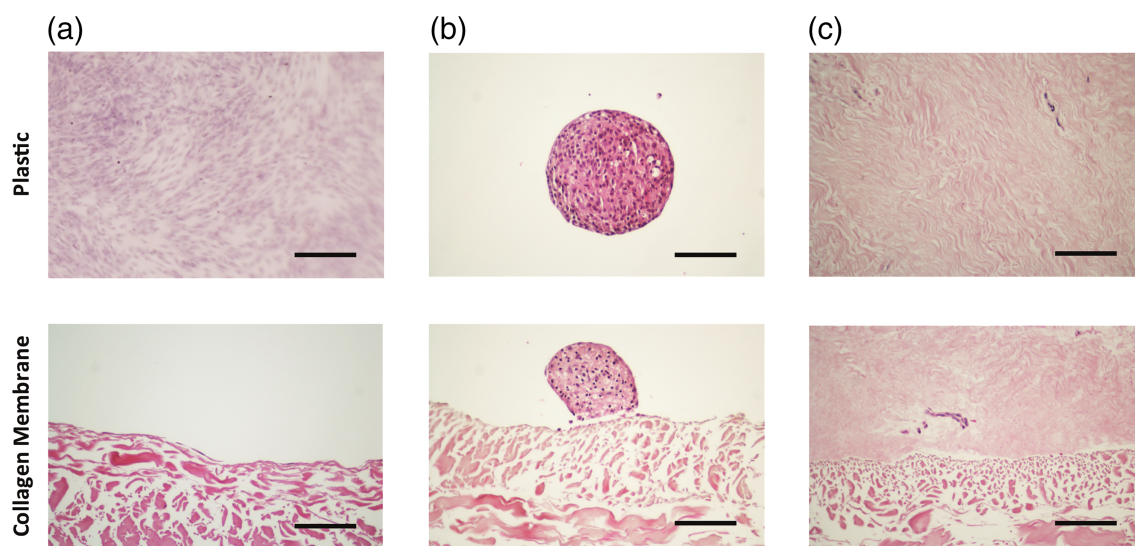

FIGURE 1 Morphology of gingiva monolayers, spheroids and ex vivo tissue cultures. (a) Hematoxylin–eosin staining was performed on gingiva cell monolayers, (b) gingiva cell spheroids, (c) and gingiva ex vivo tissue cultures on plastic or collagen membranes to visualize morphology. The length of the black bars equals 100 μm [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Proliferation and apoptosis

GC monolayers (Figure 2a) were positive, GC spheroids (Figure 2b) and gingiva ex vivo tissues (Figure 2c) were negative for KI67. GC spheroids were positive for CASP3 (Figure 2b), whereas GC monolayers (Figure 1a) and gingiva ex vivo tissues were negative for CASP3 (Figure 2c).

3.3 | Metabolic activity

Metabolic activity of GC monolayers on collagen membranes was similar to the metabolic activity of the GC monolayers on the corresponding plastic control (Figure 3a). Metabolic activity in GC monolayers on bone substitute was significantly reduced compared with the GC monolayer plastic control (Figure 3b).

No significant change in metabolic activity was observed in GC spheroids on collagen membranes or bone substitute compared with GC spheroids on the plastic control (Figure 3a,b).

Metabolic activity of gingiva ex vivo tissues showed a trend for decrease on collagen membranes and a trend for increase on bone substitute compared with gingiva ex vivo tissues on the plastic control, but the trends did not reach the level of significance (Figure 3a,b).

3.4 | Relative mRNA expression

In GC monolayers on collagen membranes, *COL1A1* production significantly decreased (Figure 4a) and *IL8* expression significantly increased (Figure 4e) compared with the plastic control, whereas *VEGF* (Figure 4b), *ANG* (Figure 4c) and *IL6* (Figure 4d) expression was not significantly altered. In GC monolayers on bone substitute, *COL1A1*

expression significantly decreased (Figure 4f) and *VEGF* (Figure 4g), *IL6* (Figure 4i) and *IL8* (Figure 4j) expression significantly increased compared with the plastic control, whereas *ANG* (Figure 4h) expression was not significantly altered.

