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Formation of human periodontal ligament cell spheroids on chitosan films

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59855R1
Full Title:	Formation of human periodontal ligament cell spheroids on chitosan films
Keywords:	Spheroids; Three-dimensional; Cell Culture; Periodontal ligament cells; Chitosan films; Periodontal regeneration
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Dear Dr. Stephanie Weldon,

On behalf of my co-authors, I am submitting our original article manuscript entitled "Formation of human periodontal ligament cell spheroids on chitosan films" to *JoVE*.

Periodontal ligament (PDL) cells hold great promise for periodontal tissue regeneration. Conventionally, PDL cells are cultured on two-dimensional (2D) substrates such as tissue culture polystyrene (TCPS). To improve conventional PDL cell culture models, we have recently developed a three-dimensional (3D) cell culture method, which is based on spheroid formation of PDL cells on chitosan films. Here, we present detailed cell spheroid culture protocols based on chitosan films. The 3D culture system of PDL cellular spheroids overcome some of the drawbacks related to conventional 2D monolayer cell culture, and thus may be suitable for producing PDL cells with an enhanced therapeutic efficacy for future periodontal tissue regeneration.

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Yours sincerely,

Xiangzhen Yan
(on behalf of all authors)

TITLE:

Formation of Human Periodontal Ligament Cell Spheroids on Chitosan Films

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KEYWORDS:

Spheroids; Three-dimensional; Cell culture; Periodontal ligament cells; Chitosan films;

Periodontal regeneration

SUMMARY:

Here, we present protocols of culturing human periodontal ligament (PDL) cell spheroids by chitosan films. The culture of three-dimensional (3D) cellular spheroids provides an alternative to conventional tissue culture polystyrene (TCPS) culture system.

ABSTRACT:

Periodontal ligament (PDL) cells hold great promise for periodontal tissue regeneration. Conventionally, PDL cells are cultured on two-dimensional (2D) substrates such as tissue culture polystyrene (TCPS). However, characteristic changes of PDL cells have been observed during in vitro culture. This phenomenon is probably because the 2D TCPS differs from the in vivo three-dimensional (3D) microenvironment. Compared to cells cultured on 2D substrates, cells grown in a 3D microenvironment exhibit more similarities to in vivo cells. Therefore, 3D cell culture models provide a promising alternative for conventional 2D monolayer cell culture. To improve conventional PDL cell culture models, we have recently developed a 3D cell culture method, which is based on spheroid formation of PDL cells on chitosan films. Here, we present detailed cell spheroid culture protocols based on chitosan films. The 3D culture system of PDL cellular spheroids overcome some of the limitations related to conventional 2D monolayer cell culture, and thus may be suitable for producing PDL cells with an enhanced therapeutic efficacy for future periodontal tissue regeneration.

INTRODUCTION:

Periodontitis, initialized principally by dental plaque[1], is characterized by the damage of periodontal tissues including periodontal ligament (PDL), alveolar bone, and cementum. Current treatments for periodontitis are usually successful in preventing the progress of the active disease, but the regeneration of lost periodontal tissues remains a clinical challenge. Recently, important progress has been made in cell-based approaches for periodontal tissue regeneration to overcome the drawbacks of current treatments[2-4].

Our previous systematic review revealed that PDL cells showed great potential for periodontal regeneration[5]. Conventionally, PDL cells are cultured on two-dimensional (2D) substrates such as tissue culture polystyrene (TCPS). However, characteristic changes of PDL cells have been observed during in vitro culture[6]. This phenomenon is probably because the 2D TCPS differs from the in vivo three-dimensional (3D) microenvironment[7]. Compared to cells cultured on 2D substrates, cells grown in a 3D microenvironment exhibit more similarities to in vivo cells[8]. Therefore, 3D cell culture models provide a promising alternative for conventional 2D monolayer cell culture.

