ORIGINAL RESEARCH PAPER

Ophthalmology

3D BIOPRINTING OF CORNEAL STROMAL EQUIVALENT

KEY WORDS: Bioprinting, collagen, gelatin, 3D cornea.

Dr Anand Goyal* 3rd year resident, Ophthalmology Department, GMC kota PG Hostal 2, room no 1, behind MBS hospital, kota *Corresponding Author

Dr Nikita Mandawat 3rd year resident, Ophthalmology Department, GMC kota PG Hostal 2, room no 23, behind MBS hospital, kota

INTRODUCTION

The WHO estimate the patients of corneal blindness require surgery around 10 million after trachoma and other 5 million people due to other causes. Corneal diseases (eg. Corneal opacity) are major cause of blindness[1]. The unmet need for cornea donors has lead to increasing efforts in the development of artificial corneal substitute.

Previously construct biosynthetic corneal model :-[2]

a) The study by Li et al.-

- For study of corneal cellular regeneration.
- They use the plastic contact lens molds into which hybrid collagen hydrogels were injected and crosslinked and over which epithelial cells are cultured.

b) The karamichos et al.-

- For study of corneal fibrosis.
- They plated corneal fibroblast onto porous polycarbonate membrane bearing and secreted ECM.

Above both are not for clinical use as they are not anatomically and geometrically related to human cornea.

c) 3D bioprinting-

- Is a technique that has gain interest for tissue engineering application for its ability to direct the assembly of 3-dimensional and biological structures of cornea by Newcastle University. 3D bioprinting is an additive manufacturing technology in which cells are combined with a suitable biomaterial and deposited within micrometer precision, layer-by-layer, to generate tissue constructs for a variety of applications.

The artificial corneal substitute to be functionally mimic the native cornea must meet the specific criteria .

The native cornea is having 2 features-

1) Transparent to light
2) Around 70 % of refractive power of eye

These 2 are due to –

1) Near perfect spherical anterior surface so, the ability to recapitulate the symmetric curvature is necessary and fundamental to design framework (corneal geometry).
2) Distinct arrangement of collagen lamellae in the corneal stroma that provide the strength and shape (Organotyping).

Corneal Stroma[3]

Around 200-250 lamellae are present. In anterior stroma lamellae interwoven with one other and In mid & posterior stroma these are lying parallelly. So, complexity of stroma is a challenging.

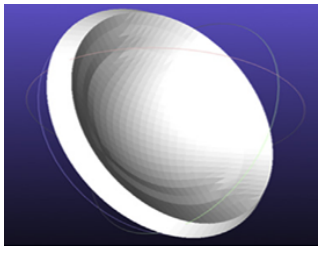
Pre-printing steps[2] :-

1) Digital model-

- For Geometry and Organotype assessment of the patients normal cornea. Printed constructs were anatomically analogous to a human corneal model derived from the topographic data. It is construct by using a rotating scheimpflug camera with a placido disc.

The vertical and horizontal diameters of the model measured 12.377mm×12.385 mm, respectively. While its thickness measured approximately 500 um at the centre and 823 um towards the periphery.

Figure no.1) showing model cornea



2) Digital Support system generation-

- A support structure is required to print cornea and preserve the shape of printed cornea construct after bioprinting. The corneal model was used as a template to build a digital support structure in order to start the 3D bioprinting process. This dome shaped corneal model ®-6.5 mm) inverse shape (anterior surface downward) is substracted from the centre of the square face of digital cuboid.

Figure no.2) showing corneal model used as template

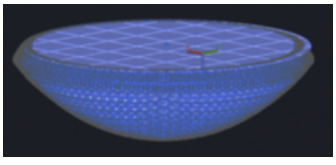
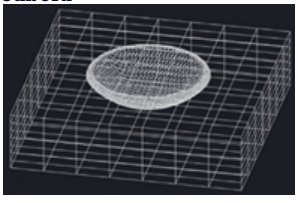


Figure no.3) showing corneal model substracted from centre of cuboid



- This digital cuboid is sit neatly in a petri dish. This all information is exported as an STL (steriolithography) file.

Figure no.4) showing digital cuboid with central template

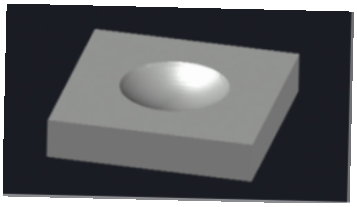
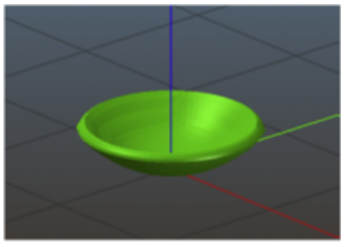


Figure no.5) showing digital cuboid placed in petri dish

Printing setup[2] :-

- Pneumatic 3D extrusion bioprinter, (*INKREDIBLE* bioprinter) having a 3D printing software Slic3r. STL file is imported onto software and from which G- code is subsequently exported. G- code is final command to what to be form.
- Printing is from the centre of the support and then outwards and upwards towards the rim (concentric).