In GC spheroids on collagen membranes, *ANG* expression significantly decreased (Figure 4c) compared with the plastic control, whereas *COL1A1* (Figure 4a), *VEGF* (Figure 4b), *IL6* (Figure 4d) and *IL8* (Figure 4e) expression was not significantly modulated. In GC spheroids on bone substitute, *IL6* expression was significantly decreased (Figure 4i) compared with the plastic control, whereas *COL1A1* (Figure 4f), *VEGF* (Figure 4g), *ANG* (Figure 4h) and *IL8* (Figure 4j) expression was not significantly altered.

In gingiva ex vivo tissues on collagen membranes, *IL8* expression significantly decreased (Figure 4e) compared with the plastic control, whereas *COL1A1* (Figure 4a), *VEGF* (Figure 4b), *ANG* (Figure 4c) and *IL6* (Figure 4d) expression was not significantly altered. In gingiva ex vivo tissues on bone substitute, *COL1A1* (Figure 4f), *VEGF* (Figure 4g) and *IL8* (Figure 4j) expression significantly decreased compared with the plastic control, whereas *ANG* (Figure 4h) expression was not altered.

3.5 | Protein levels

In GC monolayers on plastic, the following protein concentrations were measured: 131,249 ± 413 pg/ml COL1A1, 0.151 ± 0.016 ng/ml VEGF, 64.909 ± 5.761 pg/ml ANG, 2,300.585 ± 69.806 pg/ml IL6 and 22.750 ± 3.042 pg/ml IL8. On collagen membranes (Figure 5a) and bone substitute (Figure 6a), production of ANG significantly decreased compared with the plastic control, whereas production of COL1A1, VEGF, IL6 and IL8 was not significantly modulated.
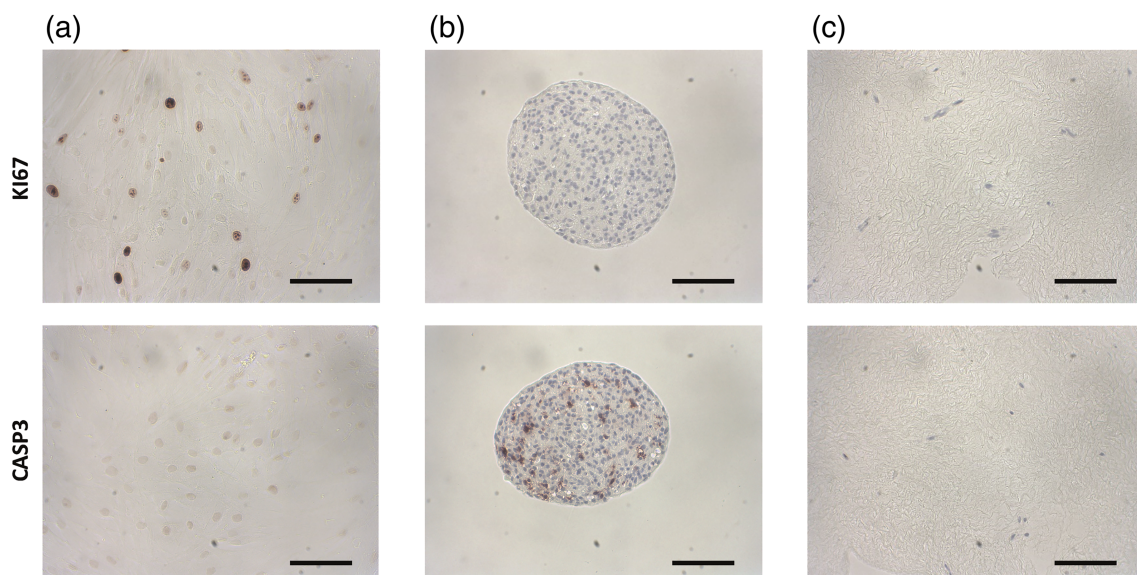