Conventional 3D culture method is encapsulating cells in 3D biomaterials. Compared with cells encapsulated in 3D biomaterials, cellular spheroids mimic the in vivo situation more closely because spheroids are aggregates of cells growing free of foreign materials[9-12]. It is reported that cellular spheroids promoted MSC bioactivities via the preservation of extracellular matrix (ECM) components including fibronectin and laminin[13]. To improve

conventional PDL cell culture models, we have recently developed a 3D PDL cell culture method, which is based on spheroid formation of PDL cells on chitosan films[14]. Spheroid formation increased the self-renewal and osteogenic differentiation capacities of PDL cells[14]. Here, we present detailed PDL cell spheroid culture protocols based on chitosan films. The 3D culture system of PDL cellular spheroids overcome some of the shortcomings related to conventional TCPS cell culture, and thus may be suitable for producing PDL cells with an enhanced therapeutic efficacy for future periodontal tissue regeneration.

PROTOCOL:

The study protocol was approved by the Ethics Committee of School and Hospital of Stomatology, Tongji University. All patients provided written informed consent.

1. PDL cell isolation

1.1 Make proliferation medium for culture of PDL cells: α-MEM medium supplemented with 10% FCS and 100 U/mL pen/strep.

1.2 Prepare a container with ice to transfer isolated third molars.

1.3 Sterilize surgical instruments by using an autoclave.

1.4 Extract normal human impacted third molars from adults (18-28 years of age) at the Dental Clinic of School and Hospital of Stomatology, Tongji University.

1.5 Place third molars into the proliferation medium and transfer into the laboratory at 4 °C.

1.6 Place the extracted tooth in a sterile 100 mm Petri dish, working within a biohazard laminar flow hood.

1.7 Add 10 mL of PBS to wash the extracted tooth. Repeat the washing step twice.

1.8 By using a sterile scalpel, gently separate the PDL from the root.

NOTE: PDL should be scraped from the middle third part of the root to avoid contaminations from gingival or apical tissues.

1.9 Mince PDL into 1-2-mm fragments with the help of a sterilized scalpel blade.

1.10 Place PDL fragments in a T-25 culture flask, filled with 3-4 mL of proliferation medium.

1.11 Culture the samples in an incubator at 37 °C in 5% CO_2 with humidified air.

1.12 Change the culture medium after outgrowth of PDL cells was observed. It usually takes 1-2 weeks for the outgrowth of PDL cells. Observe the outgrowth of PDL cells via an inverse phase contrast microscope.

1.13 Change the culture medium twice per week thereafter.

1.14 When PDL cells have reached confluence, sub-culture cells at ratio of 1:4 (passage 1).

1.15 Observe cell growth daily and change the culture medium twice per week.

1.16 Perform each subsequent passage at ratio of 1:4 after the cells achieve 80% confluence. Use third- to fifth-generation of PDL cells for cell seeding.

2. Preparation of chitosan films

2.1 For the preparation of a 1% (v/v) acetic acid solution, add 5 mL of acetic acid to 495 mL of double-distilled water in a clean glass beaker.

2.2 For the preparation of 1% w/v chitosan solution, weigh 5 g of chitosan powder (average molecular weight 400 kDa, 85% degree of acetylation) and add to 495 mL of 1% (v/v) acetic acid solution.

2.3 Stir the prepared solution for 24 hours with stirring bar and magnetic stirring plate at 60 °C.

2.4 Autoclave the prepared solution to ensure complete dissolution of chitosan.

NOTE: The experiment can be paused at this stage.

2.5 To form chitosan films, add 1% w/v chitosan solution into tissue culture polystyrene (TCPS) dishes at 0.25 mL/cm^2. For example, add 0.5 mL of 1% w/v chitosan solution to one well of 24 –well plate, working within a laminar flow hood.

2.6 Dry TCPS dishes in an oven at 60 °C for 24 hours to form chitosan films.

2.7 Prepare 0.5 N sodium hydroxide (NaOH). Stir 10 g of NaOH, a little at a time, into a large volume of double-distilled water by using the magnetic stir bar, and then dilute the solution to make 0.5 L.

NOTE: Add NaOH to water--do not add water to solid NaOH. Do not touch NaOH! It could cause chemical burns.

2.8 Neutralize chitosan films with 0.5 N NaOH. Add 0.5 mL of 0.5 N NaOH solution to one well of 24-well plate for 2 hours.

2.9 Wash chitosan films three times with double-distilled water.

NOTE: The experiment can be paused at this stage.

