PROSPECTS OF 3D BIOPRINTING AS A POSSIBLE TREATMENT FOR CANCER CACHEXIA

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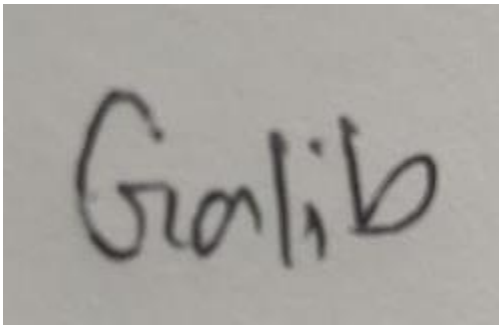
Declaration

It is hereby declared that

1. That the research work reported in this thesis title '**Prospects of 3D bioprinting as a possible treatment for Cancer cachexia**' has been carried out under the supervision of Dr. Iftekhar Bin Naser, Assistant Professor, Program Coordinator, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka and Salman Khan Promon, Lecturer, Department of Life Sciences, Independent University, Bangladesh.
2. This research work presented here is my original work while completing my degree at BRACU University.
3. The thesis has not been submitted to any other institution for any degree or diploma.
4. All the main sources of help have been acknowledged.

Student's Full name & Signature



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Approval

The project titled “Prospects of 3d bioprinting as a possible treatment for cancer cachexia” submitted by Mustafa Galib (18136075) of Spring 2018 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on (28/07/2021)

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Abstract

Cancer cachexia is a multifactorial syndrome that is identified by ongoing muscle atrophy, along with functional impairment, anorexia, weakness, fatigue, anemia, reduced tolerance to antitumor-treatments. Ultimately, reducing the patients' quality of life. Cachexia alone causes about 22-25% cancer deaths. This review covers the symptoms, mediators, available treatment and future prospects of 3D bioprinting for cancer cachexia. Studies about cachexia have shown several factors that drive this disease – protein breakdown, inflammatory cytokines activation and mitochondrial alteration. Even with proper nutrition, physical exercises, anti-inflammatory agents, chemotherapy and grafting attempts, standard treatment has been unsuccessful for cachexia. But use of 3D-bioprinting shows much promise compared to conventional methods by attempting to fabricate 3D-constructs mimicking the native muscle tissues. In this review, some 3D-bioprinting techniques with their advantages and drawbacks, along with their achievements and challenges in in-vivo applications have been discussed. Constructs with neural integration or muscle-tendon units aim to repair muscle atrophy. But it is still difficult to properly bio-print these complex muscles. Although progress can be made by developing new bio-inks or 3D-printers to fabricate high resolution constructs. Using secondary data, this review study shows prospects of why 3D-bioprinting can be a good alternate approach to fight cachexia.

Keywords

Cancer cachexia, muscle atrophy, tissue regeneration, 3D Bioprinting

Dedicated to my family

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List of acronyms

	ACRONYM	FULL FORM
1	MDSCs	Myeloid-derived suppressor cells
2	APR	Acute phase response
3	TGF	Transforming growth factor
4	LAB	Laser assisted bioprinting
5	SLA	Stereolithography
6	PEG	Poly (ethylene glycol)
7	ECM	Extra cellular matrix
8	RGD	Arg-Gly-Asp
9	EHD	Electrohydrodynamic
10	dECM	decellularized extracellular matrix
11	PCL	sized poly(ε-caprolactone)
12	mdECM	Muscle derived extracellular matrix
13	MPCs	mdECM bio ink-printed constructs
14	CPCs	collagen bio ink-printed constructs
15	ITOP	Integrated tissue-organ printer
16	hMPC	human muscle progenitor cell
17	TA	Tubagus anterior
18	hNSCs	human neural stem cells
19	MTU	muscle–tendon unit
20	PU	polyurethane
21	MTJ	muscle-tendon junction
22	CVD	cardiovascular diseases
23	VEGF	vascular endothelial growth factor

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Chapter 1

Introduction

Weight-loss is indicated as a common prognostic factor among cancer patients. But when it occurs for no apparent reason, even if the patient is consuming adequate nutrition, the patient is left wondering what went wrong. Cancer cachexia is a disease combined of several abnormalities, that relates to weight-loss (1). Along with weight-loss, abnormalities like loss of muscle and resistance to insulin is seen as well. Almost 2 million people die annually due to cachexia (2,3). 22-25% of deaths by cancer are reported to be caused by cancer cachexia alone (4).

Cachexia is a multiorgan condition that causes skeletal muscle protein tissue loss or muscle atrophy. The muscle loss can go as high as 75% and 85% loss of total body-fat (5). As a result, it causes functional impairment as the body's skeletal muscle can regenerate lost tissue upon injury till a certain threshold (6). Furthermore, the patient starts to lose 30% of their body mass and without the use of therapeutics it can be fatal (7). Due to how Cachexia acts, the abnormal metabolism affects fat tissues which can target skeletal-muscles, so oncologists are required to estimate the loss of muscle instead of weight (8). A nitrogen flux may occur in the liver from the skeletal-muscle. This decreases the supply of branched-chain amino-acids in the plasma required to activate the muscle protein synthesis (9). Cancer cachexia negatively impacts a patient's quality of life due to decrease of mobility, fatigue and physical activities (5,10).

Molecular mechanism studies regarding cancer cachexia have been undergoing for some time now and it's still not clear exactly what is responsible for its development. Patients face asthenia, anemia, tiredness and anorexia due to increased exposure to surgical, radiotherapeutic, and chemotherapeutic treatment complications (8,11). In several cases, timing of advanced drugs and therapy administration was the reason why there were no beneficial clinical results (12). Patients may experience 3 stages of cancer cachexia according to experts - pre-cachexia, cachexia and refractory cachexia. So, their treatments are suggested to be initiated quickly so that they can prevent or delay refractory cachexia progression (10,13).

At present there is no therapy, medicine or surgery available that is quite effective against cancer cachexia i.e., exempt from side effects. And so, it is recommended for people to strive to implement healthy lifestyles to prevent this condition. In our aging society, there is a huge medical

need for therapies against degenerative muscle disease like cachexia, which is rapidly increasing. Furthermore, cachexia lacks disease-modifying medication (14).

Since its discovery, success of organ and tissue transplantation for saving patients with incurable diseases has been impeccable. But its biggest drawback is the demand has surpassed quantity of donors; especially regarding muscle tissue donors. But alongside availability, limitations of responding to immune system and organ rejection also plays a role. The concept of tissue engineering with 3D bioprinting works to overcome this very limitation (15). 3D bioprinting has become the most promising method in tissue engineering because of its ability to control geometry. Recent advances in 3D bioprinting technologies enable us to bioengineer various functional skeletal muscle tissue constructs with complex geometry. It is capable of fabricating a wide selection of biomaterials with or without cells in a precise and controlled placement (16,17). A 3D printed structure can also stimulate cellular activities which can enhance activity of electrically stimulated muscles tissues. The 3D printed constructs can help to repair or attempt to replace the loss of muscle that is caused by cachexia (15). Despite experiments being limited to rats or time constraints, 3D bioprinting does pose a good and impressive alternative to solving cachexia and its muscle loss.