Figure no.6) showing concentric printing process



- 30 G high precision blunt & straight profile needle
- Speed- 6 mm/s
- Resolution- 100 um
- Nozzle- 200um & 300um.
- Bioprinting is completed in < 10 min.

Figure no.7) showing real image of printing process by Newcastle University

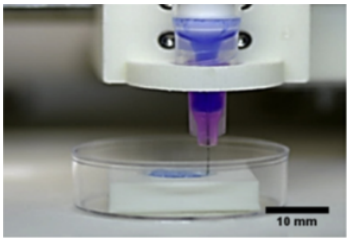
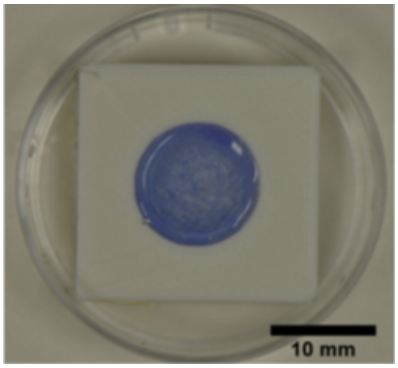


Figure no.8) showing 3D printing constuct form



- Central and Peripheral corneal thickness was significantly reduced when a nozzle diameter of 300 um was applied relative to the 200 um nozzle due to the decrease in bio-ink deposition.

- Structures deposited by 200um nozzle is showing

1) Greater stability
2) High cell viability

Table no.1) showing relation of Corneal thickness with Nozzle diameter

Nozzle diameter	Central corneal thickness	Peripheral corneal thickness
200um	609.4um	711.2um
300um	518.5um	584.7um

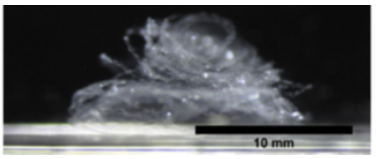
Bio-ink preparation

- Six bio-inks were formulated-

1) 3% (w/v) sodium alginate only.
2) 8 mg/ml methacrylated collagen only.

If only alginate bio-ink used, resulting printed corneal structures began to twisted when corneal keratocytes cells were incorporated. So, collagen bio-ink in place of alginate, but found that concentrations used were not viscous enough i.e. unable to maintain their shape and get diffuse.

Figure no.9) showing 3D printing constuct if only alginate bio-ink is used



So, composite bio-inks comprising both collagen and alginate in order to combine their respective material and mechanical properties.

- Sodium alginate and Methacrylated type I collagen were used to prepare all bio-inks.
- Both of these are high viscosity agent.
- *Alginate provide stiffness* and mechanical support.
- *Collagen provide tensile* strength.

final four bio-inks, termed Coll-1 to Coll-4, had various combinations of methacrylated collagen mixed with 2% (w/v) sodium alginate to the following ratio.

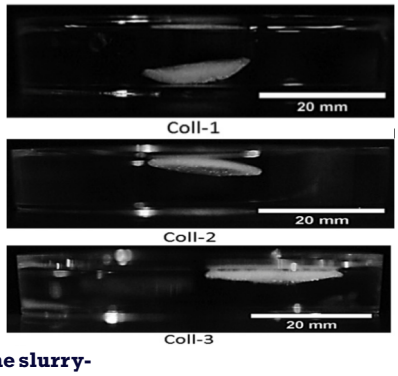
3) Coll-1: one part 8 mg/ml collagen to two parts alginate.
4) Coll-2: one part 8 mg/ml collagen to three parts alginate.
5) Coll-3: one part 6 mg/ml collagen to two parts alginate.
6) Coll-4: one part 6 mg/ml collagen to three parts alginate.

Structures printed with,

- Coll-4, presented with little structural integrity and fragmented easily.
- Coll-3, remained intact but were unable to maintain an appropriate degree of curvature.
- Coll-2, displayed more curvature than those printed with Coll-3.

Best preservation of corneal shape was obtained with the Coll-1 bioink because enhanced stability of the corneal structures printed with Coll-1 was attributed to the combined tensile strength of collagen and alginate.

Figure no.10) showing 3D printing constuct using different preparation



Gelatine slurry-

- Accommodating the continuous extrusion of bio-ink while retaining the shape of previously deposited bio-ink. Gelatine

slurry provide stiffness to cornea so that corneal curvature is maintained after detachment. In the absence of the gelatine slurry cornea did not maintain their shape and clumped together. After aspiration of gelatine the shape of cornea is maintained by crosslinking.

Stem Cell culture

- Corneal keratocytes are the most abundant cell type of stroma and suitable cell type for bio-ink formulation. Stromal cells were isolated from cadaverous corneal tissue (age 60–80 years and with no prior history of corneal diseases or ocular trauma, research consent taken). The epithelia-depleted corneal tissues were finely chopped using a scalpel, transferred to DMEM/F12 medium, supplemented with serum, collagenase type 1 and penicillin/streptomycin. Media were changed every 2–3 days, and cultures were maintained until reaching 70–80% confluence. At this point fibroblasts underwent serum starvation for a period of 3 days to promote their differentiation into keratocytes.

