Evaluating the Viability of Bioprinting Skin Organotypic Through a Comparison of the Contraction of Collagen Hydrogels Prepared Using Extrusion-Based Printing and Traditional Skin Grafting Methods

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Rationale:

Multiple methods through tissue engineering have been applied to construct skin grafts for burn treatment. However, several disadvantages accompany these methods, including immune reactions, transmission of diseases, and shortages of donor skin. A novel technique - bioprinting - would alleviate many of these complications.[1] Bioprinting skin holds great potential for application in treatments as it would not only eliminate the need for donors, but it would also prevent homogeneity issues accompanied by current methods. Bioprinting allows for faster integration with the host tissue, lower risk of rejection, and uniform tissue growth in vivo.[2]

Currently, extrusion-based printing is found to be the most feasible bioprinting technique in terms of vertical configuration and allowing usage of a larger variety of bioinks, including more cell-dense bioinks; however, present limitations include lower cell survivability due to the shear stress that occurs during printing.[2] During our experiment, we aim to evaluate the cell survivability and functionality of keratinocytes and fibroblasts, comparing poured and printed samples of a collagen gel solution.

Research Question:

Does the shear stress applied on keratinocytes and fibroblasts by the bioprinter affect their functionality in collagen contraction?

Hypothesis:

If the shear stress of the bioprinter is applied to the components of the extracellular matrix (collagen and fibroblasts) or the keratinocytes, then there will be reduced functionality of such components in collagen contraction.

Null Hypothesis:

If the shear stress of the bioprinter is applied to the components of the extracellular matrix (collagen and fibroblasts) or the keratinocytes, then there will be enhanced functionality of such components in collagen contraction.

Engineering Goals:

The engineering goal of this project is to determine whether the shear stress applied on keratinocytes and fibroblasts by the bioprinter will affect their functionality in collagen contraction. We hope to create an accurate skin equivalent using the bioprinter for an efficient skin grafting method to replace traditional methods. Perfecting this bioprinting technique will allow for enhanced medical outcomes with skin grafting patients.

Expected Outcomes:

Due to prior research finding shear stress to cause lower cell survivability, it is expected that collagen contraction rates of printed samples will be less than those of plated samples. It is believed that the shear force of the printer applied on the cells will alter cell functionality and lower the total cell count, reducing contraction ability.

Materials:

General:

- Cellink Heartware Inkjet Bioprinter
- Biosafety Cabinet
- Air compressor
- 5mL, 10mL, 1mL, and 20mL pipette tips along with pipette gun
- Aspirator
- 24mm Diameter inserts
- Petri Dishes
- 70% Ethanol spray
- Washer

Collagen Gel Components:

- Collagen (2mg/mL)
- NaOH
- PBS
- FBS
- L. Glutamine
- 10x EMEM
- .05% Trypsin-EDTA
- Na_2CO_3
- DMEM
- Medium: Cascade Biologics Medium 106
- Keratinocytes
- 3T3 (matrix for KC)
- K+ Medium
- KC Medium

Experimental Setup:

Experimental Group:

The experimental group in our experiment is the collagen gel samples that will be passed through the bioprinter and therefore will have an applied shear force. There will be three inserts per trial and each insert will be observed and the appropriate values will be measured each day for eleven days.

Experimental Layer Conditions:

1. Printed FB + Poured KC
2. Printed FB + Printed FB

3. Poured FB + Printed KC

Control Group:

The control group in our experiment is the collagen gel samples that are not passed through the bioprinter and, rather, are plated using 5 mL pipette tips. Just like the experimental group, there will be three inserts per trial and each insert will be observed and the appropriate values will be measured each day for eleven days.

Control Layer Condition:

1. Poured FB + Poured KC

Constants:

- Conditions under which collagen gel solution is set:
    - 37 °C Incubator
- Frequency by which media is changed
    - Every 2 days for Collagen Contraction
- Media Used:
    - DMEM (Collagen Contraction)
    - KC Medium

Independent Variable:

Method of preparation (Printed vs Poured)/Applied pressure (kPa) on Fibroblasts/Collagen Matrix

Dependent Variable

Rate of collagen contraction (mm/day)

Procedure:

Plating Collagen:

1. Using standard values for the amounts of each collagen gel component per 5 mL, calculate the appropriate value of each component per 13 mL, accounting for the 1 mL needed per each of the four conditions and each of their three samples (4 x 3 = 12) as well as an extra mL in case of errors.
    a. *13/5 = 2.6; Multiply each value by 2.6 to get the appropriate amount needed

Component:	Per 5 mL:	Per 13 mL
10x EMEM	0.5 mL	1.3 mL
L. Glutamine	0.0416 mL	0.10816 mL
FBS	0.5 mL	1.3 mL
Na_2CO_3	0.142 mL	0.3692 mL
Collagen (4.82 mg/mL)	2.84 mL	7.384 mL
PBS (add until reach desired amount of mL)	Approximately 1 mL	Approximately 2.6 mL
NaOH	Until a pH of 7---red	Until a pH of 7--red

2. Combine the above components, making sure to add NaOH and PBS last to ensure that a pH of 7 is reached and a total of 13 mL are made. Plate in the inserts using the different conditions for the first layer (Shown on the left side): *All printed components are printed at a pressure of 10 kPa
3. Prepare the collagen gel solution for the second layer, which contains the fibroblasts, accounting for 56 mL of solution needed. (3 mL per insert x 12 inserts + 3 mL x 6 flasks + 2 extra mL in case of error.) (Multiply all values by 11.2 to get appropriate amounts needed)

a.