2.10 Prior to cell seeding, sterilize the chitosan-coated 24-well plates in 70% alcohol overnight at room temperature.

2.11 Rinse chitosan-coated plates three times with PBS at room temperature.

2.12 Sterilize chitosan-coated plates by treatment under ultraviolet light overnight at room temperature.

3. **Cell seeding**

3.1 Pre-warm proliferation medium PBS solution and trypsin/EDTA solution to 37 °C.

3.2 Discard the supernatant from the T-75 cell culture flask.

3.3 Wash confluent PDL cells with 10 mL of PBS solution.

3.4 Discard the PBS solution.

3.5 Add 1 mL of trypsin/EDTA solution to T-75 cell culture flask.

3.6 Incubate cells with trypsin/EDTA solution for 3 min at 37 °C.

3.7 Terminate the digestion process of trypsin by adding 3 mL of proliferation medium to T-75 cell culture flask.

3.8 Transfer the prepared cell suspension into a 15 mL conical centrifuge tube.

3.9 Centrifuge the suspension for 5 min at 300 x *g* and room temperature.

3.10 Discard the supernatant from the 15 mL conical centrifuge tube and re-suspend the cell pellet in 500 µL of proliferation medium.

3.11 Count PDL cells with a hemocytometer.

3.12 Seed PDL cells at the densities of 0.5 x 10^4, 1 x 10^4, 3 x 10^4, and 6 x 10^4 cells/cm^2.

3.13 Culture PDL cells on chitosan films in an incubator at 37 °C in 5% CO_2 with humidified air.

3.14 Change the culture medium twice per week thereafter.

3.15 On days 1, 2, 3, 4, and 5, observe the cell morphology daily via an inverse phase contrast microscope.

4. Cell survival

4.1 After 1, 3, and 6 days of culture, determine the survival of cells using a commercial viability/cytotoxicity kit.

4.2 In a 15 mL tube, prepare 2 mL of a fresh solution of 2 mM calcein-AM and 4 mM ethidium homodimer in PBS by vortexing for 5 s.

NOTE: Keep the process in the dark.

4.3 Remove the culture medium and wash spheroids in PBS.

4.4 Incubate spheroids in of PBS containing 2 mM calcein-AM and 4 mM ethidium homodimer for 30 min at room temperature in the dark.

4.5 Rinse spheroids twice in PBS. For spheroids from one well of 24-well plate, use 2 mL of PBS for each time.

4.6 Observe the samples by using a fluorescence microscope using 10x or 20x magnification.

REPRESENTATIVE RESULTS:

Using the present protocol, viable PDL cell spheroids were successfully formed. **Figure 1** showed that suspended cells or spheroids instead of attached cells were mainly observed on chitosan films. For the seeding density of 0.5 x 10^4 cells/cm^2, attached PDL cells were occasionally found on day 1 and 3, and PDL cell spheroids were rarely observed. On the contrary, for the seeding densities of 3 x 10^4 and 6 x 10^4 cells/cm^2, various sizes of PDL cell spheroids were found since day 1. PDL cell spheroid formation was observed from all the seeding densities after 3 days. As shown in **Figure 1**, 3 x 10^4 cells/cm^2 was the optimal PDL cell seeding density because the size of PDL cell spheroids was homogeneous at this cell seeding density. After 5 days of culture, larger spheroids were formed for all the cell seeding densities. Suspended PDL cells were rarely found on day 5.

The viability of PDL cells in spheroids was assessed after 1, 3 and 6 days. As shown in F**igure 2**, the majority of cells in spheroids were living cells on day 1, 3, and 6. While on day 6, the number of dead cells was increased in the central part.

FIGURE LEGENDS:

Figure 1. The morphology of PDL cellular spheroids.

For the seeding density of 0.5 x 10^4 cells/cm^2, PDL cell spheroids were rarely observed on day 1 and 3. For the seeding densities of 3 x 10^4 and 6 x 10^4 cells/cm^2, various sizes of PDL

cell spheroids were found since day 1. PDL cell spheroid formation was observed from all the seeding densities after 3 days. After 5 days of culture, larger spheroids were formed for all the cell seeding densities. Suspended PDL cells were rarely found on day 5. Scale bar: 200 µm. This figure has been modified from Yan et al.[14].

Figure 2. The viability of PDL cellular spheroids on chitosan films.
The viability of PDL cells in spheroids was assessed after 1, 3 and 6 days. As shown here, the majority of cells in spheroids were living cells on day 1, 3, and 6. While on day 6, the number of dead cells was increased in the central part. Scale bars: 200 µm. This figure has been modified from Yan et al.[14].

