Investigation of viability, morphology and differentiation potential of dental stem cells in the presence of scaffolds

Ph.D. Thesis

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Budapest
2020

1. INTRODUCTION

Replacing dead or lost tissues is one of the most important issues in medical science today. In order to achieve the exact same tissue structure as the original one, many diverse chemical and biological aspects need to be considered. Tissue engineering technique is the exploration and exploitation of these aspects, which was formerly part of bio-material development, but has now grown into a completely separate field within regenerative medicine. The essence of tissue engineering is to create a combination of cells, tissue scaffolds and bioactive molecules that can produce healthy, functional tissue.

The main pillars of tissue engineering are stem cells, which have unlimited self-renewal capacity and are capable of producing differentiated progeny cells. Stem cells can be isolated from adult or embryonic tissues, but the use of the latter both raises ethical and technical issues. Therefore, the focus of regenerative research today is on mesenchymal stem cells that are found in the adult body. Such a group of multipotent stem cells (capable of producing every adult tissue cell type, excluding gametes) was first discovered in the bone marrow, but has since been isolated from several adult tissues, such as tooth-associated tissues. The great advantages of the latter include, among other things, their relatively easy and large availability, high proliferation activity and the differentiation ability into many cell types. Dental stem cells can also be isolated from dental pulp, apical papilla, dental follicule, or periodontal ligament. Accurate exploration of the properties and behavior of these cells (their characterization) is very important as this knowledge will determine whether they can be used later in regenerative therapy, such as dentistry.

Most human tissue cells are surrounded by a support network called the extracellular matrix (ECM), made up of carbohydrates and proteins. One important area of regenerative medicine is to mimic the ECM with tissue supports (scaffolds), however, they are difficult to produce. The ideal scaffold must meet several criteria: it must allow the cells to adhere, migrate, proliferate and differentiate. It also has to facilitate the transport of growth factors to the appropriate location and the optimal supply of nutrients and oxygen, in addition to being biocompatible and biodegradable. Because different cell types prefer different environments during their proliferation and differentiation, optimizing the abovementioned scaffold properties for a given cell type is also crucial.

At present, most of the scaffolds in the literature are based on some kind of polymer molecule. Since natural ECM contains high levels of proteins, i.e. amino acid based polymers (collagen, fibronectin), poly (amino acid) based tissue supports can be promising in

regenerative therapy. Poly (amino acid) based hydrogels form a special class of this type of scaffolds: they are combinations of water and polymeric molecules and thus have the properties of liquid and solid materials. Some examples of these beneficial properties: they are form-fitting, deformable, they support cell migration, nutrient and oxygen diffusion. In addition, they may respond to the changes of an environmental parameter (such as temperature and pH), in which case they are called intelligent materials. However, the preparation of polyamino acid-based gels can be difficult and expensive, so their use as scaffolds in the literature is rare. Focusing on dental stem cells has been carried out by our team exclusively on these types of hydrogels.

Therefore, in my PhD research I investigated the biocompatibility of polyamino acid-based hydrogel scaffolds with different mechanical and chemical properties using stem cells derived from dental origin. I observed the effect of different crosslinkers (cystamine, diaminobutane, lysine) and stiffness modification on cell morphology, viability, and differentiation potential in vitro. I also examined the effect of changes in thiol groups and dopamine content on cell proliferation, migration and differentiation. My goal was to explore and optimize the critical parameters of polyamino acid-based gels, which can be used to create a scaffold that can subsequently be used in regenerative medicine.

2. OBJECTIVES

In my research I investigated the biocompatibility of polyaspartic acid-based hydrogels with different physical and chemical properties in vitro, using stem cells derived from periapical ligament. During my work, my aim was:

1. Cultivation and investigation of morphology and viability of human mesenchymal stem cells derived from periodontal ligament (PDLSC) on polyaspartic acid-based hydrogels crosslinked with **cystamine and diaminobutane.**
2. Cultivation and investigation of morphology and viability of human mesenchymal stem cells derived from periodontal ligament (PDLSC) on polyaspartic acid based hydrogels containing **different amounts of thiol groups.**
3. Investigation of **osteogenic differentiation potential** of periodontal ligament stem cell stem cells on polyaspartic acid-based hydrogels with different physico-chemical properties.

4. Cultivation and investigation of morphology and viability of human mesenchymal stem cells derived from periodontal ligament on polyaspartic acid-based hydrogels crosslinked with **cystamine and lysine.**
5. Cultivation and investigation of morphology and viability of human mesenchymal stem cells derived from periodontal ligament on **dopamine** containing polyaspartic acid-based hydrogels.

