DEVELOPMENT OF BLOOD SERUM-BASED REGENERATIVE THERAPIES FOR THE TREATMENT OF DEGENERATIVE DISORDERS OF THE KNEE JOINT

Doctoral thesis

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Budapest
2019

1. Introduction

Regeneration is the essential ability of the living organisms to replace or re-grow lost and damaged parts of the body. The ability of the human body to regenerate is much more limited than that of the inferior organisms. The only sure cure for diseases involving extensive tissue destruction or even total organ loss is organ transplantation. However the number of organs available for transplantation is limited and the risk of immunological incompatibility and organ rejection is high. Therefore researches developing various regenerative therapies are more and more popular year after year. Cellular and cell-free therapeutic groups can be distinguished within regenerative therapies. Blood separation products is one of the cell-free therapeutic approaches and it is able to induce tissue remodeling at the site of the injury, similar to wound healing. The process is triggered by platelets circulating in the vasculature, which are being activated upon tissue injury causing the release of growth factors stored in the α-granules of platelets into the environment. Fibrin clot resulted from the coagulation cascade provides an appropriate scaffold for immune and somatic cells migrated into the site of injury.

After the proliferation and differentiation of the somatic cells the regeneration is completed by tissue remodeling. Numerous blood separation products are used in medicine for the induction of tissue regeneration, of which platelet rich plasma (PRP) and platelet rich fibrin (PRF) are the most widespread. PRP is isolated using plastic tubes containing anticoagulants. Platelet number is increased by multiple centrifugation of the plasma, and as a last step platelets are activated by bovine thrombin and/or calcium-chloride or freeze-thawing. Finally, the growth factor containing plasma is injected into the site of the injury. Although the application of PRP affects numerous clinical areas, it is most widely used in orthopedics. The drawbacks of PRP include the externally added anticoagulants and platelet activators, furthermore there is no standardized protocol for the isolation procedure. In contrast, PRF is isolated in glass blood drawing tube without any anticoagulant by centrifuging the blood immediately after blood drawing. Platelets are activated in contact with the glass surface, resulting the fibrin clot, which can be used as a cell free scaffold. It is most commonly used in oral surgery and orthopedics, however the drawback of PRF is the loose structure, which is resulted in the disruption of the membrane during suturing.

To eliminate the disadvantages of PRP, hyperacute serum was created which is the serum squeezed out from the PRF clot. Hyperacute serum does not contain any externally added materials, such as anticoagulant or platelet activator. On the basis of our previous results, hyperacute serum enhanches the proliferation of osteoarthritic bone marrow derived mesenchymal stem cells, chondrocytes and osteoblasts significantly higher, than PRP. Osteoarthritis (OA) is the most common degenerative joint disorder, but the causes of the disease are still unknown. Initially, OA has been considered to be a disease of the articular cartilage, but recent researches have indicated that the entire joint is involved in the patomechanism of OA. The enhanced synthesis of inflammatory proteins leads to an increased level of matrix metalloproteinases (MMP) and to a decreased level of matrix protein synthesis, which is resulted in enhanced tissue destruction. The conservative therapy of osteoarthritis is able to reduce pain and inflammation only, while degraded cartilage can be replaced by surgical treatments. Its regenerative therapy including the intraarticular injection of hyaluronic acid and/or PRP aims to decrease the inflammation of the synovium and to regenerate the cartilage and bone tissue.

2. Aims

On the basis of our previous results, hyperacute serum increases cell proliferation of osteoarthritic bone marrow derived mesenchymal stem cells, osteoblasts and chondrocytes significantly higher, that PRP. Moreover, dispite of the similar production method, the composition of PRP and hyperacute serum differs from each other.

On the basis of these results, our current aims were:

1. The quantitative comparison of the composition of PRP, hyperacte serum, blood serum and plasma including ion content, the number of blood cells and the concentration of inflammatory and anti-inflammatory proteins.

2. The development of a complex, three dimensional osteoarthritic knee tissue model, which includes the bone, cartilage and synovial tissues.

The investigation of the effect of hyperacute serum on the protein composition of the tissue culture supernatant and on the cell proliferation rate in our three dimensional knee tissue model. Hyperacute serum was isolated by hypACT Inject device, which is a medical device developed by our research team.

