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A Review On 3D Bioprinting Of Bone Tissues For Healing Human Bone Defects.

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Abstract*:* Bone is a metabolically active organ. Excessive stress or traumatic accidents may cause fractures, and diseases like Osteoporosis and osteomalacia lead to the weakening of bones respectively which further causes defects in them. Although bone has a high capacity for healing, large bone defects cannot be self-healed. They need various surgical interventions such as joint arthroplasty or bone grafting. Such surgical procedures can heal the defect but may cause inflammation at the surgical site, infection, donor-site morbidity, and various other limitations. To avoid these post-surgical problems a three-dimensional bioprinting (3D) of bone tissue is introduced. In this technique, a bone scaffold of precise size and shape is engineered with the help of bioinks comprising suitable biomaterials, cells, and growth factors. This bone scaffold is then implanted in the patient's body through surgical procedures. Bone scaffolds, bio-inks, and growth factors are the important elements of this technique. This technique enables us to construct personalized scaffolds according to patients' needs. High-accuracy simultaneous printing of cells with scaffolds is one of the advantages of 3D printing. In this review, bone defects have been discussed followed by an introduction to 3D bioprinting and its type. Later the following review emphasizes the process of bone tissue bioprinting and the properties of an ideal bone scaffold. Bioinks and the composition used for the process and various growth factors involved in it are mentioned. And at last, the current challenges of 3D bioprinting in the treatment of bone defects in humans were also acknowledged

Keywords: Scaffolds, Bio-inks, GelMa, Bone Regeneration, Bio-compatibility, Extrusion-based Bioprinting, Ink-jet Bioprinting, Laser-assisted Bioprinting, Stereolithography Bioprinting.

Introduction:

Bone is a dynamic tissue crucial for body structure and organ protection, including the brain, heart, and lungs. Bone stores minerals like calcium and phosphorus. bone cells are of three types:

1. Osteoblasts – Form bone by producing its organic matrix.
2. Osteocytes – Mature cells maintaining bone and responding to mechanical stress.
3. Osteoclasts – Break down bone tissue to release minerals, aiding in bone remodeling.

Bones also contain mineralized tissue, marrow, blood vessels, nerves, cartilage, and linings like the endosteum and periosteum

Excessive stress or trauma, such as falls or sports injuries, can cause fractures, including stress fractures. Conditions like osteoporosis, which weaken bones, also increase fracture risk by reducing mechanical stability. Types of fractures include:

1. Open fractures: Bone breaks through the skin.
2. Closed fractures: Bone breaks without piercing the skin.
3. Complete fractures: Bone splits into two or more pieces.
4. Displaced fractures: Bone fragments leave a gap but remain aligned.

Fracture repair methods are chosen based on the type and location of the injury:

1. **External fixation**: Involves surgically implanting devices like pins or screws outside the body to stabilize the fracture. These devices are removed once the bone has healed. This method is commonly used for complex fractures and corrective procedures such as osteotomies.

2. **Internal fixation**: Uses permanent implants, such as plates, screws, or prosthetics, to maintain alignment and stability while the bone heals. Internal fixation is further divided into:

- **Open Reduction and Internal Fixation (ORIF)**: Involves surgically exposing the fracture, realigning the bones, and securing them with hardware.

- **Total Joint Arthroplasty**: A common intervention for severely damaged or osteoarthritic joints. While effective, it carries risks such as inflammation (5% failure rate), infection, implant loosening, pain, or decreased function.

Bone grafting is another technique to repair or replace bone. It involves transplanting bone tissue from another part of the body or a donor to the fracture site to promote regeneration and structural support. However, it also has risks, including infection, graft rejection, insufficient healing, and complications at the donor site. This brings us to the use of 3D bioprinting as an alternative method for producing synthetic bone tissue.

Three-dimensional (3D) bioprinting is an advanced technology that fabricates complex tissue-like structures by depositing layers of living cells, biomaterials, bioinks, and growth factors. It is currently used for printing tissue and organ models to aid in drug research and potential treatments. A notable achievement in 2021 was the production of steak-like cultured meat, composed of bovine cell fiber, offering a sustainable alternative to traditional meat harvesting.