CHAPTER 2

Causes and Mediators

Dysregulation of metabolism, increasing catabolic drives for breaking down fat/protein and dysregulation of neurohormones are 3 main factors that drive this disease (Figure 1) (8).

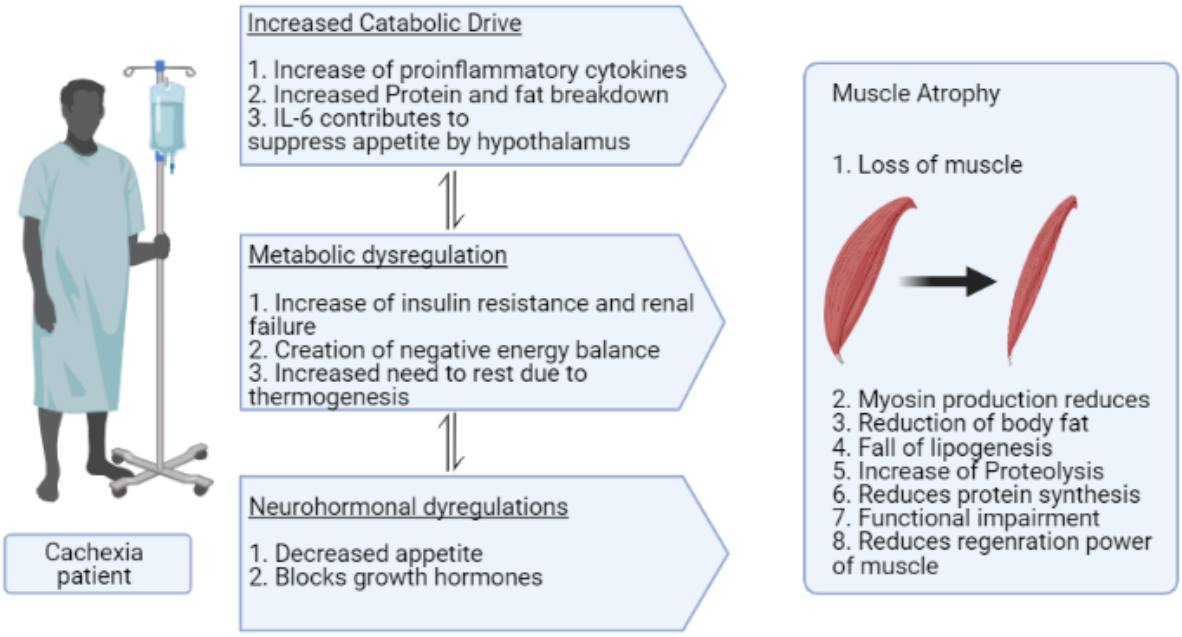

Figure 1. 3 factors that drive cancer cachexia – Increased catabolic drive, Metabolic dysregulations, Neurohormonal dysregulations (8).

Muscle loss usually occurs due to protein breakdown. Cancer cachexia makes myofiber of cell-membrane weak, reduces dystrophin levels and causes muscle dystrophy (18). Cancer cachexia patients mostly have a negative energy balance with increasing need to rest. Their need to rest increases frequently due to constant thermogenesis, i.e., energy used is increased or energy intake is reduced. So, patients with a good diet and nutrition intake will still lose weight. This in turn makes them unable to do physical activities (19,20).

Blood in our body also plays an active role in cancer cachexia. They are means of transportation for tissue wasting tumor mediators that include factors contributing to systemic inflammation (Figure 2) (21). Additionally, suppressor cells derived from myeloid (MDSCs) that expand during cancer development were deemed to be a contributor of murine cancer cachexia. This inducted acute phase response (APR) and changed energy metabolic states (22).

The presence of inflammatory cytokines like TNF-α, IL-6, and IL-1b are mediators that contribute to cancer cachexia (23,24). The activation of TNF plays a role in suppressing appetite which leads to degradation of proteasomal pathway or breakdown of muscle protein with apoptosis (9). This is a kind of alteration in mitochondria of skeletal-muscle, where activation of NF-κB is responsible

for reducing the capacity for muscle oxidation with factors that express mitochondrial biogenesis (9,19). A very recent study had concluded that for cachexia-inducing properties to be expressed, the Fn14 in tumors are required (25).

There is a muscle differentiation and growth regulator which is a negative autocrine, called the myostatin. This signals and activates through pathways associated with ActRII/SMAD2,3 (23,26). Due to tumor burden, activin-A is expressed and secreted in skeletal muscle (27). In recent studies, it was found that TGF-β family members, GDF11 and MIC-1/GDF15 showed signs as cachexia mediators. They exerted additional effects on appetite control through its recently identified receptor GFRAL. TGF-β also causes cancer-associated muscle weakness (27–29). The Malignant tumors are also contributors to muscle atrophy as they are able to deprive tissues of substrates (30).

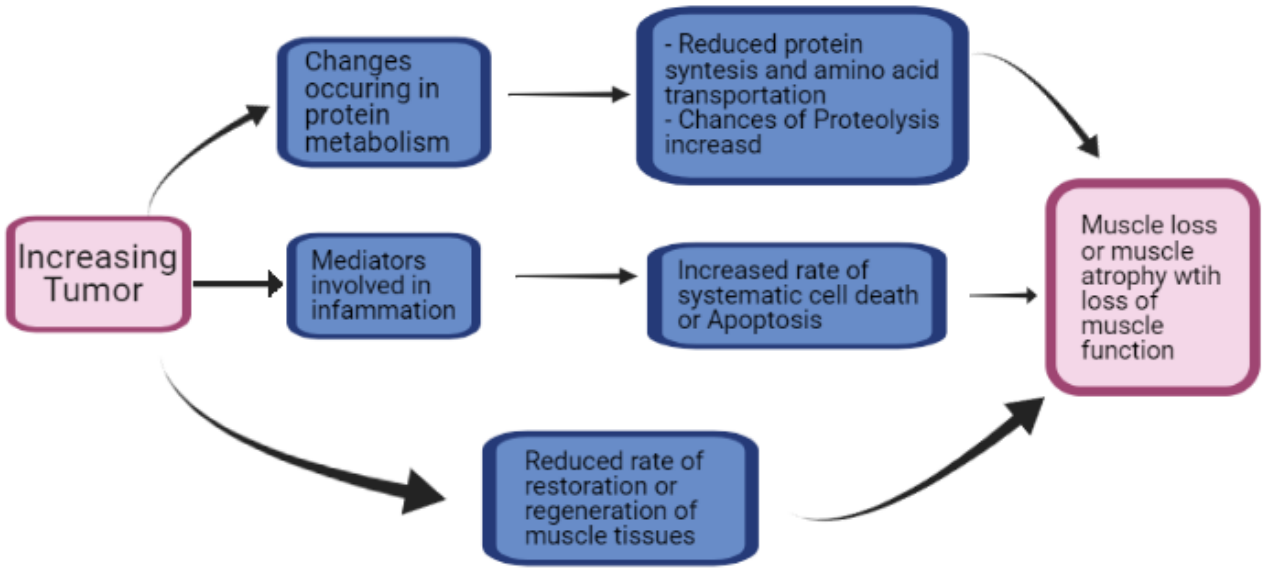

Figure 2. Cancer cachexia causing muscle wasting. Cachexia causes alteration in the protein metabolism and also reduces regeneration ability of muscles. This causes functional impairment due to systemic inflammation (19,21).