3. The comparison of mechanical and biological properties of PRF membranes isolated by hypACT Inject or blood glass drawing tube. The effect of freeze-thawing and freeze-drying on the mechanical and biological properties of the membranes was also investigated. The final aim was to develope the most appropriate PRF membrane for surgical use.

3. Methods

3.1. Quantitative comparison of the composition of blood serum, plasma, PRP and hyperacte serum

Plasma and PRP were isolated using plastic tubes containing EDTA or citrate as anticoagulent, while serum and hyperacute serum were isolated by glass blood drawing tube.

Laboratory testing of blood derived products

Cell counter was used for the quantitative determination of erythrocyte, leukocyte and platelet number in the serum and plasma fractions. The concentration of ions, such as calcium, magnesium, copper, zinc, iron, sodium, phosphate, and potassium ion and the activity of ALP enzymes were measured using an automated laboratory machine (n=4).

Quantitative measurement of protein composition

The concentration of inflammatory and anti-inflammatory proteins was determined by ELISA and multiplex ELISA assay. (n=8).

3.2. Investigation the effect of hypACT Inject derived hyperacute serum in osteoarthritic knee tissue model

Isolation of hyperacute serum by hypACT Inject device

Hyperacute serum was isolated by hypACT Inject device (n=5, pooled).

Development of the knee tissue model

After harvesting the tissue samples 5 pieces of bone and 4 pieces of cartilage were put into the bottom of each well in a 12 well plate, while 2 pieces of synovial membrane per well were placed into transwell inserts with 0.4 μm pore polyester membrane. The synovial fluid was replaced by cell culture medium. Tissues were stimulated with IL-1β cytokine for 2 days and medium was exchanged and tissues were grown in culture medium containing human serum albumin or hyperacute serum for 5 days.

Tissue viability measurements

Tissue viability was determined on days 2, 5 and 7 using Cell Proliferation Kit II according to the manufacturer's instructions.

Isolation of synovial fluid samples

Excess synovial fluid was harvested from grade 2 and 3 osteoarthritic patients on the basis of Kellegren-Lawrence scale (n=19). Samples were stored at -80 °C.

Cytokine measurement of the tissue culture supernatant and the synovial fluid samples by multiplex immunoassay

A literature review on cytokines in osteoarthritic synovial fluid was conducted and 39 proteins were identified, which were described to play a role in the patomechanism of osteoarthritis. These proteins were measured in the culture supernatant (n=12) and in the synovial fluid samples (n=19) after IL-1β stimulation and on the 3. and 5. day of the treatment by multiplex immunoassay.

3.2. Comparison of PRF membranes produced by glass blood drawing tube and hypACT Inject device

Sample preparation

Fresh, freeze-thawed, and freeze-dried PRF membranes were used during the experiments. Fresh membranes were used immediately after isolation. Freeze-thawed membranes were frozen at −20 °C, overnight and thawed at +4 °C. Freeze-dried membranes were frozen at −80 °C for 30 min and freeze-dried (−54 °C, 12 Pa) overnight.

Tensile Strength Measurement

The tensile strength of the PRF membranes was assessed using universal testing machine. The maximum load at specimen failure was recorded and tensile strength was calculated (n=4).

Surface and structural observation by scanning electron microscopy

The surface microstructure of the membranes was examined by scanning electron microscope. Before microscopic observation the membranes were fixed by glutaraldehyde and dehydrated by increasing concentrations of ethanol.

Live/dead cell staining

Blood cells embedded into the PRF membranes were observed by confocal micropscopy after live/dead staining by Calcein-AM and ethidium-homodimer.

Cell viability measurements of mesenchymal stem cells

MSCs were seeded onto fresh, freeze-thawed, and freeze-dried PRF membranes in 24 well low attachment plates at a density of 35,000 cells/membrane. Cell viability on 1, 7, and 14 days was determined using XTT Cell Proliferation Kit II according to the manufacturer's instructions. The wet weight of the PRF membranes were measured on 1, 3, 6, 8, 10, and 13 days during cell culture period using a digital analytical balance (n=4).

