The Use of Human Blood Derived Protein Components and Crosslinked Hyaluronic Acid in Tissue Engineering and Regenerative Medicine

PhD thesis

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1. Introduction

The aim of regenerative medicine and tissue engineering is to repair or replace damaged, missing, old, or diseased cells, tissues, and organs to restore their functions. Generally, the three main areas of tissue engineering are cells, scaffolds, and growth factors. Stem cells are in widespread use in this field because of their ability to form *de novo* tissue and promote innate repair, while scaffolds are applied to arrange them in a three-dimensional architecture. Growth factors complete the role of scaffold materials by contributing to the regulation of stem cell fate. Human blood derivatives are prevalent sources of growth factors; hence, they are often applied in tissue engineering.

The main types of human blood derivatives without red blood cells are serum products and plasma products. The advantage of serum derivatives is that no anticoagulant is needed for their isolation, thus the cytokine milieu is natural. SPRF (serum from platelet rich plasma) is a serum product with noted proliferative and regenerative effect. It may be used for the indication of treating osteoarthritis in earlier stages to ease pain and

improve the joint function, besides, it may be used as a supplementing material in cell culture replacing FBS (fetal bovine serum). However, its aseptic isolation is difficult, thus a medical device, HypACT Inject Auto was developed for serum isolation in a closed sterile system.

The advantage of plasma products is that they are stable due to the added anticoagulants. Their fibrinogen concentration can be multiplied by cryoprecipitate isolation, which makes them promising base materials for scaffold development.

For scaffold preparation, another widely applied material is high molecular weight hyaluronic acid (HA). HA is naturally present in mammalian body, it has advantageous physical and biological properties, like biocompatibility, biodegradability, and non-immunogenicity, besides, it can interact with cells promoting regeneration.

HA degrades *in vivo* rapidly; thus, chemical modification of the chains is necessary to prolong its presence after implantation. Divinyl sulfone (DVS) and 1,4-butanediol diglycidyl ether (BDDE) are crosslinking reagents, which react on the hydroxyl groups of HA.

Crosslinked hydrogels are more resistant against enzymatic degradation, and they are water-insoluble, with increased mechanical properties.

Crosslinked HA was found to be bioinert, thus it needs supplementation to enhance cellular attachment on the hydrogel. Hybrid scaffolds can be fabricated with collagen, chitosan, or silk fibroin among others, or the scaffolds can be coated. The supplementation with human blood derived protein components is another option to improve cell adhesion on the hydrogels.

The structure of HA based hydrogels mimics natural tissues; therefore, they can serve as a synthetic extracellular matrix filling up the space of the missing tissue. They can help organizing the attaching cells into a three-dimensional structure and inducing the remodeling and vascularization of the scaffold. Thus, these scaffolds may be applicable in the cases of soft tissue defects like congenital malformation, extirpation, or trauma, or they may be used as a wound dressing for non-healing skin defects.

2. Objectives

Based on the reported regenerative effects of blood derivatives and biocompatible scaffolds, our aims were the following:

1. Investigating the safety of the medical device HypAct Inject Auto by examining the clinical equivalence of the device derived serum and manually isolated serum.
2. Comparing the effect of differently isolated serum types and fetal bovine serum on the viability and proliferation of mesenchymal stem cells.
3. Developing a crosslinked hyaluronic acid-based scaffold supplemented with human blood derived protein components.
4. Examining the physical properties, biodegradability, and biocompatibility of these scaffolds *in vitro* and *in vivo.*

3. Methods

3.1. Clinical equivalence testing of the medical device, and examining SPRF as medium supplementation

Serum was isolated from whole blood manually and using the medical device. The device derived SPRF contains a few red blood cells, which can be removed by a second centrifugation step. These serum types, and FBS were used as a supplementing material in the medium of mesenchymal stem cells. The viability of the cells was determined by XTT (Cell Proliferation Kit II) measurement, or they were visualized by live-dead staining.

3.2. Development of a biocompatible crosslinked hyaluronic acid-based scaffold

Swelling ratio and enzymatic degradation measurement

High molecular weight hyaluronic acid was crosslinked with BDDE or DVS in 2 and 5% concentrations. The swelling ratio of the crosslinked gels was quantified by weight measurement, while the *in vitro* enzymatic degradation was investigated using Ehrlich's solution, which measures the N-acetyl glucosamine concentration, one side product of HA degradation.

Human blood derived protein supplementation of the scaffolds

Two types of human blood derivatives were used to induce cellular attachment on the scaffolds: SPRF was crosslinked to the matrices using DVS, and fibrin was polymerized into the gels. Fibrin originated from cryoprecipitate, which was isolated from human plasma, and in this case the natural polymerization process of fibrinogen was utilized to immobilize it into the hydrogel.

Structure analysis

The cross-section of the crosslinked and protein containing matrices was visualized by scanning electron microscopy, and the structural changes were followed by Fourier-transform infrared spectroscopy.

In vitro cytotoxicity and cell attachment tests

In vitro cytotoxicity test was conducted with MSCs to examine if the toxic residuals of the crosslinkers were removed. The viability of the cells was measured by XTT. The cell adherence capacity of the hydrogels was determined by MSCs cultured on the hydrogels, and the cells were visualized by live-dead staining. The cell attachment properties of the different hydrogels were compared to each other.