DISCUSSION:

The present study introduced a 3D cell culture system to overcome some limitations related to conventional 2D monolayer cell culture. According to the protocol, PDL cellular spheroids were successfully formed by culturing cells on chitosan films. Our previous study reported that spheroid formation increased the self-renewal and osteogenic differentiation capacities of PDL cells [14]. Instead of using an enzyme to harvest cells from TCPS, PDL cell spheroids could be harvested from chitosan films by simply pipetting the medium a few times[14]. Thus, ECM and intercellular junctions can be well preserved.

The critical steps of this protocol include: (1) making sure that the surgical instruments and chitosan films are sterilized for cell culture; (2) scraping the PDL from the middle third part of the root to avoid contaminations from gingival or apical tissues; and (3) requiring higher cell seeding densities (≥3 x 10^4 cells/cm^2) for the successful spheroid formation of PDL cells.

However, one limitation for this method is that decreased proliferation of PDL cells was observed after spheroid formation[14], which is similar to some previous studies that performed on adipose stromal cells[10] and hepatocellular carcinoma cell line[15]. This is probably caused by the difference in the regulation of cyclin-dependent kinase inhibitors between spheroid and monolayer cells[16]. To benefit clinical application, further studies to promote the proliferation of cell spheroids are required.

Another drawback of the cellular spheroid is the inadequate diffusion in the core. Although PDL cells were mainly alive in spheroid culture, the number of dead cells was increased in the central part of spheroids on day 6. This phenomenon was probably because the diffusion of oxygen and nutrients is compromised in the spheroid core as PDL cell spheroids become larger[17,18].

Other methods that reported to induce cellular spheroids include a non-adherent surface[19], microwells[20], spinner flasks[21], micropatterned surfaces[22,23], hanging drops[24], and a forced-aggregation technique[25]. Compared to aforementioned methods, chitosan films are relatively cost-effective and easy to reproduce. Another advantage of this method is the antimicrobial properties of chitosan[26], which is significantly beneficial for in vitro cell culture.

In summary, here we present detailed 3D cell spheroid culture protocols based on chitosan films. The culture of PDL cellular spheroids overcome some of the limitations related to conventional 2D TCPS cell culture, and thus may be suitable for producing PDL cells with an enhanced therapeutic efficacy for future periodontal tissue regeneration.

ACKNOWLEDGMENTS:

This study was sponsored by National Natural Science Foundation of China (NSFC 81700978), Fundamental Research Funds for the Central Universities (1504219050), Natural Science Foundation of Shanghai (17ZR1432800), and Shanghai Medical Exploration Project (17411972600).

DISCLOSURES:

The authors have nothing to disclose.