Cell viability measurements of gingival fibroblasts

The same experiment was repeated using gingival fibroblasts. Moreover, the concentration of pro-collagen type I was determined in the culture supernatant on days 3, 5 and 7 (n=4).

Measurement of plasmin enzyme activity

The endogenous degradation potential of the membranes was determined by plasmin enzyme activity test using N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (n=4).

Statistical analysis

The dataset was normally distributed on the basis of the D'Agostino and Pearson omnibus normality test, thus the data were analyzed by Pearson correlation test and by one-way analysis of variance (ANOVA) with Tukey's post-hoc test. The correlation was considered to be very strong when $r>0.75$ and $p<0.05$. Prism 7 software (Irvine, CA) was used for statistical analysis.. Data are presented as mean+ SEM.

4. Results

4.1. Quantitative comparison of the composition of blood serum, plasma, PRP and hyperacte serum

5. Laboratory testing of blood derived products

The ionic balance of PRP was disturbed by EDTA, particularly in case of calcium ion and chelating ions and the activity of ALP enzyme was also inhibited by PRP. Citrate also caused minor differences in the ionic composition of the plasma samples, while the ionic balance and the activity of ALP enzyme remained at the physiological level. The number of leukocytes was the highest in citrate PRP. The number of platelets was significantly higher in EDTA PRP, than in citrate PRP, while in plasma, serum and hyperacute serum the platelet number was extremely low. Obviously, the MPV values were in serum and hyperacute serum zero, because the membranes of the activated platelets were disrupted. There was not any difference in platelet function between EDTA and citrate PRP.

Quantitative measurement of protein composition

The concentration of the systemic inflammatory proteins was similar in EDTA PRP and in hyperacute serum, but the concentration of platelet derived inflammatory and angiogenic

proteins was significantly higher in EDTA PRP compared to hyperacute serum.

5.1. Investigation the effect of hypACT Inject derived hyperacute serum in osteoarthritic knee tissue model

Comparison of the protein composition of osteoarthritic synovial fluid and tissue culture supernatant

Among the investigated proteins the highest concentrations were measured in case of CD163, osteonectin, and matrix metalloproteinase-2 (MMP-2) both in vitro and in vivo. After transforming the dataset by logarithm, a very strong correlation ($r=0.77$, $p<0,05$) was found between the cytokine content of the in vitro and in vivo samples ($n_{synovial\ fluid} = 19$, $n_{supernatant} = 12$).

Tissue viability measurements

Cell viability in bone, cartilage, and synovial membrane increased significantly in response to hyperacute serum treatment during the 5 days long culture period, compared to the human serum albumin control (n = 12).

Cytokine measurement of the tissue culture supernatant

The 39 selected proteins were classified into five different groups according to their function in osteoarthritis:

The concentration of the main osteoarthritic inflammatory cytokines:

The concentration of IL-8 increased significantly in response to IL-1β stimulation, while the concentration of IL-2, TNF-α, IL-8, IL-12, IL-15, IL-17 and IL-18 decreased significantly after the 5 day long hyperacute serum treatment compared to day 0.

The concentration of the osteoarthritic inflammatory chemokines:

After the 5 day long hyperacute serum treatment the concentration of CCL-1, CCL-3, CXCL-10 és CX3CL1/fractalkine was presented in significantly lower concentration, than before the treatment.

The concentration of the osteoarthritic anti-inflammatory cytokines:

The level of IL-4 receptor α and IL-13 was significantly lower in response to the 5 day long hyperacute serum treatment compared to day 0.

The concentration of the osteoarthritic matrix metalloproteinases and aggrecan:

The concentration of MMP-3, MMP-13 and aggrecan decreased significantly in response to hyperacute serum treatment at day 5 compared to day 0.

Biomarkers of tissue remodeling:

The concentration of collagen I alpha 1 and osteonectin increased significantly in response to hyperacute serum teratment until day 3, while the level of RANKL decreased after hyperacute serum treatment until day 5 compared to day 0.