Component:	Per 5 mL:	Per 56 mL
10x EMEM	0.5 mL	5.6 mL
L. Glutamine	0.0416 mL	.46592 mL
FBS	0.5 mL	5.6 mL
Na_2CO_3	0.142 mL	15.904 mL
Collagen (4.82 mg/mL)	2.84 mL	7.384 mL
PBS (add until reach desired amount of mL)	Approximately 1 mL	Approximately 26. 84 mL
NaOH	Until a pH of 7---red	Until a pH of 7--red
Fibroblast Cells		Concentration: 5×10^5 cells/mL

b. To isolate/prepare the Fibroblast cells:

i. Suction all liquid from the given cell culture

ii. Add 1 mL of PBS

iii. Suction again

iv. add 2 mL of Trypsin to make sure that no cells are stuck to the bottom of the flasks

v. Place in incubator at 37℃

vi. add 4 mL of DMEM medium to neutralize trypsin

vii. Suction again

viii. Centrifuge cells @ 1200 rpm for 5 min

ix. Count cells per 1 mL using an optical microscope (as seen in table above) and add the corresponding amount of the cell culture to the above components (Needed 7.5 x 10^4 cells/iinsert----2.5 x 10^4 cells/mL x 28 mL printed and 28 mL poured----1 mL cells in HuFb media to each 28 mL of collagen gel solution)

c. Use the above conditions for the second layer (on the right) to accordingly place the solution on the 1st layer--Printed or poured

4. Add DMEM media to the collagen gel inserts every other day, suctioning old media first and then adding 3 mL to the bottom of the insert and 2 mL to the top of the insert.
5. After the first two days, add washers to the center of each collagen insert to weigh the gel down and prepare to add the keratinocytes to the layers.
6. Prepare Keratinocytes:
    a. Remove the medium from the given flasks with keratinocytes and 3T3 cells
    b. Add 5 mL of PBS with edta to remove the 3T3 cells from the flask and incubate for 5 min--Suction
    c. Add 2 mL of trypsin and incubate for 4 min to ensure that none of the keratinocytes are stuck to the bottom of the flask
    d. Change media to JG1 on day one, then two days later change media to JGII. Two days after that begin to change the media daily to JGL adding 160 microliters of each aliquot, after filtering, when doing so.
    e. When adding media as described above, also add 4 mL of K+ media

f. Separate 1 mL of the above solution on day one, centrifuge, and resuspend in 2 mL of media to get the desired concentration of 600 x 10^4 cells/mL

7. Add 50 µL of keratinocytes to the centers of these washers after another 2 days to allow for differentiation of skin layers.

Evos Imaging and Determination of Collagen Contraction Rates:

1. Take images of the prepared Collagen Gels after they have fully gelled over the 4 week period using EVOS Imaging. Do this every day for 11 days.
2. Upload the resulting images into Image J and determine a scale for measuring the diameter of the collagen gels using the known diameter of the petri dish to be 24mm.
3. Since the collagen gels will not all be perfect circles, find the average diameter of each gel by taking 5 measurements for each image. Record the measured diameters and compare over an 11 day period.
4. Determine the rate of collagen contraction using the change in diameter and time.

We hope to conduct at least two trials for the above experiments

Data Analysis:

Using ImageJ, we will determine the average diameters for each insert per day and use these values to calculate the average area (mm^2) per day. Graphing these values over the 11 day period, we should be able to determine an average rate of contraction, which will be indicated by the slope of the graph. We plan to analyze data taken in this form through a Two Sample t-test Assuming Unequal Variances, comparing the values for Average Area (mm^2) covered between the printed and poured samples for collagen contraction and determining p values to determine whether the differences in rates for printed and poured samples were significant. In this case,

larger p values, greater than 0.05, are preferred because these signify that the two rates present few differences. In order to conduct such statistical analysis, we plan to use Microsoft Excel, in which, by inputting our data and navigating to data analysis, we should be able to obtain the desired variance and p values. Given that we obtain the desired larger p values, we would be able to accept our hypothesis, confirming that the shear stress applied on the cells by the bioprinter has little effect on the rate of collagen contraction.

Risk and Safety:

For this project, personal protective equipment like lab coats, gloves, and goggles are obviously needed. In addition, the participants have to be aware of the many biohazards present in a bio lab such as the toxicity of cell culture and media. Various chemicals such as Trypsin which will be used in the preparation of each type of cell have high potential for harm and must be handled with caution. We must also make sure most of our work is done in biosafety cabinets. The research will involve the participants using 70% ethanol, which is flammable. Finally, training is required so that the participants know the proper methods of pipetting and sterilizing. In regards to general areas of concern, we must ensure that our samples are placed in a stable environment at all times. The slightest change in the environment can affect our samples' ability to function as skin cells and this can skew our results.

Post Summary:

*No changes were made to our experimental procedure throughout the course of this research, however, we were unable to conduct two trials.

Bibliography:

[1] Augustine, Robin. "Skin bioprinting: a novel approach for creating artificial skin from synthetic and natural building blocks." Progress in biomaterials vol. 7,2 (2018): 77-92.

doi:10.1007/s40204-018-0087-0

[2] Kačarević, Željka P et al. “An Introduction to 3D Bioprinting: Possibilities, Challenges and Future Aspects.” Materials (Basel, Switzerland) vol. 11,11 2199. 6 Nov. 2018, doi:10.3390/ma11112199

[3] Falguni P., Jinah J., Lee J.W., Dong-Woo C. Essentials of 3D Biofabrication and Translation. Elsevier; Amsterdam, The Netherlands: 2015. Extrusion Bioprinting; pp. 123–152.

[4] Lee JS, Hong JM, Jung JW, Shim JH, Oh JH, Cho DW Biofabrication. 2014 Jun; 6(2):024103.