Chapter 3

Symptoms and Consequences

The features of cancer cachexia can range from loss of weight/muscle to abnormalities in metabolism. Most common symptoms include fatigue and anemia that tires out the patient more

than usual due to progressive depletion of the body's energy and protein reserves (7,31). Furthermore, it can make patients more susceptible to develop toxicity related to drugs, which also shows poor prognosis (32,33). Along with loss of skeletal muscle, cancer cachexia also causes cardiac-muscle wasting and subsequently causes remodeling and dysfunction of cardiac muscle. This increases the chances of cardiac mortality (34–36). Cancer cachexia also causes alterations in the functions of the liver by increasing energy loss in the process of tumor glycolysis producing and converting lactate to glucose (Figure 3) [37].

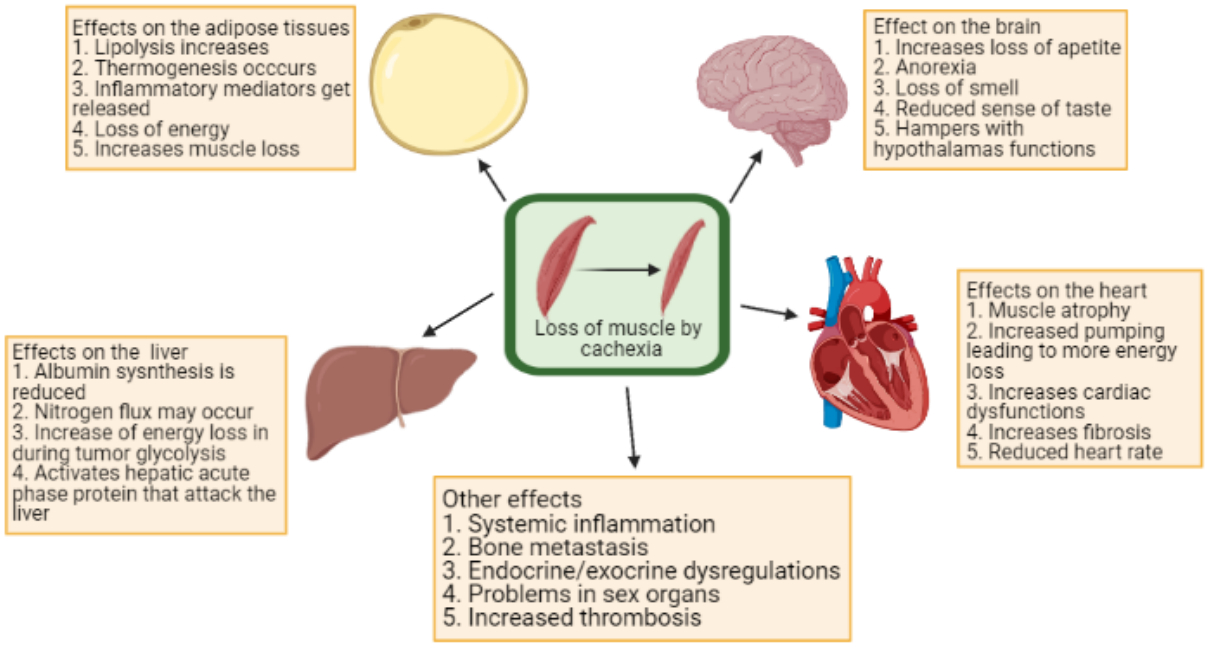

Figure 3. Cancer Cachexia is a multifactorial disease that causes skeletal loss, which in result contributes to several other problems (5,6).

Due to reduced food intake, the patients face chemosensory distress, hyper catabolism and systemic inflammation by abnormal metabolism (13). During chemotherapeutic sessions, patients experience side-effects like anorexia, dry mouth, anemia, asthenia, malabsorption, diarrhea, nausea. They also encounter problems like low-intake of food, body pain, anxiety, depression with insomnia (7,32). Another problem that lies with cachexia is that it cannot be fully reversed by nutritional methods as the anabolic response is altered (11,13). Due to abnormal metabolism, a

study showed that dysfunctional fat storage can lead to hepatic steatosis, insulin resistance and sarcopenia (27,38).

Cachexia also activates hepatic acute phase protein in patients that may promote macrophages produced from IL-1 and IL-6 to infiltrate the liver. This can greatly influence systemic inflammation to escalate in cachexia (27,39,40). Cachexia also promotes loss of bone (Figure 3) (41,42). The metastatic bone invasion due to release of transforming growth factor (TGF)-β contribute to muscle wasting (43)

CHAPTER 4

Available Remedy and Treatments

The role of a proper nutritional diet is very important. Without adequate energy and nutrient supply, it is not possible to increase or stabilize mass and body weight. So, the patients are nutritionally monitored early on before they face a huge degree of body weight loss. These monitoring consists of providing nutritional and metabolic aid to the patients according to their needs (10,13,44). It was seen that fish oil from fatty acids possess the potential to regulate pro-inflammatory cytokines and to increase sensitivity to insulin (45). The branched chain amino acids decrease muscle loss and protein degradation (46). But as it was mentioned before, that this disease cannot be reversed just by providing proper nutrition. It is much more complex than that.

Again, with physical exercises, modulation of skeletal muscle metabolism can help to improve insulin sensitivity, regulate cellular homeostasis and promote myogenesis (47–49). Exercising is necessary for skeletal muscle metabolism (50). But patients with cachexia face frequent difficulty as they have very limited physical capacity. They are also subject to fatigue, anemia, cardiac dysfunctions as well, so physical exercise puts quite a toll on them (51).

There are many anti-inflammatory agents that help to reduce inflammation by cachexia. Corticosteroids are one such anti-inflammatory drug that help to reduce fatigue and increase appetite for a short time (52,53). But they are not recommended as extended use can cause muscle wasting side-effects (54,55). In Addition to that, even though thalidomide has both immunomodulatory and anti-inflammatory properties, it is not recommended due to its severe side effects (56–58). A study showed that by using ActRIIB decoy receptors, activin type II B receptor

pathway can be blocked to bring in resistance to muscle wasting. But it was not successful as it caused patients to suffer from internal bleeding (59,60).

During chemotherapy or chemo-radiotherapy sessions, weight loss of nearly 4-12kg is a common observation mostly due to muscle atrophy. The consequences of using cytotoxic and targeted cancer therapies have direct effects to cause muscle wasting (32).

Autologous muscle transfer is done when muscle atrophy occurs in larger areas except this can cause trauma or even nerve injury hampering motor functions (61,62). Again, grafting of healthy muscle, received from a donor site free of any type of injury, is usually used for restoring the impaired function (63). But such grafting leads to morbidity (64). In addition to that, most grafting procedures can or may fail due to necrosis or even infection from the donor itself (65).