FIGURE 2 Proliferation and apoptosis in gingiva spheroids and ex vivo tissue cultures. Immunohistochemical staining of KI67 and Caspase 3 (CASP3) was performed on (a) gingiva cell monolayers, (b) gingiva cell spheroids and (c) gingiva ex vivo tissue cultures to visualize proliferation (KI67) and apoptosis (CASP3). The length of the black bars equals 100 μm [Colour figure can be viewed at wileyonlinelibrary.com]
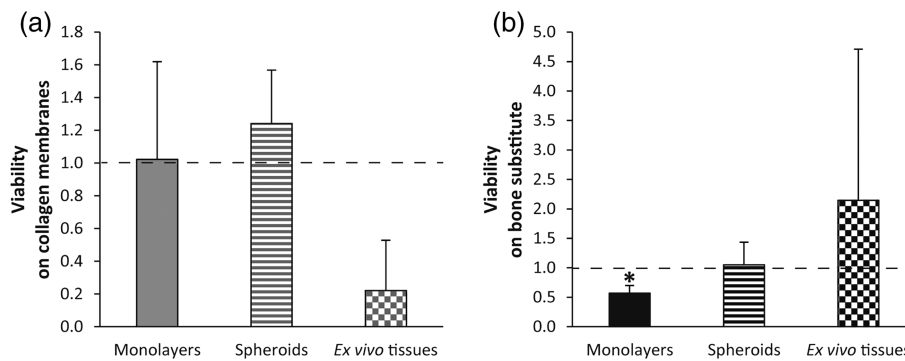
FIGURE 3 Metabolic activity of gingiva monolayers, spheroids and ex vivo tissue cultures. Viability of gingiva cell monolayers, gingiva cell spheroids and gingiva ex vivo tissue cultures on (a) collagen membranes or (b) bone substitute was determined by measuring the metabolic activity of resazurin conversion. Fluorescence intensity at 540/34 nm excitation and 600/40 nm emission is given on the y-axis. Data are displayed as mean + SD, relative to the respective controls on plastic (dashed line). Significance of $p < 0.05$ is indicated with *. $n = 6$

In GC spheroids on plastic, the following protein concentrations were measured: 1,026 ± 94 pg/ml COL1A1, 0.948 ± 0.160 ng/ml VEGF, 169.007 ± 8.684 pg/ml ANG, 3,821.612 ± 1198.239 pg/ml IL6 and 832.744 ± 12.107 pg/ml IL8. On collagen membranes (Figure 5b), production of VEGF significantly increased and production of ANG significantly decreased compared with the plastic control, whereas production of COL1A1, IL6 and IL8 was not significantly modulated. On bone substitute (Figure 6b), production of ANG and IL6 significantly decreased compared with the plastic control, whereas production of COL1A1, VEGF and IL8 was not significantly modulated.

In gingiva ex vivo tissues on plastic, the following protein concentrations were measured: 380 ± 81 pg/ml COL1A1, 54.882 ± 28.400 ng/ml VEGF, 2,301.754 ± 36.699 pg/ml ANG, 5,512.046 ± 863.179 pg/ml IL6 and 4,795.266 ± 1487.073 pg/ml IL8. On collagen membranes (Figure 5c), production of IL6 significantly decreased compared with the plastic control, whereas production of COL1A1, VEGF, ANG and IL8 was not significantly modulated. On bone substitute (Figure 6c), none of the measured proteins were significantly modulated compared with the plastic control.

4 | DISCUSSION

Development of cell culture systems, such as 3D cell culture models, has advanced in the past years, now offering a wide range of approaches to study various aspects of mammalian tissues in a more in vivo-like environment (Shamir & Ewald, 2014). Monolayer cultures have been the golden standard for in vitro cell culture for decades, providing a myriad of reference literature and established methods. Meanwhile, 2D and 3D cell cultures have been compared in colon cancer cells (Riedl et al., 2017), (Imamura et al., 2015), hepatocytes (Schyschka et al., 2013), embryonic stem cells (Baharvand et al., 2006), skin cells (Sun et al., 2006) and others, demonstrating that 2D and 3D cell cultures deliver different results. Finally, which one is recommendable? To answer this question, cell culture systems have to be compared with the in vivo situation to assess which one matches best with the situation inside the living body. The in vivo

equivalent for this study would be the human gingiva, which cannot be used in situ for ethical reasons. Animal models such as mice are popular in in vivo research because of their genetic similarity to humans. In the field of dentistry, however, animals do not entirely reflect the anatomy and physiology of human denture, but rather bear species-specific differences that make them not comparable with the human situation. For this reason, this study compared GC monolayer and spheroid cultures to gingiva ex vivo tissues, which we considered the most adequate and feasible model to represent the in vivo structure and behavior of human gingiva.