5.2. Comparison of PRF membranes produced by glass blood drawing tube and hypACT Inject device

6. Tensile Strength Measurement

On the basis of our measurements the material of hypACT PRF membrane is more homogenous, than the structure of PRF membrane isolated by glass blood drawing tube. On the basis of the tensile strength values, freeze-thawed hypACT PRF membrane was the strongest.

Surface and structural observation by scanning electron microscopy

The structure of freeze-thawed and freeze-dried membrane was more compact with smaller pores between fibers, than in case of fresh membrane. The isolation method did not cause differences in the structure of the membranes.

Live/dead cell staining

Living cells were observed in fresh membranes, while only dead cells were observed in freeze-thawed and freeze-dried samples.

Cell viability measurements of mesenchymal stem cells

Significantly higher number of cells were able to adhere onto the freeze-thawed and freeze-dried samples, than onto the fresh one. Moreover, the cell proliferation rate after one week was also higher in case of freeze-thawed and freeze-dried samples, compared to fresh membrane. No significant differences were found in the weight changes of the membranes.

Cell viability measurements of gingival fibroblasts

Significantly higher number of cells were able to adhere onto the freeze-thawed and freeze-dried samples, than onto the fresh one. Moreover, the cell proliferation rate after one week was also higher in case of freeze-thawed and freeze-dried samples, compared to fresh membrane. There was not any difference pro-collagen synthesis of fibroblasts between samples.

Measurement of plasmin enzyme activity

The plasmin enzyme activity in freeze-thawed PRF membranes was significantly higher, than in fresh and freeze-dried samples.

5. Conclusions

5.1. Quantitative comparison of the composition of blood serum, plasma, PRP and hyperacute serum

Comparing the composition of blood derivatives, the ionic content of hyperacute serum seemed to be more balanced and more similar to the physiological conditions, than plasma derivatives. Moreover, hyperacute serum containes significantly lower number of platelet derived inflammatory proteins, than EDTA PRP.

5.2. Investigation the effect of hypACT Inject derived hyperacute serum in osteoarthritic knee tissue model

During our experiments a complet knee tissue model was developed, which is the first model contaning all of the three major elements of the knee joint. The cytokine composition of the model is very similar to the composition of the osteoarthritic synovial fluid, thus, it can bridge the gap beetween in vitro cellular and in vivo human experiments. We have shown, that hyperacute serum has strong cell proliferative effect on osteoarthritic bone, cartilage and synovial tissues, furthermore, it is able to decrease the osteoarthritic inflammation and it is able to decerase inflammation and tissue degradation caused by IL-1β cytokine.

5.3. Comparison of PRF membranes produced by glass blood drawing tube and hypACT Inject device

The cell adhesion and proliferation capacity, the structure and the degradation time of hypACT PRF membrane is similar to the PRF membrane isolated by glass blood drawing tube. The material of hypACT PRF membrane is more homogenous, thus it is more suitable for suturing than the membrane isolated by glass blood drawing tube. Freeze-thawing has positive effect on the mechanical and biological properties of PRF membranes. We have shown, that the adhesion of mesenchymal stem cells and gingival fibroblasts was the highest on freeze-thawed PRF membrane, and the activity of plasmin enzyme was also lower in freeze-thawed membranes indicating a longer degradation time. The tensile strength of freeze-thawed membranes was also higher compared to the fresh and freeze-dried samples, especially in case of hypACT PRF membrane. Summarizing our results, the freeze-thawed hypACT PRF membrane is the most suitable for surgical use in tissue regenerative therapies.

6. List of publication

Publications of the current dissertation:

Kardos, D.; Simon, M.; Vácz, G.; Hinsenkamp, A.; Holczer, T.; Cseh, D.; Sárközi, A.; Szenthe, K.; Bánáti, F.; Szathmary, S.; Nehrer, S.; Kuten, O.; Masteling, M.; Lacza, Z.; Hornyák, I. (2019) The Composition of Hyperacute Serum and Platelet-Rich Plasma Is Markedly Different despite the Similar Production Method. Int. J. Mol. Sci., 20: 721

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Cytokine Changes in Osteoarthritic Knee Joint Tissues in Response to Hyperacute Serum Treatment. Cells 8*:* 824.

IF: 5,656

Independent publications:

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