Recent advancements in 3D bioprinting techniques include:

1. **Extrusion-based Bioprinting**: Uses controlled ejection of bio-inks through nozzles to stack layers and build predefined 3D constructs. It supports a wide range of bio-inks.

2. **Inkjet Bioprinting**: Delivers biological inks precisely, allowing the creation of multifunctional and microscopic structures with droplet sizes as small as picoliters.

3. **Laser-assisted Bioprinting**: Enables high-resolution tissue printing with precise cell patterning and small-scale structures.

4. **Stereolithography Bioprinting**: Uses digital micromirror arrays to control light intensity and polymerize light-sensitive materials for detailed structures.

This review highlights the potential of 3D bioprinting in bone tissue regeneration, focusing on its role in healing bone defects. It also discusses bioinks, biomaterials, the importance of scaffolds, and relevant studies supporting the technique's efficacy.

BIOPRINTING IN BONES

Using bio-printed bone grafts instead of allografts could decrease the chance of graft rejection and chronic inflammation. Bone tissue can be created using 3D scaffolds which are then seeded with cells. The development of scaffold is the most important step in the bioprinting of bone and hence is much focused, bone tissue engineering using 3D printing involves several steps:

1. The first step is to capture the death of the defective bone of each patient using CT or MRI scans.
2. A scaffold with precise shape, size, mechanical, and biological is designed using various software tools like 3D slicers.
3. Once the scaffold is printed, cells, and tissues are seeded into it. Finally, the scaffold is implanted into the patient’s body through a successful surgical procedure.

The 3D printing technique consists of the following elements:

1. **Bone Scaffolds.**
2. **Bioinks.**
3. **Growth factors.**

Bone Scaffolds

Bone defects caused by injury or disease often cannot fully heal with mechanical supports alone. Autologous bone grafting, which uses the patient's own bone, is the current standard but has significant drawbacks, such as pain, inflammation, and complications at the donor site. Allografts, derived from other humans, offer an alternative but carry risks like immune rejection. To address these concerns, engineered bone scaffolds have been developed in recent years.

Scaffolds are vital in bone tissue engineering, acting as 3D structures that replicate the essential properties of the natural extracellular matrix. They provide a framework for cells to adhere to, proliferate, migrate, and differentiate into bone tissue. Scaffolds are primarily used to offer mechanical and physical stability to bone. A well-designed scaffold should maintain its mechanical integrity for 1-3 months after implantation, after which it should be absorbed by the body without hindering bone growth.

Porosity is a key feature of scaffolds, as they need to be highly porous with an interconnected pore structure to support cell activity. The ideal pore size for bone tissue engineering is around 325 micrometers, promoting effective tissue regeneration.

Mechanical Properties

The mechanical properties of a scaffold depend on its structure and must be strong enough to endure the implantation process while being suitable for the bone. These properties vary depending on the size and type of bone being repaired.

Chemical Properties

The chemical properties of scaffolds are essential for addressing complications in bone tissue engineering. Since bone tissue repair is a prolonged process, scaffolds must maintain their integrity for at least 3 months. Materials like bio-ceramics (calcium sulfate, silicate-based ceramics), biopolymers (polylactide, polylactide-co-glycolic acid), and hybrid materials are often used, as they support cell growth and scaffold integrity. Hydrogels are particularly useful as they optimize the properties of scaffolds and provide a supportive microenvironment for cells to grow, aiding the formation of a 3D tissue model.

Biological Properties

The scaffold must be biologically compatible and non-toxic to promote cell growth and matrix reproduction. Since scaffolds are not permanent implants, biodegradability is crucial. This allows the scaffold to degrade over time as cells create their own extracellular matrix. The scaffold must be constructed with precise shape and size to match the patient's defect, captured through CT or MRI scans, to ensure effective implantation and functionality.