The human gingiva is formed by layers of oral gingival epithelium, junctional epithelium, sulcular epithelium and gingival connective tissue. GC as well as the gingiva ex vivo tissues used in this study were harvested from the gingival connective tissue of human donors. This is the part of gingiva that gets into touch with a collagen membrane during guided tissue regeneration or would have contact with bone material in the case when a collagen membrane collapses. Gingival connective tissue typically consists of a collagen fiber network, produced by GC. HE staining of gingiva ex vivo tissues on plastic and collagen membranes confirmed a fibrous network (Figure 1c), showing mainly eosinophilic fibers and small vessels. Compared with that, GC spheroids (Figure 1b) formed an eosinophilic network, whereas GC monolayers (Figure 1a) remained more separate. COL1A1 is the main component of the extracellular matrix produced by gingival fibroblasts. The mRNA data of this study revealed that *COL1A1* production in GC monolayers on collagen membranes (Figure 4a) and bone substitute (Figure 4f), compared with plastic controls, resembled the *COL1A1* production in gingiva ex vivo tissues, whereas in GC spheroids, an opposite, but not significant, trend was observed. At protein levels (Figures 5 and 6), the highest concentrations of COL1A1 were found in GC monolayers, independent of the surface. Visualization on bone substitute was not possible as the material took on such a dark color during the HE staining that GC and ex vivo tissues were not visible anymore. Further experiments will be required to find out how to overcome this technical hurdle. Taken together, from a morphological point of view, our results suggest that GC spheroids better mimic the three-dimensionality of the gingiva ex vivo tissue, but GC in

FIGURE 4 mRNA production in gingiva monolayers, spheroids and ex vivo tissue cultures on collagen membranes or bone substitute. α-1 type I collagen (*COL1A1*; A, F), vascular endothelial growth factor (*VEGF*; B, G), angiogenin (*ANG*; C, H), interleukin (*IL*)6 (D, I) and *IL8* (E, J) relative mRNA expression was measured in gingiva cell monolayers, gingiva cell spheroids and gingiva ex vivo tissue cultures on (a–e) collagen membranes or (f–j) bone substitute with quantitative polymerase chain reaction, relative to the reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and respective controls on plastic (dashed line). Data are displayed as mean + SD, significance of $p < 0.05$ is indicated with *. n = 6
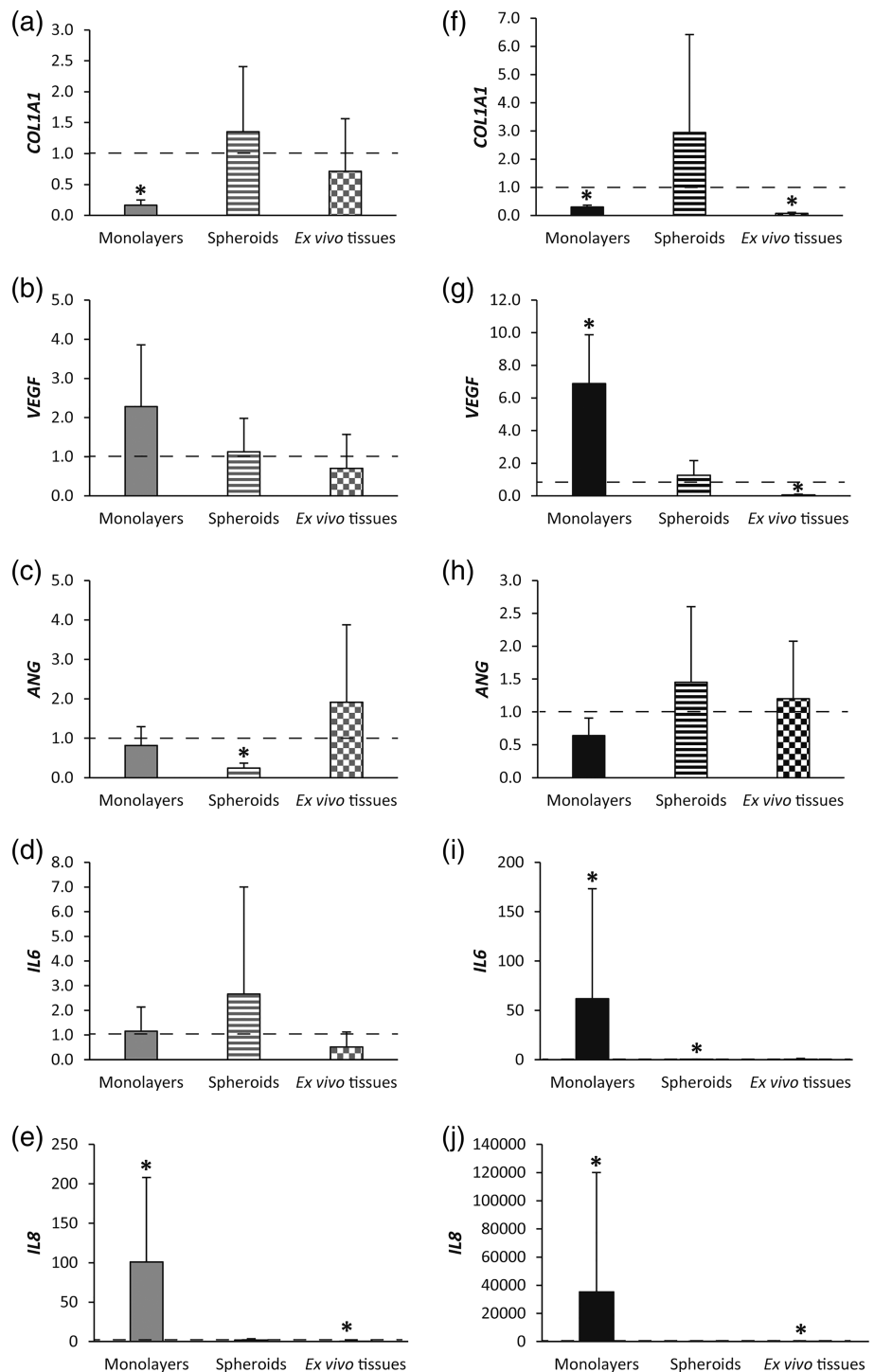
monolayers closer represent mRNA expression of extracellular matrix component *COL1A1* than GC spheroids.

