

INVESTIGATION OF TISSUE ENGINEERING AND 3D BIOPRINTING STRATEGIES FOR THE REGENERATION OF SOFT AND HARD TISSUES

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by

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Submitted

**In fulfilment of the requirements of the degree of Doctor of
Philosophy**

to the



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Dedicated to my parents...

Lt. Mr. Pradip Chakraborty

Mrs. Jayasree Chakraborty

for their infinite love, support, and encouragement

ॐ सर्वेशां स्वस्तिर्भवतु । सर्वेशां शान्तिर्भवतु ।
सर्वेशां पुर्णभवतु । सर्वेशां मङ्गलंभवतु ।
ॐ शान्तिः शान्तिः शान्तिः ॥

Om Sarveshaam Svastir-Bhavatu | Sarveshaam Shaantir-Bhavatu |
Sarveshaam Purnnam-Bhavatu | Sarveshaam Manggalam-Bhavatu |
Om Shaantih Shaantih Shaantih ||

May there be Well-Being in All,
May there be Peace in All,
May there be Fulfilment in All,
May there be Auspiciousness in All,
Om Peace! Peace! Peace!

*“All outstanding work, in art as well as in science, results
from immense zeal applied to a great idea.”*

– Santiago Ramón y Cajal

CERTIFICATE

This is to certify that the thesis entitled **“Investigation of Tissue Engineering and 3D Bioprinting Strategies for the Regeneration of Soft and Hard Tissues”** being submitted by **Ms. Juhi Chakraborty** to the **Indian Institute of Technology Delhi** for the award of the degree of **“DOCTOR OF PHILOSOPHY,”** is a record of the original research work carried out by her under my supervision and guidance. She has fulfilled all the requirements for submission of this thesis, which to the best of my knowledge, has reached the required standard.

The material in this thesis has not been submitted in part or full to any other University or Institute for the award of any other degree.

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Juhi Chakraborty

ABSTRACT

Since the last three decades, extensive efforts have been undertaken towards the regeneration and/or replacement of damaged or diseased organs by tissue engineering. However, there have only been a sporadic success with organ transplantation regarding the development of healthy tissues such as cartilage, bone, skin, cornea, etc., owing to the intricacy of the human tissues. Furthermore, it is acknowledged that developing designed organs and tissues for transplantation is likely complex because our knowledge of organ regeneration and related technologies is still minimal. The conventional tissue engineering methods fail to configure the desired tissues with intricate biomimetic architecture. Three-dimensional (3D) bioprinting, also known as 3D bioprinting, is highly promising that overcomes the constraints of traditional tissue engineering practices. The advantage of 3D bioprinting is that it permits the development of layer-by-layer custom-made, patient-specific constructs by fabricating anatomically relevant implants akin to the complexity of the native tissue using the cells from the patient itself. In light of this, the present study highlights the application of 3D bioprinting and decellularization techniques for soft and hard tissue regeneration, explicitly focusing on cartilage, bone, skin, and cornea.

Most of the articular cartilage is comprised of a collagen II fiber network arranged in arches. The lack of suitable microfabrication techniques capable of developing 3D fibrous structures is one of the biggest obstacles to *in vitro* replication of cartilaginous tissue with an arch-like structure. As a result, in contrast to the conventional wood-pile pattern, we provide in the first study a 3D bioprinting technique for producing constructs that resemble the arches of collagen II using two different types of bioinks: gelatin methacryloyl (GelMa) and silk fibroin-gelatin (SF-G). SF-G bioink laden with mesenchymal stem cells derived from human bone marrow (BM-MSCs) exhibited its prominent role compared to GelMA in the fibrous collagen network formation and chondrogenesis as ascertained from the biochemical assay, gene, and protein expression. This was further validated by a protein-protein interaction study using Metascape, GeneMANIA, and STRING analysis. These findings demonstrated the role of SF in the modulation of Wnt/ β -catenin and TGF- β signaling pathways. Therefore, the arch-like 3D bioprinted constructs exhibit a significant promise for the regeneration of articular cartilage.

In our second study, we developed a shear-thinning bioink multicomponent in nature composed of Alginate and Gellan Gum with acceptable mechanical and printing capabilities.