Recent research has focused on improving scaffolds' mechanical and biological properties. Prof. Derekshanfar and associates found that increasing the surface coverage of the scaffold enhances its fracture toughness and elastic modulus. Prof. Park and colleagues showed that mesenchymal stem cells (MSCs) helped improve outcomes in osteoporosis treatment by enhancing the connection between tendons and bones. Foroutan and associates used electroconductive filaments in scaffolds to take advantage of magnetic properties, preventing the spread of cancer cells and offering a potential treatment for bone cancer.

Bioinks

Bioinks are essential materials used to fabricate 3D tissue-like structures in bioprinting. These materials are formulations of living cells that are designed to be processed by automated fabrication technologies. When developing bioink formulations, several factors must be considered:

1. **Cell Viability**: Ensuring that the living cells in the bioink remain alive and functional.
2. **Printability**: The ability of the bioink to be printed accurately and consistently.
3. **Mechanical Properties**: The strength and stability of the printed structure.
4. **Choice of Biomaterial Inks**: Selecting appropriate biomaterials for the bioink formulation.

Bioinks are not standalone substances but rather complex mixtures, typically based on standardized materials that provide consistency, reliability, and functionality. Some commonly used base materials include:

1. **Alginate**: A plant-based biomaterial known for its excellent biocompatibility and mechanical properties.
2. **GelMA**: A modified form of gelatin that is easily tunable, photo-cross-linkable, and biocompatible.
3. **Collagen**: Known for its biological compatibility and support for cell adhesion, proliferation, and tunable properties.

In bone tissue printing, **stiffness** is particularly important for promoting cell attachment and proliferation, which are necessary for the differentiation of human mesenchymal stem cells (hMSCs) into osteogenic cells. The bioink must mimic the native environment of the target tissue. For bone tissue, the ideal stiffness of the extracellular matrix (ECM) produced by the bioink is estimated to be between 25-40 kPa.

A bioink is typically composed of biomaterials, cells, and growth factors, providing the necessary components to support tissue regeneration and growth.

BIOMATERIALS-

These are the materials with specific biological, chemical, and physical properties and have appropriate functionality. These materials should be biocompatible as they often remain within the body. In the context of bone tissue engineering, a biomaterial should show controlled degradation that is degradation without the generation of harmful byproducts. Vascularization is the next pivotal property of biomaterials in the case of bone. Researchers stated that an ideal biomaterials should exhibit the following properties: -

1. Osteoconductive: The property of biomaterial structure that provide a framework that focuses on new bone formation
2. Osteoinduction: The stimulating property of biomaterial that actively induces new bone formation. Biomaterials containing calcium phosphate, alumina ceramic, titanium, or glass ceramic were classified as osteoinductive.
3. Osteogenesis: Biomaterial properties that actively promotes the formation of new bone tissue by attracting osteoblasts. This could be achieved through a biomaterial stiffness that facilitates osteogenic differentiation, creating voids within the biomaterials that are conducive to cell attachment and differentiation, and implementing stress relaxation patterns that promote bone formation.

Bio-ceramics poses similarities to natural bone structure, osteoconductive, and bioactivity. Therefore, they are one of the highlighted material groups in bone tissue engineering. Also, polymeric-based biomaterial such as hydrogels are used in cell-laden constructs due to their properties of high-water content, easy process, injectability, and favorable microenvironment offering for cell encapsulation such cell-laden hydrogels are the primary building blocks for bioprinting.

Gellan gum hydrogel is investigated as a candidate material for cartilage tissue engineering due to its characteristics such as high biocompatibility, low cytotoxicity, and a similar structure to cartilage. Recardo Levato and his associate studies show articular cartilage resident chondroprogenitor cells (ACPCs) when combined with biomaterials are suitable for tissue engineering. Recardo Levato et al constructed a bio-printed model of articular cartilage by combining ACPCs and MSC-laden bioinks.

Cells in Bioprinting

In addition to printability, bioinks must maintain **cell viability** during and after the printing process, closely mimicking natural bone tissue. To create a functional bio-printed bone structure, various types of cells should be incorporated. These typically include:

- **Bone Marrow Stromal Cells**: These cells support bone formation and repair.
- **Endothelial Cells**: Critical for forming blood vessels, enabling tissue vascularization.
- **Induced Pluripotent Stem Cells (iPSCs)**: These cells can be reprogrammed to differentiate into various cell types, including bone-forming cells.