Biological scaffolds are sometimes used in regenerative surgical procedures to repair muscle atrophy. This can help to provide a structural framework (66). However, allograft and xenograft can activate severe response from the immune system causing rejection. This occurs due to the presence of antigens in the donor tissue (67–70).

Cancer cachexia, being a multidimensional syndrome, makes most unimodal techniques unlikely to succeed. All in all, there are no agents, no effective therapy or surgery nor any medicines that are seen to be completely effective for cancer cachexia.

CHAPTER 5

3D bioprinting

3D bioprinting technology is a fairly new strategy that is able to yield positive results regarding regenerative medicine by creating tissue constructs. The disadvantages of scaffold-based tissue engineering technologies can be overcome by the use of printing bio ink layer-by-layer. These mimics the structure of the tissue targeted naturally (71).

Normally a bioprinting process consists of 3 steps which are pre-processing, processing, and post-processing. The first Pre-processing step deals with the digital design that is retrieved via medical images and selected materials. After that, the images designed are transferred to the bioprinting system with the bio-inks loaded. Lastly, for tissue maturation, the printed constructs are put into a bioreactor during the post processing phase [72]

Pre-processing

A blueprint is designed for the tissue or organ along with detailed information its printed structure and it cell locations in 3D. This step is accomplished with the help of a computer. Using many imaging steps, important information of anatomy, histological structure, composition and human organ topology is obtained [73]. Using MRI or CT scans, it is possible to create 3D computer models. CT is frequently used as it is reproducible, nondestructive and it can be used to quantitatively measure biological parameters [74]. The use of computational models can help to improve the design of the constructs as well. [75]. A Bio-CAD system is used to mimic 3D anatomic structures to create the desired tissue models [76]. This combined use of Bio-CAD and Bio-CAM helps in accelerating bioprinting process and printed tissues quality [72].

Processing

A suitable bioprinter loaded with appropriate bio-inks is used to print desired structures during the processing stage. Optimal bio-ink is very important for a smooth printing process. The physical and chemical properties of bio-ink are important to maintain in order to produce constructs [77].

Post-processing

The final step is the maturation of bio-printed tissue constructs before they are implanted into a host. For this a proper bioreactor is required that is able to provide a dynamic environment for maturation and for scaling up. But till now it is quite difficult to get the materials for bioreactors that avoid tissue damage [78,79].

There are three approaches to bioprinting – biomimicry, autonomous self-assembly and mini-tissues (80). Biomimicry helps to reproduce a specific cellular functional component of tissue by mimicking the cellular microenvironment (81). Autonomous self-assembly uses a guide for creating more complexity. This guide has the properties of stem cells and embryonic organs as 3D biostructures (82). Mini-tissues help to print smaller functional building blocks on scaffolds and integrate them into a large macrostructure (83,84).

Inkjet printers are used for both non-biological and biological applications (85). With the availability of commercial products and ease of modification, inkjet bioprinters are often used in bioprinting of tissues and organs (Figure 4). Some major advantages of this are easy accessibility to a bioprinting platform, a high processing speed with a cost that is fairly low and modifiable. But the one major drawback lies in the choice of bio ink material which is quite limited. The material needs to be liquid and viscous enough to be shot out of the nozzle. Cell density is also another

issue because too much can clog the nozzle and damage cells (15,17). The laser assisted bioprinting (LAB) works based on modified laser direct writing and laser induced forward transfer techniques. It can print a wide range of cells and their viability is well retained (Figure 4) (80). At high resolution, LAB can position the small drops of biomaterial to print high cell densities and hydrogel precursors. This can be done with any desired viscosity (86,87). For printing at high resolutions, the time taken is rather slow and not convenient for rapid fabrication (88). The use of lasers with UV light can also affect the cells negatively (89). So, tests are done on recipient cells and tissue for in situ and in vivo bioprinting. Extrusion bioprinters have a broader range of biomaterials. This includes biocompatible copolymers, hydrogels and cell spheroids. They are viscous enough to be printed (Figure 4). But it does have limitations such as cell death via shear stress, limited materials choice due to viscosity and rapid encapsulation of cells (15,17). Stereolithography (SLA) is a process which is powered by a laser assisted bioprinting system. This system creates 3D structures by photocuring photopolymerizable liquid polymers which produces realistic microstructures (Figure 4) (90).