Spheroids form a tight 3D structure with multiple cell layers compared with 2D monolayer cell culture. Previously, it was reported that spheroid cores of different cell types are hypoxic and apoptotic because oxygen and nutrient diffusion is limited (Mittler et al., 2017). In contrast to current literature, we found apoptotic cells spread over the whole cross-section of GC spheroids, thus not accumulating in a certain layer (Figure 2b, lower panel), whereas proliferative cells were only found in GC monolayers (Figure 2b, upper panel). The extent of

positive staining for CASP3 differed between donors. The donor displaying the strongest CASP3 staining is included in Figure 2b. This does not resemble the situation in the gingiva ex vivo tissue (Figure 2c). Thus, when using GC spheroids as cell culture model, it has to be considered that apoptotic conditions prevail inside the spheroids, which could have a potential influence on their behavior (Cheng et al., 2015). Besides CASP3 as an indicator for apoptosis, KI67 staining was only positive in GC monolayers, suggesting proliferation activity in this cell model, but not in GC spheroids or gingiva ex vivo tissues. To circumvent the formation of apoptotic areas,
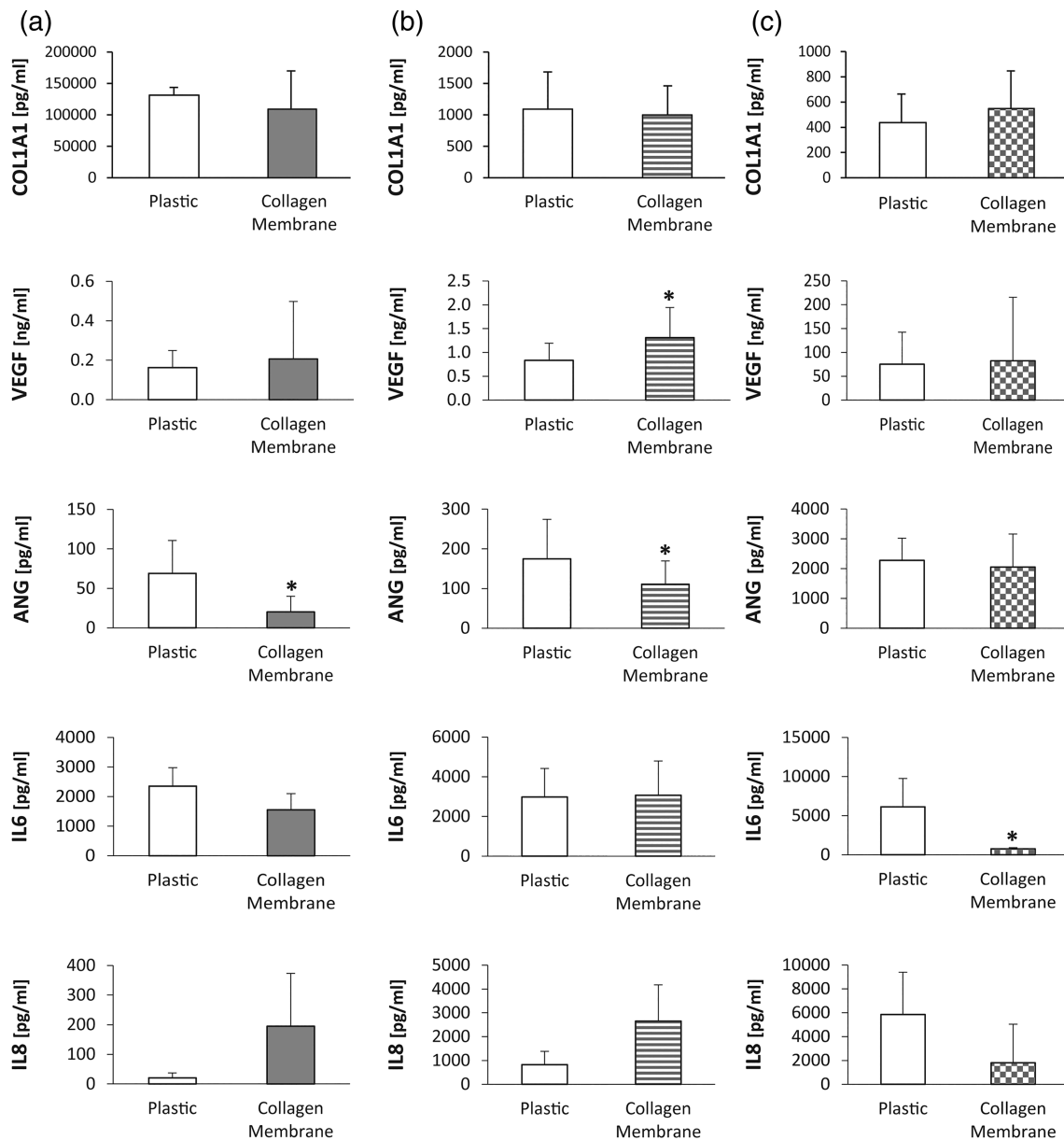
FIGURE 5 Protein production in gingiva monolayers, spheroids and ex vivo tissue cultures on collagen membranes. Protein production of α-1 type I collagen (COL1A1), vascular endothelial growth factor (VEGF), angiogenin (ANG), interleukin (IL)6 and IL8 was measured in (a) gingiva cell monolayers, (b) gingiva cell spheroids (c) and gingiva ex vivo tissue cultures on collagen membranes with ELISA (gray bars) and a respective plastic control (white bars). Data are displayed as mean + SD, significance of $p < 0.05$ is indicated with *. n = 6

spheroids can be formed using a smaller cell number, resulting in smaller spheroids with smaller diffusion distances (Mittler et al., 2017). For a more biological approach, future experiments will be based on approaches aiming for vascularization of spheroids as it was attempted by forming spheroid co-cultures with endothelial cells (Shoval et al., 2017) or using microtissue shapes that permit more diffusion like toroids (Manning, Thomson, & Morgan, 2018).