For bone tissue, osteogenic (bone-forming) and angiogenic (blood vessel-forming) cells are essential. Mesenchymal Stem Cells (MSCs), which have osteoinductive properties, are particularly important. These cells can differentiate into osteoblasts (bone-forming cells), supporting the creation of new bone tissue and aiding in the growth and healing of existing bone.

Cell-laden hydrogels offer a significant advantage over cell-free constructs, as they provide a more supportive and functional environment for cell growth, differentiation, and tissue development.

Growth Factors in Bone Bioprinting

Bone is a highly vascularized tissue with strong healing capabilities, but in cases of large bone defects, the natural healing process may not be sufficient. In these situations, bioinks are often formulated with various **growth factors** to support tissue regeneration and enhance healing. These growth factors play a vital role in stimulating the processes necessary for bone repair. Some key growth factors used in bone bioprinting include:

1. **Bone Morphogenetic Proteins (BMPs)**: These are natural growth factors that promote osteogenic differentiation, making them potent bone inducers. BMPs are essential for initiating and supporting bone regeneration.
2. **Statins**: Known for their anabolic effects on bone tissue, statins (like **simvastatin**) can induce osteoblastic differentiation of mesenchymal stem cells (MSCs), enhancing osteogenesis and supporting bone healing. They also work synergistically with BMPs and vascular endothelial growth factors (VEGFs) in bone regeneration.
3. **Fibroblast Growth Factors (FGFs)**: FGFs, particularly **FGF-2** and **FGF-9**, stimulate osteoblast proliferation and play a crucial role in angiogenesis, supporting the formation of new blood vessels in the bone tissue.
4. **Parathyroid Hormone (PTH)**: PTH has the potential to enhance bone regeneration by increasing the number of MSCs and promoting their differentiation into osteogenic cells, aiding in bone repair and regeneration.
5. **Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)**: These drugs can be incorporated to manage inflammation and post-surgical pain, improving the healing environment for bone tissue.

Additionally, **calcium phosphate biomaterials** are ideal for formulating bioinks due to their ability to carry **BMPs**, **platelet-rich plasma**, **strontium ranelate**, and statins. These compounds activate native cells and encourage the generation of functional bone tissue, improving the overall healing process.

Case Studies in 3D Bioprinting for Bone Tissue Engineering

1. **Mingkui Shen's Bioprinting Strategy for Vascularized Bone Tissue**: Mingkui Shen and his team developed a bioprinting approach that allows for the formation of an extracellular matrix (ECM) to support in-situ vascular networks in bone tissue scaffolds. Two bioinks were used:
   - Photo-crosslinked ECM hydrogel combined with bone mesenchymal stem cells (BMSCs) as the primary matrix bioink.
   - Thermo-sensitive hydrogel supplemented with ECM for promoting angiogenesis, used as a secondary bioink.

The team used an extrusion-based 3D printer to create a 3D scaffold with interconnected structures, featuring photo-crosslinking for stability. By incorporating BMSCs and angiogenesis-promoting agents, the scaffold effectively supported vascular tissue formation, crucial for bone healing. This printed scaffold showed potential as a bone graft for large bone defect repairs, helping to regenerate bone tissue by inducing blood vessel formation.