The bioink was reinforced by adding two kinds of silk nanoparticles (SNP), i.e., native and regenerated, improving the printability. The bioink with the SF solution acts as a control set. The rheological and mechanical properties of the Alginate-Gellan Gum bioink were enhanced by the addition of native SNP. However, the control group exhibited better chondrogenesis with BM-MSCs as determined by the RT-PCR, immunofluorescence, and histology analysis. Hence, adding the SNP by us improved the overall printability while maintaining the chondrogenesis of articular cartilage.

Hydroxyapatite (HAP) shows promising applications for treating bone abnormalities. However, traditional foaming procedures could minimally regulate the pore size, geometry, and interconnectivity of 3D porous HAP scaffolds, which limits their clinical success. It is challenging to replicate the complex 3D framework and the effective dynamics of skeletal distortions, emphasizing the need for a personalized, coveted tissue substitute, for which 3D printing offers an alternative. Hence, the third study describes the fabrication of a 3D-printed HAP scaffold and their potential for *in vivo* bone regeneration in tibial defects of rat. Micro-computed tomography (micro-CT) and histomorphometry analysis were used to determine bone ingrowth. Implants at the defect site showed significant bone ingrowth. Micro-CT measurements revealed that the implant's bone mineral density (BMD) at the defect site was $1024 \text{ mgHA ccm}^{-1}$, which was almost 61.5 percent greater than the BMD at the sham control site. The hematoxylin and eosin micrographs also showed no sign of any immunoinflammatory response. The findings imply that the 3D-printed HAP scaffolds provided a sufficient matrix for producing patient as well as defect-specific bone growth.

Developing an *in vitro* aging skin model is challenging since aging is a complex activity throughout a person's lifetime. Moreover, skin is composed of more than a single cell type; hence it is challenging to replicate this composite phenomenon within a short time. Therefore, in our fourth study, we describe the development of a 3D bioprinted skin aging model utilizing SF-G bioink, recapitulating the dual layer of the dermis and epidermis present in native-aged skin. We have exploited the use of the senescent or late passage fibroblast cells, keratinocytes, and melanocytes in co-culture. This was compared with the early passage cells as a control. Reduced fibronectin expression in gene and protein analysis accompanied by a decline in the expression of collagen I, II, and IV demonstrate the potency of our 3D bioprinted model equivalent in recapitulating the attributes of extrinsic aging *in vitro*. Further, we developed two pathological conditions: oxidative stress induced by H_2O_2 and high glucose as other prospective alternative agents in inducing senescence. Our developed 3D bioprinted skin aging

model has numerous uses in basic research on aging, disease modeling, and the screening of active pharmaceutical ingredients.

Decellularized corneas derived from different species have become extremely popular in tissue engineering due to donor tissue scarcity. The decellularized cornea is discovered to elicit an immune reaction despite removing the cellular constituents and antigens since the collagen fibrils are distorted, exposing specific antigenic regions that frequently result in transplant rejection. Hence, our final study highlights the modulation of decellularized corneas from goats using chondroitin sulfate and their implantation into rabbit stroma, citing the distorted collagen conformation getting restored. Healthy monocytes and differentiated macrophages were studied with their surface marker in an *in vitro* immune response study using pHrodo red, Lysotracker red, ER tracker, and CD63, LAMP-2 antibodies that established that when compared to decellularized matrices, cross-linked ones produced the weakest immunological response. Further, histological staining after three months of implantation *in vivo* into the stroma of rabbits validated our *in vitro* findings. As a result, we draw the conclusion that the chondroitin sulfate cross-linked to the decellularized corneal matrix may function effectively as a replacement for allograft and human cadaveric corneas.