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401

Figure 1

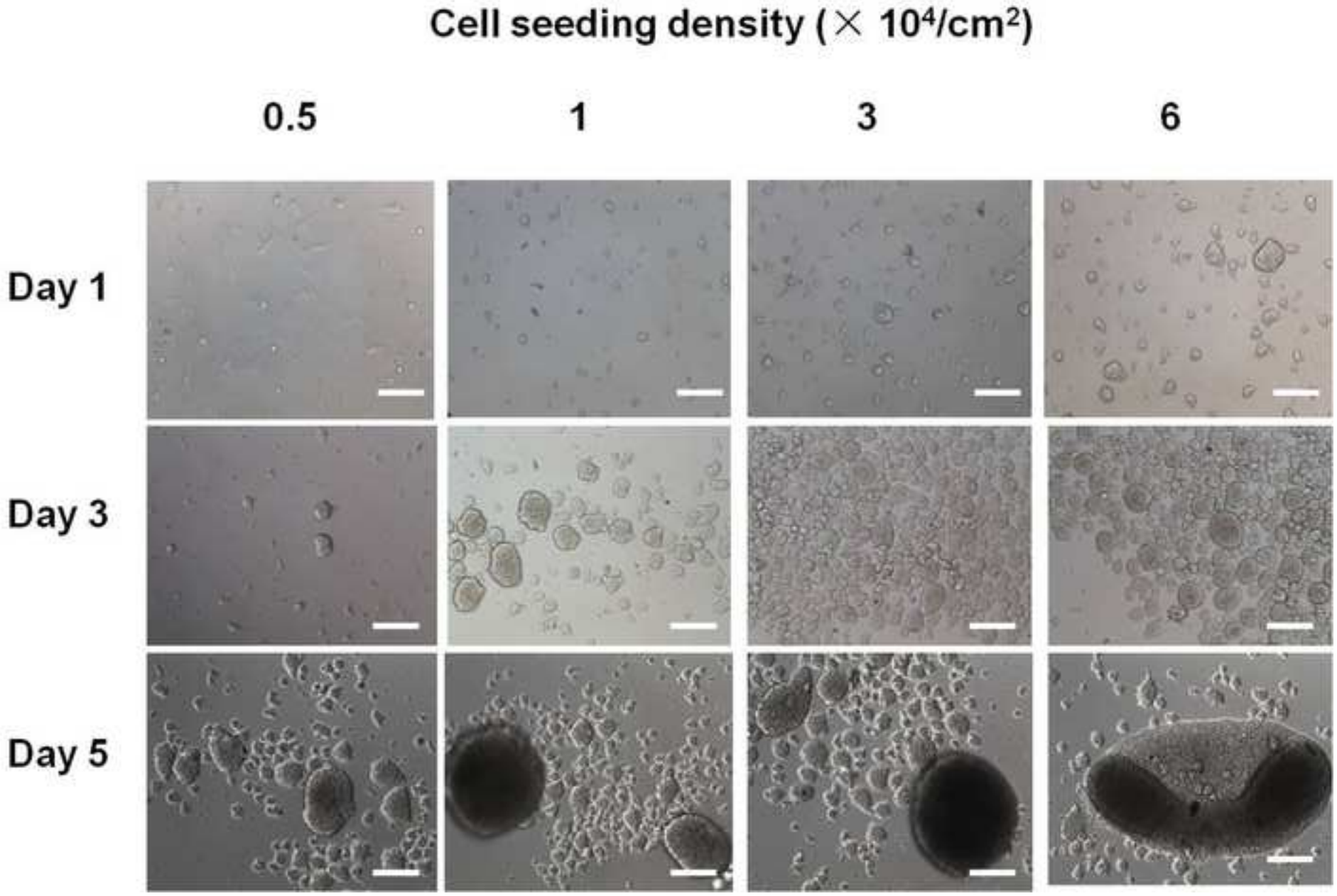
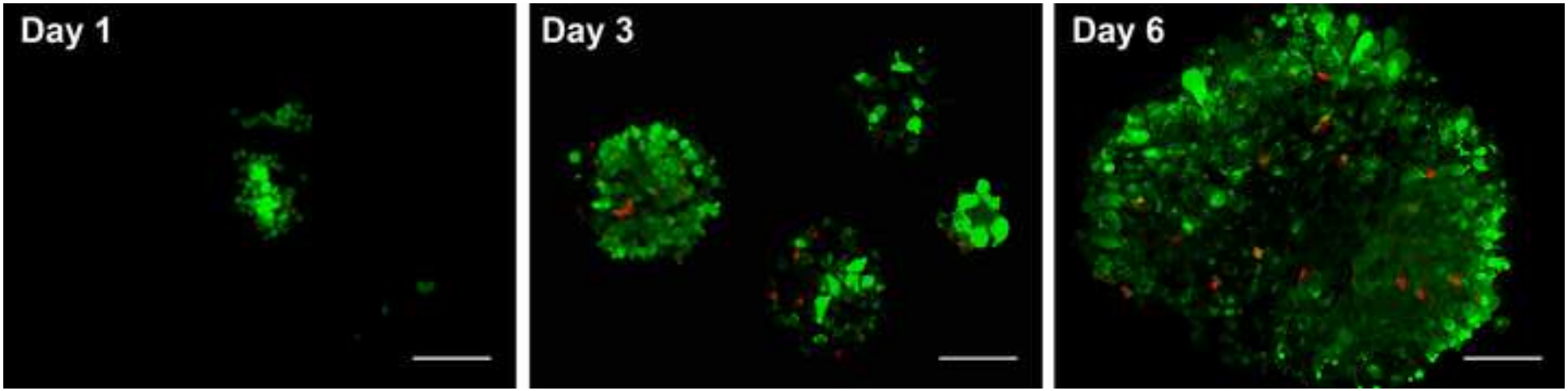