3D bioprinting process[2]

- Gelatine slurry was prepared using the *Freeform Reversible Embedding of Suspended Hydrogels (FRESH)* technique. The hollowed-out section of the 3D printer was filled with gelatine slurry in order to facilitate the printing of high viscosity collagen and alginate bio-inks. The support was then returned to the printing plate. Corneal structures were extruded at air pressure-

Table no.2) showing air pressure value for different preparation

Alginate	collagen	Col 1	Col 2	Col 3	Col 4
180kpa	15kpa	40kpa	20kpa	15kpa	10kpa

-Then gelatine slurry was aspirated and crosslinked.

Crosslinking

Corneal structures printed using,

1) alginate-based bioink- crosslinked with cacl2 and incubated.
2) collagen alone is just incubated.

- After printability had been optimized, corneal keratocytes were incorporated into Coll-1 at a concentration of 2 million cells/ml. These were printed using a nozzle diameter of 200 μm and were maintained in serum-free medium for 7 days subsequent to printing.

Printing accuracy

Printing accuracy was evaluated by :-

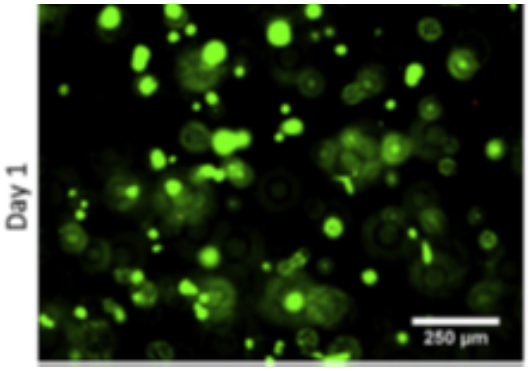
1) Quantifying central and peripheral thickness of the corneal construct by OCT sectioning.
2) The viability of encapsulated corneal keratocytes.

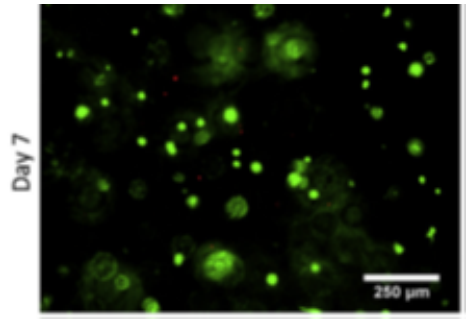
Cell viability evaluation

Done by using 2 methods-

1)Leica DM IL LED microscope

Figure no.11) showing cells in corneal construct by *Leica DM IL LED microscope*





2) Brightfield image

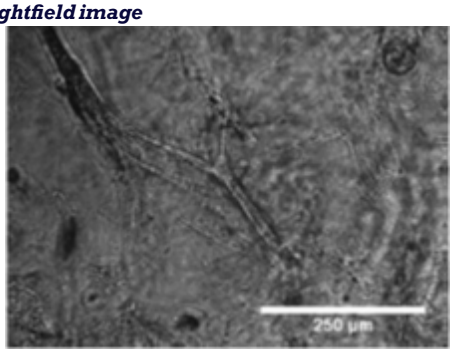


Figure no.12) showing cells in corneal construct by brightfield image

- Viable cell number and percentage viability of corneal keratocytes were assessed at day 1 and day 7.
- Using coll 1 and 200um nozzle observed cell viability on
  day 1 - 92%
  day 7 - 83%

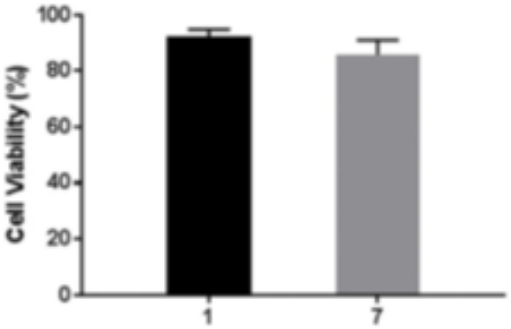


Figure no.13) showing cells in corneal construct at Day 1 and Day 7

Success of corneal 3D bioprinting depend on the ability of encapsulated cells to mediate ECM remodelling. A distinctive feature of corneal fibroblasts is when they are seeded at the base of a curved surface they migrate in lattice formation and align collagen in a way that closely resembles its arrangement in the cornea

Thus, cells seeded at the base of a scaffold bearing a close resemblance to corneal anatomy would potentially be capable of remodelling the ECM in a way that is presently unachievable with non-curved geometries.

Cause of cell damage-

1) *Extrusion 3D bioprinting is the generation of shear stress induced cell deformation at the needle wall.*
2) *Dehydration due to gelatine slurry*.

Future aspect

Support epithelial cell growth, limbal zone and Bowman's layer formation with appropriate soluble & insoluble factors and Biocompatibility of the construct following transplantation.

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