सार

पिछले तीन दशकों से, ऊतक इंजीनियरिंग द्वारा क्षतिग्रस्त या रोगग्रस्त अंगों के पुनर्जनन और/या प्रतिस्थापन की दिशा में व्यापक प्रयास किए गए हैं। हालाँकि, मानव ऊतकों की जटिलता के कारण, उपास्थि, हड्डी, त्वचा, कॉर्निया आदि जैसे स्वस्थ ऊतकों के विकास के संबंध में अंग प्रत्यारोपण में केवल छिटपुट सफलता मिली है। इसके अलावा, यह स्वीकार किया गया है कि प्रत्यारोपण के लिए डिज़ाइन किए गए अंगों और ऊतकों को विकसित करना संभवतः जटिल है क्योंकि अंग पुनर्जनन और संबंधित प्रौद्योगिकियों के बारे में हमारा ज्ञान अभी भी न्यूनतम है। पारंपरिक ऊतक इंजीनियरिंग विधियां जटिल बायोमिमेटिक वास्तुकला के साथ वांछित ऊतकों को कॉन्फ़िगर करने में विफल रहती हैं। त्रि-आयामी (3डी) बायोप्रिंटिंग, जिसे 3डी बायोप्रिंटिंग के रूप में भी जाना जाता है, अत्यधिक आशाजनक है जो पारंपरिक ऊतक इंजीनियरिंग प्रथाओं की बाधाओं को दूर करता है। 3डी बायोप्रिंटिंग का लाभ यह है कि यह रोगी की कोशिकाओं का उपयोग करके मूल ऊतक की जटिलता के समान शारीरिक रूप से प्रासंगिक प्रत्यारोपण तैयार करके परत-दर-परत कस्टम-निर्मित, रोगी-विशिष्ट निर्माणों के विकास की अनुमति देता है। इसके प्रकाश में, वर्तमान अध्ययन नरम और कठोर ऊतक पुनर्जनन के लिए 3डी बायोप्रिंटिंग और डीसेल्यूलराइजेशन तकनीकों के अनुप्रयोग पर प्रकाश डालता है, जो स्पष्ट रूप से उपास्थि, हड्डी, त्वचा और कॉर्निया पर ध्यान केंद्रित करता है।

अधिकांश आर्टिकुलर उपास्थि मेहराब में व्यवस्थित कोलेजन II फाइबर नेटवर्क से बनी होती है। 3डी रेशेदार संरचनाओं को विकसित करने में सक्षम उपयुक्त माइक्रोफैब्रिकेशन तकनीकों की कमी आर्क जैसी संरचना के साथ कार्टिलाजिनस ऊतक की इन विट्रो प्रतिकृति में सबसे बड़ी बाधाओं में से एक है। परिणामस्वरूप, पारंपरिक लकड़ी-ढेर पैटर्न के विपरीत, हम पहले अध्ययन में दो अलग-अलग प्रकार के बायोइंक: जिलेटिन मेथैक्रिलॉयल (जेलमा) और रेशम फ़ाइब्रोइन का उपयोग करके कोलेजन II के मेहराब से मिलते-जुलते निर्माण के लिए एक 3डी बायोप्रिंटिंग तकनीक प्रदान करते हैं। जिलेटिन (एसएफ-जी)। मानव अस्थि मज्जा (बीएम-एमएससी) से प्राप्त मेसेनकाइमल स्टेम कोशिकाओं से युक्त एसएफ-जी बायोइंक ने रेशेदार कोलेजन नेटवर्क निर्माण और चॉन्ड्रोजेनेसिस में जेलएमए की तुलना में अपनी प्रमुख भूमिका प्रदर्शित की, जैसा कि जैव रासायनिक परख, जीन और प्रोटीन अभिव्यक्ति से पता चला है। मेटास्केप, जेनेमेनिया और STRING विश्लेषण का उपयोग करके प्रोटीन-प्रोटीन इंटरैक्शन अध्ययन द्वारा इसे और अधिक मान्य किया गया। इन निष्कर्षों ने Wnt/ β -कैटेनिन और TGF- β सिग्नलिंग पथों के मॉड्यूलेशन में SF की भूमिका का प्रदर्शन किया। इसलिए, मेहराब जैसी 3डी बायोप्रिंटेड संरचनाएं आर्टिकुलर कार्टिलेज के पुनर्जनन के लिए एक महत्वपूर्ण वादा प्रदर्शित करती हैं।