Although CASP3 production, as indicator for apoptosis, was detected in GC spheroids, all three culture models were metabolically active, demonstrating that GC in monolayers, spheroids and gingiva ex vivo tissues are vital on collagen membranes, bone substitute and the plastic controls (Figure 3). Interestingly, GC monolayers on bone

substitute had significantly lower metabolic activity compared with the plastic control (Figure 3b). It was previously reported that Bio-Oss® (Geistlich Pharma AG) does not support strong cell attachment compared with other surfaces (Tseng, Ho, Liang, Hsieh, & Jan, 2013). Because attachment is essential to viability of adherent cells as gingival fibroblasts, a possible explanation for the significant reduction might be that the bone substitute did not provide sufficient attachment capacity while GC spheroids and gingiva ex vivo tissues provide enough attachment possibilities through their three-dimensionality. This hypothesis has to be evaluated in future experiments. A study on collagen membranes already showed that attachment behavior varies between different GC cultures (Janjić, Cvikl, Schädl, Moritz, & Agis, 2019).
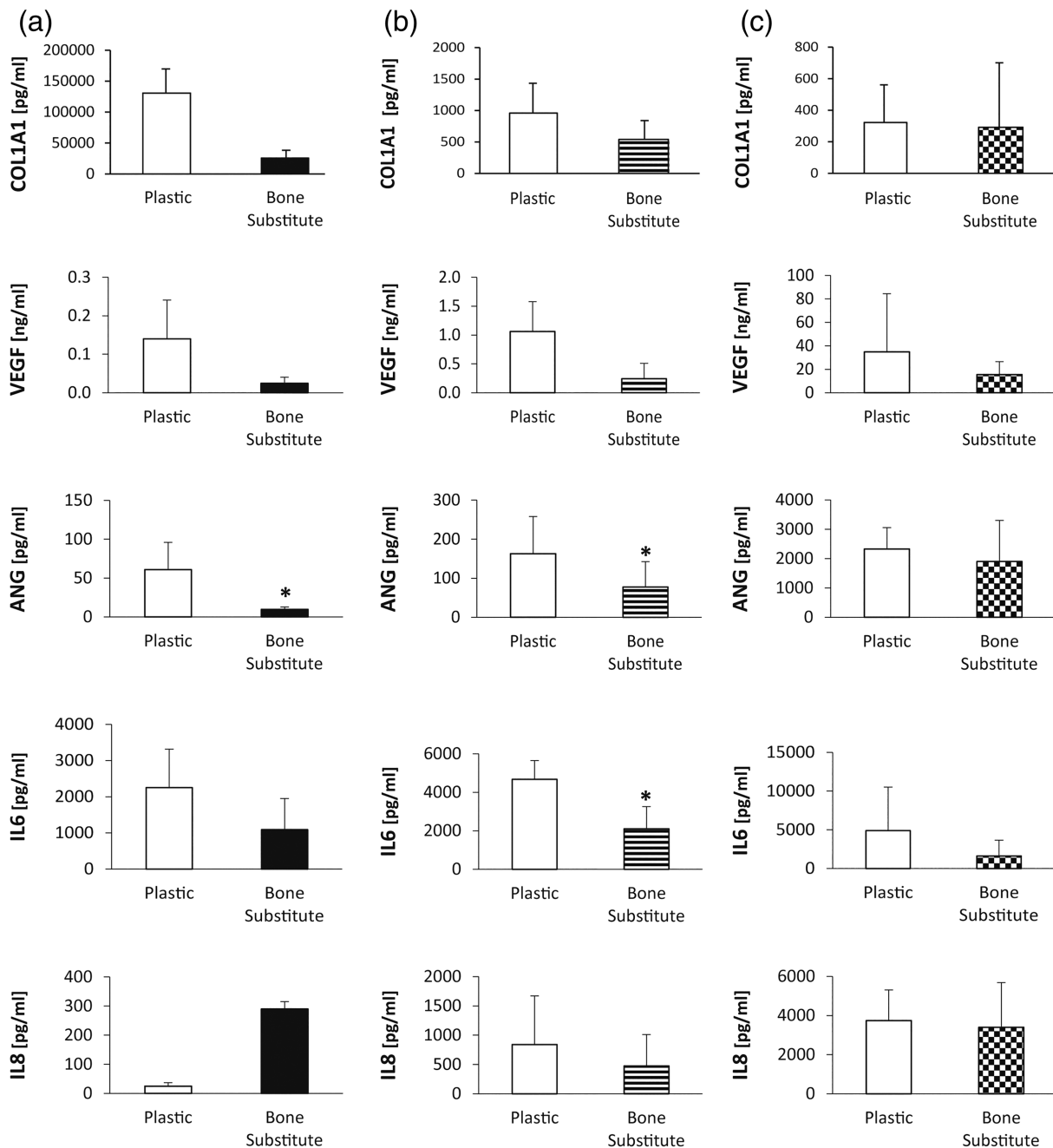
FIGURE 6 Protein production in gingiva monolayers, spheroids and ex vivo tissue cultures on bone substitute. Protein production of α-1 type I collagen (COL1A1), vascular endothelial growth factor (VEGF), angiogenin (ANG), interleukin (IL)6 and IL8 was measured in gingiva cell (a) monolayers, (b) gingiva cell spheroids and (c) gingiva ex vivo tissue cultures on collagen membranes with ELISA (gray bars) and a respective plastic control (white bars). Data are displayed as mean + SD, significance of $p < 0.05$ is indicated with *. $n = 6$