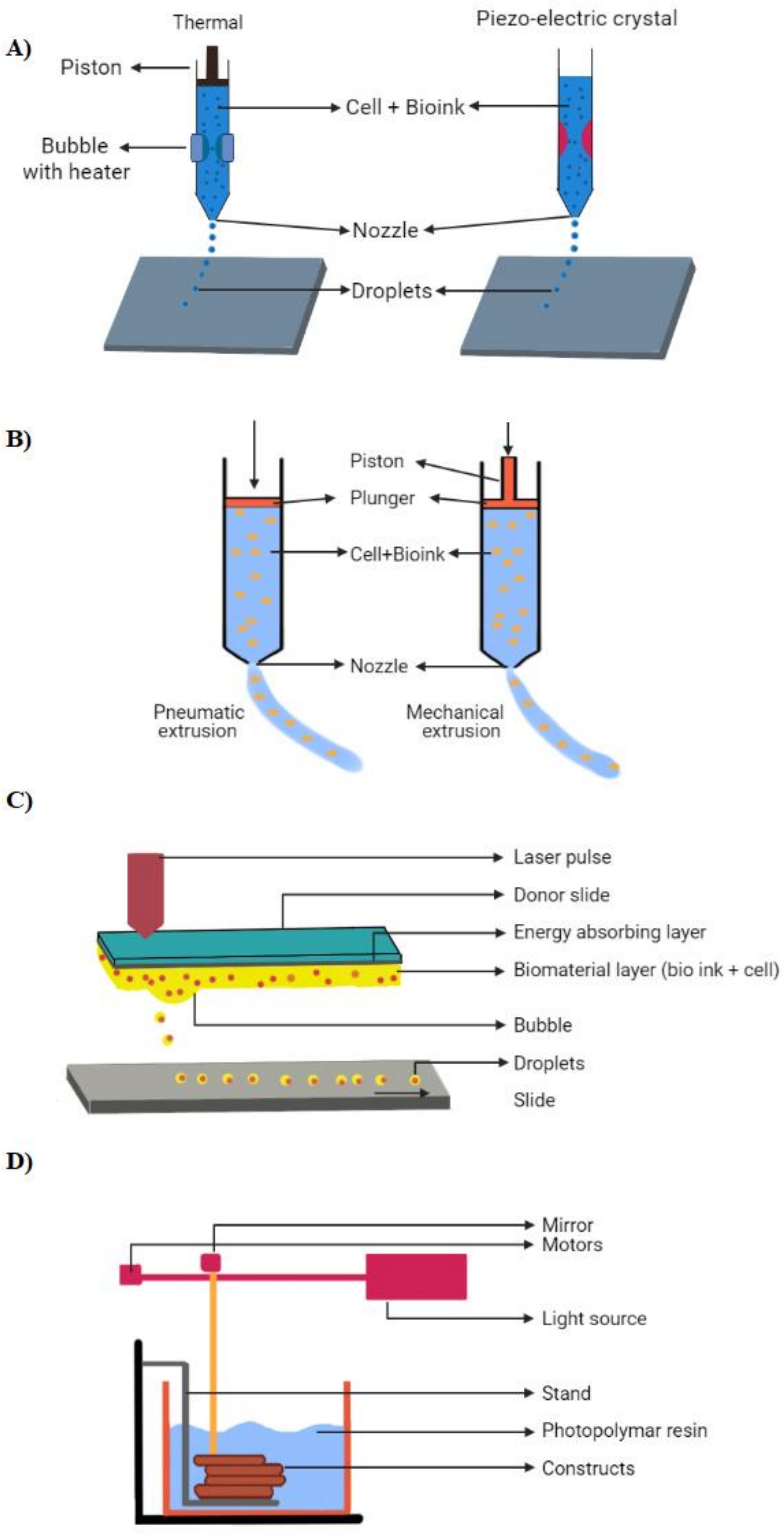

Figure 4. Different methods of 3D bioprinting. A) Inkjet Bioprinting. Droplets with cells are printed out using either thermal heaters or piezoelectric crystals. B) Extrusion bioprinting. It uses a piston to create air pressure or use of mechanical force to get the droplets. C) Laser assisted bioprinting (LAB). The light source helps create a laser that

forms bubbles in the bio-material layer and the droplets are got. D) Stereolithography. Here 3D constructs are created in a layer-by-layer step with the help of photochemical processes (15,67).

CHAPTER 6

Bio-inks

Bio-inks are living cells and biomaterials that can mimic extracellular matrix environment, cell adhesion and proliferation after 3D printing. They are usually suspended cells or tissue spheroids in a liquid solution (91). It is a bio-material that is used in 3D bioprinting to construct a live tissue. It consists of only of cells. Most contains an additional carrier material, made of biopolymer gel, that works as a 3D molecular scaffold. When cells attach to this, they are able to grow, spread and proliferate. Usually natural or synthetic polymers are selected with good biocompatibility. During printing process, it is the bio-ink that provide safety to the cells. Other than that, bio-inks can retain water, making it like a hydrogel with strong mechanical stability (92).

3D bioprinting uses several kinds of bio-inks to construct cell-laden tissue constructs that has strength and can keep cells moist while allowing to print without clogging the nozzle. The materials used are gelatin, Poly (ethylene glycol) alginate, hydrogels, collagen, and hyaluronic acid. Some of the more important features that a bio-ink needs to have are, printability, biocompatibility, mechanical property and ease of spatial arrangement (85). Some of the more important features that a bio-ink needs to have are –

a. Printability: The current 3D bioprinting processes have a limited choice of materials [86]. The bioprinting process requires low viscosity, structural integrity and some crosslinking methods like polymer crosslinking, photo crosslinking, and thermal crosslinking [88,91]. For enhancement of cell viability, the shear thinning ability of bio-inks is ideal.

b. Biocompatibility: Bio-ink should be biodegradable, should not cause inflammatory or immune response and support cell attachments with proliferation in situ. The constructs are designed such that they themselves are harmless to the subject [85].

c. Mechanical property: Bio-ink used in regenerative tissue engineering mimics and maintains the structural and mechanical properties of native tissues to support cell growth. For that, minimum tensile strength, stiffness and elasticity is required [93].

d. Ease of spatial arrangement: Viscosity, along with other properties determine the resolution and micro-scale patterns [17]. The 3D architectural structure is very important for tissue development. For example, some bio-inks have encapsulated cells in alginate [94–99].

CHAPER 7

Printable Biomaterials

A major obstacle for bioprinting is finding out new biomaterials that are printable where cells can survive with their potency intact (100). The biomaterials that are going to be used needs to have an enhanced surrounding that helps host tissue formation. The mechanical strength needs to be strong and stiff enough to provide sufficient support, handling and implantation for the cells (72,101). The biomaterials should have proper viscosity as well so that the internal structures do not break apart (102). The biomaterials should also have properties like maturation, proliferation, biocompatibility, less immunogenic, biodegradability and differentiation (101,103).

The biomaterials used for printing are categorized into synthetic and natural polymers. Synthetic polymers have the mechanical strength needed for printing and processing (104). They help to precisely control molecular weight and functional groups but they lack motifs that are cell-responsive and have cell proliferation that are hindered. On the other hand, natural polymers are biodegradable and biocompatible. On the hindsight, they are mechanically weak as well (105).

1. Some examples of bio-ink can be alginate, gelatin, collagen, fibrin, hyaluronic acid (HA), agarose, chitosan, silk, decellularized extracellular matrix (dECM), poly(ethylene glycol) (PEG), etc. (106). Poly (ethylene glycol) (PEG) is a common synthetic polymer. It has water-soluble properties that is used for cell encapsulation and is more biocompatible because it has functionalized cell adhesion motifs. But on the downside, it is nonbiodegradable [100,107].
2. Collagen is an ECM (Extra cellular matrix) protein that has a triple-helix structure of 3 polypeptide helices 52. Glycine, proline, and hydroxyproline are the 3 types of amino acids that are included in collagen. Collagen is biocompatible and biodegradable and can be used in

regeneration of skin, bone, cartilage, and islets. But is weak mechanically and doesn't support long-term tissue and organ cultures [108–112].

3. Gelatin is derived from native collagen. It consists of RGD (Arg-Gly-Asp) cell recognition signal that can bind to the cell surface receptors. Its properties include water solubility, biodegradable, noncytotoxic, and nonimmunogenic. But these unstable at body temperature [113,114].
4. Fibrin is a natural protein material that can bind to several growth factors [115]. Printed fibrin can release vascular endothelial growth factor (VEGF) and this enhances vascularization. [112,116]. It is used in printing thick vascular networks and tubular tissue structures [115,117].
5. Alginate is isolated from brown algae. It is a polysaccharide whose biodegradability can be increased by oxidation [118]. It the lacks cell response due to cell-adhesive moieties. But it is good for large tissue bioprinting [119,120].