हमारे दूसरे अध्ययन में, हमने स्वीकार्य यांत्रिक और मुद्रण क्षमताओं के साथ एलिनेट और गेलन गम से बना एक कतरनी-पतला बायोइंक मल्टीकंपोनेंट विकसित किया। बायोइंक को दो प्रकार के रेशम नैनोकणों (एसएनपी) को जोड़कर मजबूत किया गया, यानी, देशी और पुनर्जीवित, जिससे मुद्रण क्षमता में सुधार हुआ। एसएफ समाधान के साथ बायोइंक एक नियंत्रण सेट के रूप में कार्य करता है। एलिनेट-गेलन गम बायोइंक के रियोलॉजिकल और मैकेनिकल गुणों को देशी एसएनपी के अतिरिक्त बढ़ाया गया था। हालाँकि, नियंत्रण समूह ने आरटी-पीसीआर, इम्यूनोफ्लोरेसेंस और हिस्टोलॉजी विश्लेषण द्वारा निर्धारित बीएम-एमएससी के साथ बेहतर चॉन्ड्रोजेनेसिस का प्रदर्शन किया। इसलिए, हमारे द्वारा एसएनपी को जोड़ने से आर्टिकुलर कार्टिलेज के चॉन्ड्रोजेनेसिस को बनाए रखते हुए समग्र मुद्रण क्षमता में सुधार हुआ।

हाइड्रॉक्सीपैटाइट (एचएपी) हड्डी की असामान्यताओं के इलाज के लिए आशाजनक अनुप्रयोग दिखाता है। हालाँकि, पारंपरिक फोमिंग प्रक्रियाएँ 3डी झरझरा एचएपी मचान के छिद्र आकार, ज्यामिति और इंटरकनेक्टिविटी को न्यूनतम रूप से नियंत्रित कर सकती हैं, जो उनकी नैदानिक सफलता को सीमित करती है। व्यक्तिगत, प्रतिष्ठित ऊतक विकल्प की आवश्यकता पर बल देते हुए, जटिल 3डी ढांचे और कंकाल विकृतियों की प्रभावी गतिशीलता को दोहराना चुनौतीपूर्ण है, जिसके लिए 3डी प्रिंटिंग एक विकल्प प्रदान करती है। इसलिए, तीसरे अध्ययन में 3डी-मुद्रित एचएपी मचान के निर्माण और चूहे के टिबिअल दोषों में विवो हड्डी पुनर्जनन के लिए उनकी क्षमता का वर्णन किया गया है। हड्डी के अंतर्वृद्धि को निर्धारित करने के लिए माइक्रो-कंप्यूटेड टोमोग्राफी (माइक्रो-सीटी) और हिस्टोमोर्फोमेट्री विश्लेषण का उपयोग किया गया था। खराबी वाली जगह पर प्रत्यारोपण में हड्डियों में महत्वपूर्ण वृद्धि देखी गई। माइक्रो-सीटी माप से पता चला कि दोष स्थल पर इम्प्लान्ट का अस्थि खनिज घनत्व (बीएमडी) 1024 एमजीएचए सीसीएम-1 था, जो शम नियंत्रण स्थल पर बीएमडी से लगभग 61.5 प्रतिशत अधिक था। हेमेटोक्सिलिन और ईओसिन माइक्रोग्राफ में भी किसी इम्यूनोइन्फ्लेमेटरी प्रतिक्रिया का कोई संकेत नहीं दिखा। निष्कर्षों से पता चलता है कि 3डी-मुद्रित एचएपी स्कैफोल्ड्स ने रोगी के साथ-साथ दोष-विशिष्ट हड्डी के विकास के लिए पर्याप्त मैट्रिक्स प्रदान किया।

इन विट्रो एजिंग त्वचा मॉडल विकसित करना चुनौतीपूर्ण है क्योंकि किसी व्यक्ति के जीवनकाल में उम्र बढ़ना एक जटिल गतिविधि है। इसके अलावा, त्वचा एक से अधिक प्रकार की कोशिकाओं से बनी होती है; इसलिए इस समग्र घटना को कम समय में दोहराना चुनौतीपूर्ण है। इसलिए, हमारे चौथे अध्ययन में, हम एसएफ-जी बायोइंक का उपयोग करते हुए एक 3डी बायोप्रिंटेड त्वचा उम्र बढ़ने वाले मॉडल के विकास का वर्णन करते हैं, जो मूल-वृद्ध त्वचा में मौजूद डर्मिस और एपिडर्मिस की दोहरी परत को दोहराता है। हमने सह-संस्कृति में सेन्सेट या देर से पारित फ़ाइब्रोब्लास्ट कोशिकाओं, केराटिनोसाइट्स और मेलानोसाइट्स के उपयोग का फायदा उठाया है। नियंत्रण के रूप में इसकी तुलना प्रारंभिक मार्ग