2. **Weiwei Sun's Strontium-Doped Collagen Scaffold:** Weiwei Sun and colleagues developed a bone repair scaffold by integrating strontium-doped mineralized collagen, using an in-vitro biomimetic mineralization technique. The collagen was then mixed with polylactic acid (PLA) through gas extrusion 3D printing.
   The strontium doping improved the scaffold's biocompatibility and supported bone regeneration by preventing osteoclast differentiation. This scaffold promoted osteogenesis and facilitated bone healing.
3. **Xinmin Dong's Gyroid-Shaped Scaffold**: Xinmin Dong's team designed a gyroid-shaped porous scaffold using SolidWorks to optimize its porosity. The scaffold was printed using electroconductive polylactic acid (EC-PLA) and reinforced with a polymeric solution of akermanite bio-ceramic. The gyroid structure offered the necessary mechanical strength and supported the growth of bone cells and blood vessels. Additionally, the scaffold was coated with magnetic filaments to prevent cancer cell growth. The study demonstrated that this scaffold had excellent strength and strain resistance, making it a promising candidate for bone regeneration and cancer prevention.
4. **Anke M. Deleew's Human Osteoblast Scaffold:** Anke M. Deleew and his team created cell-laden scaffolds from primary human osteoblasts, isolated from surgical waste materials. Their study showed that bioprinting with higher cell densities significantly increased the mineral stiffness of the scaffold over a 10-week culture period.
   The bio-inks used were made from gelatin-alginate and graphene oxide hydrogels, which supported osteoblast differentiation and promoted bone mineralization. This research emphasized the importance of high-density cell printing for improving scaffold properties and enhancing bone regeneration.
5. **Xingge Yu's Sr-Substituted GelMa Nanocomposite Hydrogel:** Xingge Yu and colleagues developed a GelMa-based bio ink modified with strontium (Sr) and xenotlite nanocomposites to encapsulate bone marrow mesenchymal stem cells (BMSCs). This hydrogel enhanced both the printing accuracy and the mechanical strength of the scaffold.
   The addition of strontium promoted osteogenic differentiation of BMSCs, making the bioink ideal for bone marrow tissue engineering. This work led to the development of an osteogenic and immunomodulatory bioink, promoting bone regeneration.

Key Insights from the Case Studies:

- **Vascularization**: Shen's research highlights the importance of creating scaffolds that support angiogenesis, which is crucial for repairing large bone defects. Vascular tissue formation accelerates healing and promotes better bone regeneration.
- **Material Innovation**: The use of strontium-doped collagen and electroconductive scaffolds in the works of Sun and Dong underscores the significance of tailoring material properties to enhance biocompatibility and support bone regeneration.
- **Cell Density and Differentiation**: Deleew's work shows that using higher physiological cell densities can improve scaffold properties, leading to better bone mineralization and more effective healing.
- **Tailored Bioinks**: Yu's development of Sr-substituted GelMa nanocomposites demonstrates how modifying bioinks can directly affect the mechanical and biological properties of 3D printed bone scaffolds, which is key for bone tissue engineering success.

These studies emphasize the combination of **advanced bio-inks**, **optimized materials**, and **cellular components** to develop scaffolds that foster effective **bone regeneration** and healing.

CHALLENGES

3D bio-printing technology gains attraction in the field of bone tissue engineering due to its extensive capability of higher precision, ease of use and cost effectiveness for customized requirements. The 3D bio-printing technology provides a number of benefits that have not been before, such as capabilities to generate specific designs, build structure with complicated geometrics and minimize the amount of time and money spent on it. Despite this there are a number of obstacles that need to be conquered before its clinical application may become widespread. Several notable technological challenges and stability issues require resolution, such as reducing surface roughness, managing residual stress, optimizing the interaction between the electron beam and metal powders, mitigating internal structural flaws, and enhancing precision and productivity. These challenges are influenced by factors such as material characteristics, processing capabilities, and equipment capabilities. To implement 3D bioprinting technology on a large scale it is important to overcome these issues.

Furthermore, the demand persists for superior-quality raw materials in 3D printing. Given the unique molding principles employed in this technology, current raw materials typically exist as powders or filaments and must adhere to precise criteria regarding size, distribution, uniformity, oxygen content, and fluidity. These standards are often more stringent compared to those for solid materials. Moreover, these materials must meet biological criteria to mitigate potential biological hazards in medical device applications.

CONCLUSION

The field of 3D bioprinting is a relatively nascent area of study. Building upon established practices for bone replacement surgeries, research endeavors in 3D bioprinting have the potential to significantly influence advancements in bone healing. The ongoing progress in both hydrogel and 3D bioprinting domains can synergistically converge to develop innovative therapies for traumatic bone injuries. Recent advancements in these fields are prompting investigations into the application of novel hydrogel and 3D bioprinting techniques for bone replacement and regeneration.

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