Table – 1 (Source, Advantages and Disadvantages of biomaterials)

Class	Bio material	Source	Advantage	Disadvantage	Reference
Hydrogen based bio-inks	Hydrogels	They are formed in an aqueous medium, by cross-linkage of polymer chains	They maintain high level of hydration, has shear-thinning behavior	They lack mechanical strength	[121–123]
Polysaccharides	Alginate	They are biopolymers, derived from brown seaweed's cell wall	They have biocompatibility, low cytotoxicity, mild gelation process, low cost, mild cross-linking conditions, good printability	They need enhanced biological functions of bio-printed constructs, has limited degradation and cell adhesion is poor	[120,124, 125]

	Agarose	They are polysaccharides extracted from marine algae and seaweed	They have high cell viabilities, express transmembrane protein and have increased cell proliferation.	They are not degradable and has poor cell adhesion	[106,126, 127]
Protein based bio-inks	Gelatin	They are derived from denaturation of collagen	They have biocompatibility, biodegradability, low antigenicity, inclusion of intrinsic RGD motifs, have accessible active groups, absence of harmful byproducts, easy to process and low cost	These unstable at body temperature	[114,128 –131]
	Collagen	They are proteins in the extracellular matrix of mammalian cells	They are biocompatible and biodegradable	They are weak mechanically and doesn't support long-term tissue and organ cultures	[100,107]
Synthetic polymers	PEG	They are by-products from petroleum refining and derived from	They are biocompatible	They are nonbiodegradable	[100,107]

		natural gas or coal			

CHAPTER 8

Approach via 3D bioprinting

8.1 Muscle Tissue Regeneration by Electrospinning

Musculo-skeletal system injuries are quite common and their faulty healing can lead to chronic impairment (135). Several studies and experiments that were based on 3D bioprinting had shown positive results and several advantages in muscle reconstruction (136). Electrospinning is a tool that helps to obtain a fibrous structure. This allows to control arrangements, structural and biochemical properties with the use of synthetic or natural polymers. Miji Yeo and GeunHyung Kim performed a study where micro fibrous bundles were uniaxially stretched to obtain a fully aligned 3D structure. The authors developed a process of electrohydrodynamic (EHD) printing with the help of electrospinning process. They created a 3D fibrous structure consisting of micro sized poly(ε-caprolactone) (PCL) or cellulose fibers (137). There was great biocompatibility of collagen-coated surfaces as well. All the scaffolds showed high cell viability and proliferation but the differentiation was different among the scaffolds. To achieve the optimal stretching, they stretched the randomly distributed fibers where the 3D printed cells showed a homogeneous distribution. Thus, proving that this can promote cellular activities. The final structures that were retrieved were from the native muscle structure. Which meant that muscle tissue regeneration was possible (138). Patients suffering from cachexia face skeletal muscle loss. So, using electrospinning, the muscle tissue regeneration for their muscle loss may be possible with further research and experimentations. The high vitality and proliferation with homogenous distribution that increases cell activities could play a big role when muscle transplants are done to patients.

8.2 Creating 3D functional muscle constructs using Bio-ink and 3D bioprinting

Despite natural hydrogels like collagen, having properties like good proliferation and differentiation, they are mechanically weak and unstable in the mechanical loading process (139). It may not be much feasible in the long run. So, in a study conducted Choi et al, the authors

developed a functional muscle construct by using mdECM (extracellular matrix) bio ink and 3D bioprinting technology. They printed the C2C12 myoblast encapsulated mdECM bio ink to create a 3D muscle construct. They had removed the components and the preservation of extracellular molecules by decellularization process. The shape and porosity of the construct were manipulated to supply nutrients and oxygen to cells of the tissue construct. This helped to enhance cell viability and function (140). The results from the study showed that the mdECM bio ink could print sufficiently to produce various shapes of 3D muscle constructs. This meant that the bio ink can be used in designing and also producing original structures of muscles prior to implantation. High cell viability (>90%) where cell death was minimal, was able to mimic the muscle tissue architecture (141). The cell proliferation in the MPCs (mdECM bio ink-printed constructs) was seen to increase unlike the CPCs (collagen bio ink-printed constructs). The MPCs had superior myogenic gene expression that causes high cell stimulation and myogenic maturation. There was indication of formation of fundamental contractile apparatus that were structurally and functionally mature (142). In addition to that, the 3D-printed muscle constructs were also able to contract in response to electrical stimulation. This study showed that 3D cell-printing technology and mdECM bio ink can provide a biomimetic architecture and induce matured myogenic development (143). This technique via 3D bioprinting shows great promise since the ability to print different 3D muscle constructs that are similar to the original structures with enhanced vitality is present. It has the potential to develop functional engineered muscle that can fight the likes of cancer cachexia. Cachexia patient's lose muscle tissues and cells from their body in different proportions. To be able to replace the lost tissues based on the original architectural structure that was lost can be quite useful.

8.3 Treating skeletal muscle defects using 3D Bio printed Muscle Constructs

Based on their initial success using the ITOP (Integrated tissue-organ printer) system, Kim et al, conducted a study to investigate the feasibility of using 3D bio printed muscle constructs to treat skeletal muscle defects. In this study, they created skeletal muscle constructs with the structural integrity and skeletal muscle tissue organization for functional muscle tissue reconstruction. Using ITOP technology, a skeletal muscle construct was bioengineered with structural organization. The muscle construct had 3 parts- a human muscle progenitor cell (hMPC)-laden hydrogel bio ink, a sacrificing acellular gelatin hydrogel bio ink and a supporting poly(ε-caprolactone) (PCL)

polymer. In the live/dead analysis, the 3D bio printed muscle constructs had multiple myofiber bundles highly organized. It was seen that the bioprinted muscle constructs showed high cell viability compared to the non-printed muscle constructs. In the bioprinted constructs muscle contractile properties which showed that tissue maturation can be accelerated by the 3D printed organized muscle structure. Again, the microchannel structure allowed the diffusion of nutrients and oxygen that maintained cell viability in the bioprinted constructs. These results showed the ITOP system can make skeletal muscle constructs with highly viable, differentiated, densely packed myofibers over a broad range of cell densities. They created a muscle defect by excision of 30–40% of original TA (Tubagus anterior) muscles in mice (144). This defect caused irreversible functional deficits without any treatment (145). The bioprinted muscle constructs were implanted into the defect region. The created defect resulted in severe muscular atrophy in the non-treated. But it was seen that the bioprinted group maintained their original muscle volume. They also showed a significant increase in their tetanic muscle force and TA muscle weight. They had 82% restoration of their TA muscle force compared with normal TA muscle compared to non-printed groups. TA muscle weight in the bioprinted group increased as well. In H&E and Masson's trichrome staining, the bioprinted muscle group was seen to have superior muscle volume maintenance and myofiber formation with organized architecture. The other groups showed limited development. The bioprinted muscle constructs were more mature and maintained their cellular organization for reconstructing the extensive muscle defect injury. The 3D ITOP system used in this study allows current limitations of size and spatial organization for the bioengineered skeletal muscle to be overcome. By simultaneous printing of three components this study was able to create viable skeletal muscle constructs that could mimic cellular function of native skeletal muscle. A microchannel structure was created in the bioprinted muscle constructs because large-scale cell-based constructs limit supply of oxygen and nutrients (146,147). If this was done then induction of necrosis could stop muscle restoration (144,148). This study demonstrated the feasibility using 3D bioprinted muscle constructs containing human primary muscle cells. The attributes and results it showed were very positive. To be able to print a high viable muscle tissue construct from a wide range of cell densities is very impressive. The cachexia patients face loss of muscle and muscle weight. But it was that by using 3D bioprinting with PU and PCL there was an 82% restoration rate of muscle mass and with good maintenance. But further work is still needed to determine if constructs can completely replace native muscle tissues functionally and

structurally for humans. It is because the use of rat cells in this method can hinder translation of drug screening to humans (80,97,149).