Gene expression of markers representative for regeneration is another important criteria for a suitable cell culture system for research in tissue engineering. VEGF and ANG are pro-angiogenic molecules which have already been extensively researched in tissue engineering (Yamada et al., 2006), (Janjić et al., 2019). In our study, *VEGF* and *ANG* mRNA production in response to culture on collagen membranes or bone substitute (Figure 4b–h) was not comparable between different cell culture models. For *VEGF*, even a significant opposite effect was observed between GC monolayers and gingiva

ex vivo tissues on bone substitute (Figure 4g). In contrast, at protein levels, ANG production decreased in GC monolayers and spheroids on collagen membranes or bone substitute and gingiva ex vivo tissue showed a similar trend (Figures 5 and 6). For VEGF production, at least similar trends could be observed between the different culture models, whereas trends were opposite in all cultures, depending if cultured on collagen membranes or bone substitute (Figures 5 and 6). The inflammatory molecules IL6 and IL8 did not show any comparable effect between the different culture models on collagen membranes

or bone substitute. In the raw data, high variances in reaction intensities between different donors was observed, leading to high standard deviations. This suggests that cell donors have an impact on the production behavior of inflammatory molecules. Overall, the most significant results were found in GC monolayer cultures, which also seem to have a higher intensity of measured effects than in GC spheroid cultures and even more than in gingiva ex vivo tissues.

This study compared different gingiva in vitro models in the context of periodontal guided tissue regeneration, assessing the response of GC and gingiva tissue to collagen membranes and bone substitute. Soft tissue regeneration of damaged or diseased oral tissue is of importance in order to seal wounds and provide a barrier against external influences. Whole tissue regeneration in dentistry also includes healing of bone, which was not assessed in the current study. Based on our data, a next step should include comparison of different in vitro models for periodontal ligament and jawbone in osteogenic response to different biomaterials. Finally, communication between oral soft and hard tissue models in co-culture would represent the processes of in vivo oral regeneration even closer. For this, conditioned media of oral soft and hard tissue cells could be exchanged to study responses to each other (Asparuhova, Caballé-Serrano, Buser, & Chappuis, 2018), (Nagata et al., 2017). More complex approaches as direct coculture (Sinclair & Burg, 2011) or indirect coculture using inserts (Chen et al., 2015) would provide even more realistic insights into communication between oral soft and hard tissue cells during regeneration.

In conclusion, the results of this study show that different in vitro models also produce different data in many aspects. Thus, to gain scientifically and clinically significant data, the most suitable in vitro model has to be selected, including the right cell source, considering which tissue of the patient interacts with a therapeutic application, the right combination and number of cell types, being able to reproduce the in vivo morphology and the characteristic components of a tissue and the ability to be functionally similar to an in vivo tissue in terms of, for example, metabolic activity, attachment or gene expression. Our results demonstrate that there is still open potential to optimize and develop an ideal in vitro model for research in the field of periodontal regeneration, mimicking the patient situation.

CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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