Figure 2



Name of Material/Equipment	Company	Catalog Number	Comments/Description
α-MEM	Gibco	11900-073	
acetic acid	Sigma-Aldrich	64197	
Cell culture flask 25 cm2	Corning	430639	
Cell culture flask 75 cm2	Corning	430641	
Chitosan	Sigma-Aldrich	419419	molecular weight 400 kDa, degree of deacetylatic
FCS	Gibco	26140-079	
Live/Dead Viability/Cytotoxicity Kit	Molecular Probes	L3224	
NaOH	Sigma-Aldrich	1310732	
PBS	KeyGen Biotech	KGB5001	
pen/strep	Gibco	15140-122	
Trypsin/EDTA	KeyGen Biotech	KGM25200	
15 mL conical centrifuge tube	Corning	430790	
24-well plate	Corning	3524	

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Dear Dr. Stephanie Weldon,

Herewith, we submit a revised version of the manuscript “Formation of human periodontal ligament cell spheroids on chitosan films”. The original manuscript draft has been revised according to the editorial and peer review comments and all changes have been highlighted in red.
We would appreciate you considering this revised manuscript for publication.

Yours sincerely,

Xiangzhen Yan (on behalf of all the co-authors)

Response to Editorial comments:

Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: The manuscript has been thoroughly checked in the revised manuscript.

2. Please revise lines 67-69, 83-84, 193-195, 205-208, 233-235, 245-247 to avoid previously published text.

Response: The lines 67-69, 83-84, 193-195, 205-208, 233-235, 245-247 have been revised to avoid previously published text.

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Response: Explicit copyright permission with a link to the editorial policy that allows re-prints has been uploaded.

4. Authors and affiliations: Please provide an email address for each author.

Response: An email address for each author has been added in the revised manuscript.

5. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “NOTE.”

Response: Protocol to contain only action items has been revised according to the editorial comments.

6. 1.3: Please describe how to sterilize surgical instruments.

Response: The information of how to sterilize surgical instruments has been described in the revised manuscript.

7. 1.12: Please provide an estimate about how long this usually takes. How to define outgrowth of PDL cells?

Response: The information of how long and how to define outgrowth of PDL cells has been added in the revised manuscript.

8. 3.9: Please convert the centrifugation unit rpm to x g.

Response: The centrifugation unit rpm has been converted to x g in the revised manuscript.

9. Line 176: Please remove commercial language (Live/Dead).

Response: Commercial language (Live/Dead) has been removed in the revised manuscript.

10. Discussion: Please describe critical steps of the protocol.
Response: Critical steps of the protocol have been discussed in the revised manuscript (Page 6).

11. Figure 1: Please include a scale bar at the lower right corner for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.
Response: A scale bar at the lower right corner for all images has been added in the revised figure 1.

12. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.
Response: The table of materials provides information on all relevant supplies, reagents, equipment and software used and it has been revised in alphabetical order.

13. References: Please do not abbreviate journal titles.
Response: Abbreviate journal titles have been replaced by full journal titles in the revised manuscript.

Response to Reviewer Comments

Reviewer #1:

Manuscript Summary:

This manuscript presented a detailed protocol to prepare cell spheroids using chitosan films. The authors have done a good job in describing the preparation method in details. I only have one small comment: it will be helpful if the detail information of chitosan can be provided, such as distribution of molecular weight, degree of deacetylation.

Response: The detail information of chitosan has been added in the revised manuscript and table of materials.

Reviewer #2:

Minor Concerns:

- the authors describe in Introduction that spheroids superiorly mimic in vivo situation as these are 'aggregates of cells growing free of foreign materials'. The reviewer suggests to indicate that potentially formed extracellular matrix (ECM) can be found in between the cells in spheroid culture. Maybe the authors can provide information on what ECM can be found (composition, etc.).

Response: The information of extracellular matrix (ECM) found in spheroid culture has been added in the revised manuscript (Page 3).

General:

The manuscript contains multiple spelling mistakes; please carefully check English language

Response: English language has been carefully checked in the revised manuscript.

Reviewer #3:

Manuscript Summary:

Adequate

Major Concerns:

no major concerns

Minor Concerns:

no minor concerns

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