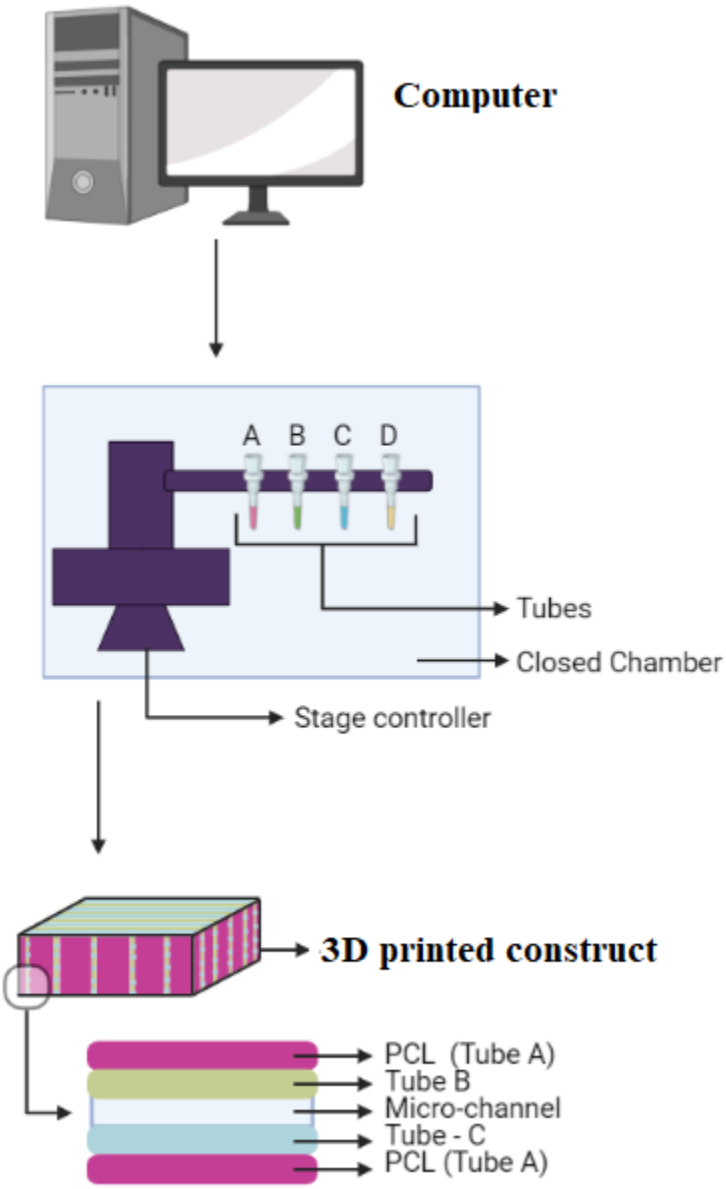

Figure 5. The ITOP system used to create 3D constructs using Bio ink and PCL (80). (Created with BioRender.com)

8.4 Restoration of muscle function by neural cell integrated 3D muscle constructs

The skeletal muscles that are deprived of nerve supply lose their contractility and face muscle atrophy (150,151). Bioengineered skeletal muscle constructs with cultured muscle cells are denervated and require rapid integration with the host nervous system (151,152). If it fails then muscle atrophy will occur and functional recovery will fail. This is something that most studies did not look into much. And so, Kim et al, developed a human skeletal muscle construct that had neural cell integration. It was done by 3D bioprinting human muscle progenitor cells (hMPCs) and human neural stem cells (hNSCs). Neural integration within the construct was able to increase long-term survivability and contribute in maturing bioengineered skeletal muscle construct. The bioprinted constructs were implanted in a rat model of tibialis anterior (TA) muscle defect injury to determine the feasibility of using this method. The 3D bioprinted skeletal muscle constructs had increased the cell survivability and maturation. The bioprinted muscle constructs were implanted in the defected sites for skeletal muscle regeneration. The non-treated group showed no sign of recovery and faced severe muscular atrophy. But the 3D printed group showed restoration of TA muscle volume and weight of TA muscle. There was a 71.42% restoration of muscle force. This showed that for subjects suffering from extensive muscle loss. Introduction of neural cell components in the 3D bioprinted skeletal muscle constructs can enhance the acceleration of muscle restoration and its function. The intervention may take up to 12 weeks in vivo. So, for constructs to restore the function of muscle in vivo, rapid innervation is critical with the host nerve. Interestingly, the muscle weight in the 3D bioprinted group was rapidly recovered. Based on the muscle force measurement, the 3D bioprinted group showed full restoration of muscle force. Thus, our results indicate that the introduction of neural cell components in the bioprinted skeletal muscle constructs could accelerate the muscle restoration. In the non-treated group, the surgically excised regions of the TA showed no sign of muscle regeneration, but fibrotic tissue was formed in the defect region, resulting in muscular atrophy. For the success of the bioengineered skeletal muscle constructs to restore the function of the injured muscle in vivo, rapid innervation with the host nerve is critical. In conclusion, the neural cell component can support bioprinted skeletal muscle constructs in vitro, resulting in rapid restoration of muscle function in the rat TA muscle defect model (153). Quite similarly like the mentioned previous study showed, this method had the same limitation of being experimented on rats. Further research is needed regarding this because this can work with cachexia. The patients lose their muscle tissue even though they are intaking enough nutrition. This problem can be solved if such 3D bioprinting technique is used. This method

showed that there is long survivability and maturation of muscle tissue. 71.42% restoration rate and that too with rapidness is something to incorporate in clinical trials for cachexia patients.

8.5 Engineering integrated muscle tendon unit via 3D bio-fabricating complex structures

Usually tissue-engineered constructs with a porous structure can be manually seeded with cells (154,155). This method has drawbacks like having difficulty to homogeneously seed a scaffold, being unable to distribute multiple cell types and poor control with scaffold microarchitecture. 3D bioprinting printing has the potential to solve these limitations (85,156). For that Tyler et al, made 3D bio fabrication of complex structures. They used multiple synthetic biomaterials and multiple cell types to engineer an integrated muscle–tendon unit (MTU). Two synthetic polymeric materials as the scaffolding component and two cell-laden hydrogel-based bio inks as the cellular component were used for the MTU construct. The scaffolding component served as the biomechanical and functional structure, while the cellular component as the biological source of tissue development. The MTU construct was constructed with thermoplastic polyurethane (PU) and C2C12 myoblasts for the muscle side and poly(ε-caprolactone) (PCL) and NIH/3T3 fibroblasts for the tendon side. These two were chosen as PU can mimic muscle's elasticity and PCL can mimic tendon's stiffness. The PU side was more elastic than the PCL side although the tensile strength did not differ. To re-create the MTU, a construct with three distinct regions was made - a muscle side with printed PU, a tendon side with printed PCL and a MTJ (muscle-tendon junction) region with overlapped PU-PCL. It was seen the cells survived the printing process and started to develop into a linearized tissue. It mimicked the biological architecture of natural muscle and tendon. In addition to that, it was observed that dense collagen deposition had formed by the NIH/3T3 cells. This marked the initial development of the tendon. This led to a high cell viability with C2C12 (92.7 ± 2.5%) and NIH/3T3 (89.1 ± 3.3%) (157). It was seen that the cells retained their original position and organized themselves into a consistent pattern. They were able to show that there was an increase of transcription of the focal adhesion markers. The advantage of having constructs made from synthetic polymers and cell-laden bio inks offers the ability to expose them to biomechanical stimulation. So, they were able to print cells with good viability. These cells are aligned into highly-aligned morphology of muscle and tendon, and have increased MTJ-associated gene expression. One limitation in this study was noticed which was the time needed for constructs to be cultured. A relatively longer time frame was needed to generate a complete integrated muscle–