In vivo biocompatibility and remodeling tests

The most promising gel type, 5% DVS containing, fibrin supplemented HA was homogenized and injected into the hindleg of male C57BL/6 mice to investigate *in vivo* biocompatibility and biodegradability. As control 5% DVS containing gels were used without fibrin. After 12 weeks the gels were harvested and examined under a light microscope, their weights were measured, and hematoxylin-eosin staining was conducted to visualize initial remodeling and vascularization.

4. Results

4.1. Clinical equivalence testing of the medical device, and examining SPRF as medium supplementation

The clinical equivalence of device derived SPRF and manually isolated SPRF was tested using mesenchymal stem cells, investigating their viability by XTT. Between manually isolated SPRF, device derived SPRF, and twice centrifuged device derived SPRF no significant difference was observed, however red blood cell containing serum had slightly but significantly lower proliferative effect than FBS. Between the other two SPRF types and FBS no significant difference was observed.

The live-dead staining showed that the density of the cells in the wells was similar in the cases of each serum types and FBS.

4.2. Development of a biocompatible crosslinked hyaluronic acid-based scaffold

Swelling ratio and enzymatic degradation measurement

The swelling ratio of the crosslinked hydrogels was measured to get information about the strength of the crosslinking. 5% crosslinker containing gels were found to have significantly lower swelling ratio than 2% crosslinker containing hydrogels. Besides, DVS containing gels were less swollen than BDDE containing gels if they contained the same amount of crosslinker.

The speed of in vitro enzymatic degradation of the hydrogels was compared to each other, and 5% crosslinker containing gels were found to be significantly more resistant against enzymatic degradation than 2% crosslinker containing ones.

Structure analysis

After protein supplementation the structure of the scaffolds was examined using scanning electron microscopy. The crosssection of the hydrogels was porous, and honeycomb shaped. SPRF was visible as a spongious layer bounding only physically, while fibrin fibers were a part of the scaffold forming a coating on the inner surfaces.

The crosslinking of hyaluronic acid and protein supplementation steps were analyzed using FTIR spectroscopy to monitor chemical changes. When SPRF was linked to the matrices, the spectra were similar to the spectra of crosslinked HA without protein addition, but fibrin linking caused major changes, amide bands were visible indicating that these scaffolds contained more protein.

In vitro cytotoxicity and cell attachment tests

The *in vitro* cytotoxicity test was conducted on MSCs which were cultured together with gel pieces, then their viability was measured. No significant difference was found between control and sample wells indicating that none of the hydrogels was cytotoxic.

In vitro cell attachment test was performed on MSCs cultured on the gel pieces for two weeks and visualized by live-dead staining. More cells could attach onto DVS containing gels than onto BDDE containing gels, besides, fibrin promoted more cell adherence than SPRF.

In vivo biocompatibility and remodeling tests

Homogenized hydrogel was injected into the hindleg of mice to examine *in vivo* biocompatibility and remodeling. The implanted hydrogels were harvested after 12 weeks, and they were found to be intact and hard, but elastic. No inflammation could be observed in the surrounding tissues. Small blood vessels were visible on the surface of the gels. The fibrin containing gels were generally more reddish colored indicating more blood vessels in their structure, besides they attached stronger to the surrounding tissues. The weight measurement did not show significant weight loss.

Hematoxylin-eosin staining was performed on the harvested gels, and both fibrin containing and control gels were infiltrated with connective tissue, but hyaluronic acid gel pieces were still visible. Small blood vessels with red

blood cells were visible especially in the fibrin containing scaffolds.

5. Conclusions

Based on the experiments, our conclusions were the following:

The medical device, HypACT Inject Auto was tested *in vitro* on mesenchymal stem cells, and it was found to be safe to be used for serum isolation, as the device derived SPRF and manually isolated SPRF were clinically equivalent regarding their effects on MSCs viability. Human serum products can also be used as medium supplementation because of their proliferative effect, but the removal of red blood cells is necessary.

Crosslinked HA with human blood derived protein supplementation was used for scaffold development with possible application in soft tissue engineering. The gels, which were crosslinked using DVS had more advantageous physical properties than BDDE containing gels: crosslinking with DVS resulted in lower swelling ratio, slower degradation, and a rougher surface, which promoted cellular adhesion. Besides, fibrin addition was

more efficient than crosslinking SPRF to the matrices based on the FTIR analysis, and *in vitro* cell attachment tests.

According to our experiments 5% DVS containing hydrogel supplemented with fibrin was the most promising scaffold for *in vivo* testing. The homogenized gel was injected into the hindleg of mice and after 12 weeks the gels were found to be biocompatible and resistant against fast degradation. Hematoxylin-eosin staining showed initial vascularization and remodeling on the harvested scaffolds.

We concluded that DVS and fibrin containing HA scaffolds are promising materials for soft tissue replacement, and after further examination they may be suitable for product development in the future.

6. Bibliography of the candidate's publications

Publications related to the thesis:

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