कोशिकाओं से की गई। जीन और प्रोटीन विश्लेषण में फाइब्रोनेक्टिन की अभिव्यक्ति में कमी के साथ-साथ कोलेजन I, II और IV की अभिव्यक्ति में गिरावट हमारे 3डी बायोप्रिंटेड मॉडल की क्षमता को दर्शाती है जो इन विट्रो में बाहरी उम्र बढ़ने की विशेषताओं को दोहराने में समतुल्य है। इसके अलावा, हमने दो रोग संबंधी स्थितियाँ विकसित कीं: H₂O₂ द्वारा प्रेरित ऑक्सीडेटिव तनाव और बुढ़ापा उत्पन्न करने में अन्य संभावित वैकल्पिक एजेंटों के रूप में उच्च ग्लूकोज। हमारे विकसित 3डी बायोप्रिंटेड स्किन एजिंग मॉडल का उम्र बढ़ने, रोग मॉडलिंग और सक्रिय फार्मास्युटिकल अवयवों की स्क्रीनिंग पर बुनियादी शोध में कई उपयोग हैं।

दाता ऊतक की कमी के कारण विभिन्न प्रजातियों से प्राप्त डीसेल्यूलराइज्ड कॉर्निया ऊतक इंजीनियरिंग में बेहद लोकप्रिय हो गए हैं। यह पता चला है कि सेलुलर घटकों और एंटीजन को हटाने के बावजूद डीसेल्यूलराइज्ड कॉर्निया प्रतिरक्षा प्रतिक्रिया उत्पन्न करता है क्योंकि कोलेजन फाइब्रिल विकृत होते हैं, जो विशिष्ट एंटीजेनिक क्षेत्रों को उजागर करते हैं जिसके परिणामस्वरूप अक्सर प्रत्यारोपण अस्वीकृति होती है। इसलिए, हमारा अंतिम अध्ययन चोंड्रोइटिन सल्फेट का उपयोग करके बकरियों से डीसेल्यूलराइज्ड कॉर्निया के मॉड्यूलेशन और खरगोश स्ट्रोमा में उनके आरोपण पर प्रकाश डालता है, जिसमें विकृत कोलेजन संरचना को बहाल करने का हवाला दिया गया है। इन विट्रो प्रतिरक्षा प्रतिक्रिया अध्ययन में स्वस्थ मोनोसाइट्स और विभेदित मैक्रोफेज का उनके सतह मार्कर के साथ पीएचरोडो रेड, लिसोट्रैकर रेड, ईआर ट्रैकर और सीडी 63, एलएएमपी -2 एंटीबॉडी का उपयोग करके अध्ययन किया गया था, जिसने स्थापित किया कि डीसेल्यूलराइज्ड मैट्रिसेस की तुलना में, क्रॉस-लिंकड वाले सबसे कमजोर प्रतिरक्षाविज्ञानी प्रतिक्रिया उत्पन्न करते हैं। इसके अलावा, खरगोशों के स्ट्रोमा में विट्रो में आरोपण के तीन महीने बाद हिस्टोलॉजिकल स्टेनिंग ने हमारे इन विट्रो निष्कर्षों को मान्य किया। परिणामस्वरूप, हम यह निष्कर्ष निकालते हैं कि डीसेल्यूलराइज्ड कॉर्निया मैट्रिक्स से क्रॉस-लिंकड चोंड्रोइटिन सल्फेट एलोग्राफ्ट और मानव कैडवेरिक कॉर्निया के प्रतिस्थापन के रूप में प्रभावी ढंग से कार्य कर सकता है।

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