tendon tissue unit. It is because the MTJ development requires collagen deposition before focal adhesions can form between the muscle and tendon (98). This study showed that it is possible to print muscle cells using 3D bioprinting. The end products that would be implanted in the cachexia patient would be structurally and biomechanically functional and have normal biological tissue development. The 3D construct after being printed becomes a linearized tissue that is able to imitate a natural muscle tissue. This can pave the way to restore the loss of muscle caused by cachexia. The time limitation seems only like a small drawback for a better life ahead.

CHAPTER 9

Use of 3D bioprinting in other fields and their limitations

3D bioprinting has been experimented and researched about for a while now. It is being used to treat cardiovascular diseases (CVD) as well. Experiments by printing 3D constructs and implementing them on mice and several other trials are being conducted. The use of tissue implants via grafting has been done earlier but the issues with tissue rejection and lack of donors causes problems [108–110]. CVD leads to cell structures of the heart to deteriorate and this requires replacement so that the prognosis of patients can be improved. 3D bioprinting technology is being used to make these replacements. The construction of cardiac patches using biomaterials and bio inks has been done to restore functions of the damaged myocardium [111]. But scaffolds via 3D bioprinting have shown rapid degeneration with mechanical instability [112,113]. Atmanli et al, constructed 3D functional cardiac patches which were able to maintain the structure of the myocardial tissue (112). In Another study led by Ong et al, they were able to make 3D biomaterial-free cardiac patches that was spontaneously beating [113]. Xu et al constructed functional cardiac pseudo tissues with structural support using ink jet printing. When it was subjected to mild electrical stimuli, they showed contractile behavior [114]. However, low viscosity is required for inkjet printers to be compatible. This results in constructs made from ink jet printers to have weaker mechanical properties [77,115–118]. In addition to that, due to a discretized flow, restriction to thin structures is also seen along with excessive thermal stress and the risk of cell lysis [117]. Such situations can have negative impacts on the viability and functionality of cells. Using the LAB system allows high cell density, cell viability and the selection of a single cell for transfer

[109,119]. LAB's resolution depends on many parameters and it also costs a lot, so this system is not commercially available [109,120]. The SLA technique in bioprinting of 3D cardiac patches and heart valves have demonstrated a lot of potential such as reduced time for printing, greater accuracy of fabrication, and higher cell viability [117]. But they also have adverse effects due to the use of lasers and the optics required are quite expensive. Hence the use of lasers can affect cell viability [82]. The construction of tissue with high oxygen-consumption rate is still difficult. When bioprinting vascularized thick tissues, printing capillaries at the submicron scale is difficult [17,121].

The study of 3D bio printed vasculature was conducted in immunodeficient mice to verify the effectiveness. Studies have been able to generate endothelium by colonizing endothelial cells but the native structure is so complicated that it is not easy to replicate them properly [122,123]. To obtain rapid gelation for 3D bioprinting, it was seen that a solution of higher than 15 wt% is best to use for the GelMA/C after numerous trials. Although it became difficult to handle when the concentration of the bio ink solution went over 30 wt%. It was mostly due to high viscosity. But the major advantage is that the 3D bio printed vasculature replicates biomimetic vessel structures that contain smooth muscle and endothelium. So, researchers are now considering 3D bioprinting of tissue constructs with some optimization that is still required to improve the methods. [124]

Similarly, for skeletal muscle regeneration, 3D bioprinting has come a long way. Several studies have been conducted as well over the years. For example, by the use of electrospinning, muscle tissue can be regenerated with future research and experimentations. The high vitality and proliferation of constructs with homogenous distribution that increases cell activities could play a big role when muscle transplants are done to patients. different shapes of 3D muscle constructs physically printed out according to their native structure has the potential to reduce muscle atrophy. The patients with cachexia encounter loss of muscle tissues even while intaking nutrition on a daily basis. Since cachexia patients lose muscle tissue in an abhorrent way, being able to make replicants of lost tissues based on the original architectural structure can give people hope and the will to keep fighting.

3D bioprinting techniques conducted on mice specimens have shown positive results. It is impressive to print from a wide range of cell densities. Features like high viable muscle tissue constructs with high rapid restoration rate of muscle mass and good maintenance can prove to be very useful. These 3D bio prints which will be implanted in the host subject is assumed to get a

normal biological tissue development which can mimic a natural muscle tissue. They have the capability to work and function like the original muscle that was lost. But since they were conducted on mice it is still not sure how it will work for human grafting and implanting. Studies have shown that it is very much possible to use a 3D bio printed muscle construct and have muscle restoration and maturation. The time constraints can be overcome with future developments

CHAPTER 10

Discussion and Conclusion

Several technologies and methods have been used to generate 3D muscle constructs, but none of these methods has succeeded to mimic the gross native morphology of muscle tissues (174–176). But among these 3D bioprinting technology have emerged as a powerful tool to build bioengineered skeletal muscle constructs. It is because these methods can generate structurally complex cell-based constructs by precise positioning of multiple cell types, bioactive factors, and biomaterials within a single architecture to mimic native tissues (80,95,143,177). 3D bioprinting has been able to construct much more accurate dense, cellularized constructs with rapid maturation (85,95,178). But further research and developments are required in 3D bioprinting for skeletal muscle for humans. In the case of skeletal muscle tissue there are many cell sources available but most of them have a limited capacity to be expanded in vitro. So even with the progression made so far, 3D bioprinting still faces tough challenges. Problems like lacking a proper biocompatible bio-ink that has supportive mechanical properties for 3D cell culture can cause cells to have reduced accuracy and structural organization (95,143). But it does offer hope and a chance for survival. Because in comparison to conventional models, 3D bioprinting can offer more freedom for the development of engineering skeletal muscle tissues (179). Available methods via 3D bioprinting may have their own drawbacks like time constraints, tests limited to mice, etc. But these are just some minor setbacks which can be outdone in the future with more research and experiments.

As methods for 3D bioprinting technology continue to become more widespread, it can be anticipated that the applications regarding 3D bioprinting will improve in the upcoming years given that cells and tissues can be constructed to create 3D bio printed muscle constructs and

tendon units. These alone are enough to take these methods into application for cachexia. Developing new bio inks and printers that are capable of projecting high resolution constructs can help improve the method. More in-depth study regarding muscle tissues and how they function, can also help in future experiments. In the end it is very plausible that 3D bioprinting will ultimately be able to fend off the muscle loss problem caused by cachexia.

CHAPTER 12

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