The ultimate goal of my work is to create a hydrogel that has ideal physicochemical properties for dental stem cells and can potentially be used as a scaffold in regenerative therapy in the future.

3. METHODS

3.1.Hydrogels

During the experiments we used poly (aspartic acid) (PASP) based hydrogels with different physico-chemical properties provided by the Nanochemical Research Group led by Prof. Dr. Miklós Zrínyi (Semmelweis University, Institute of Biophysics and Radiation Biology). The crosslinking agents used in our experiment were diaminobutane (DAB), cystamine (CYS), lysine (LYS) and gels containing dopamine (DOPA) were also synthesized. Cleavage of the disulfide bridges of cystamine produces cysteamine (CYSE) to form free thiol groups in the polymer gel. The degree of crosslinkers, which gives the molar ratio of crosslinker to monomers, appears numerically (or, in the case of gels containing lysine, as a percentage) in the name of the gels. For example, the number 1/20 represents a crosslinker for every twentieth monomers. The lower the number, the less cross-linking the gel contains, which also affects the physical properties: more cross-linkers results stiffer gels. By altering the chemical structure as well as the amount of the crosslinking molecules, it is possible to facilitate the entry of cells into the polymer matrix, thus allowing three-dimensional cell culturing. Our aim was to create the chemical structure that would support this most.

The gels used in the experiments are listed below.

Gels crosslinked with CYS and DAB: DAB 1/20, CYS 1/20, CYS-DAB 1/20, CYSE-DAB 1/20, DAB 1/40, CYS 1/40, CYS-DAB 1/40, CYSE-DAB 1/40

Gels containing thiol groups and DAB: CYSE 1/2, CYSE 1/5, CYSE 1/10, CYSE 1/20, CYSE 1/40, CYSE 1/80

Gels crosslinked with CYS and LYS: CYS 0% -LYS, CYS 20% -LYS, CYS 40% -LYS, CYS 60% -LYS, CYS 80% -LYS, CYS 100% -LYS, CYSE 20% -LYS

Gels containing dopamine : DOPA 1/10 - CYSE-DAB, DOPA 1/20 - CYSE-DAB

In accordance with the previously developed protocol, the gels were pre-treated in all cases before starting the experiments: soaking in culture medium for 3 hours, followed by drying for 10 minutes and then sterilization for 1 hour under UV light. In one experiment, 7-8 gel samples with different physical or chemical properties were tested. Five replicate experiments were performed on one sample.

3.2. Stem cell isolation and cultivation

Cells were obtained from the connective tissue adjacent to human wisdom teeth. The teeth were surgically removed from healthy young adults in the Department of Oral Diagnostics and Department of Periodontology (Semmelweis University, Budapest, Hungary) under approved ethical guidelines set by the Ethical Committee of the Hungarian Medical Research Council. Periodontal ligament cells were then isolated according to an already established protocol and maintained in a humidified incubator under standard culture conditions (37 °C, 5 % CO_2, 100 % humidity). PDLCs were cultured in alpha modification of Eagle's Minimal Essential Medium Alfa (αMEM).

3.3. Stem cell measurements on hydrogels

Cell morphology was observed under a phase contrast microscope. Microphotographs were taken by a high performance CCD camera (COHU, USA) applying Scion image software.

Cell viability was assessed utilizing the WST-1 cell proliferation reagent, a color reaction method based on the activity of the mitochondrial dehydrogenase enzyme, using a microplate reader.

In order to make the 3D migration of the cells visible under a two-photon excitation microscope, PDLCs were labeled with a vital dye, Vybrant DiD.

For detecting the expected osteogenic differentiation of the cells growing on the different gel types, ALP enzyme activity was assessed on the 3^{rd}, 7^{th} and 14^{th} day after the osteogenic induction.

To determine cell viability and ALP activity, the arithmetic mean values calculated from 15-25 independent experimental data are displayed on the diagrams. Statistical evaluation of the data was carried out by STATISTICA 10 software applying the Kruskal-Wallis non-parametric ANOVA followed by a median test. A difference was considered as statistically significant if $p < 0.05$.

4. RESULTS

4.1. Gels crosslinked with cystamine and diamonibutane

1 and 3 days after seeding of PDLSCs, phase contrast microscopy was used to observe the morphology and growth of seeded cells on 8 PASP-based hydrogels with different physical and chemical properties.

After one and three days of culturing, healthy cells with a fibroblast morphology were observed on stiffer (1/20 crosslinker ratio) DAB crosslinked gels and thiol-containing gels (CYSE). The number of the cells seemed to be the highest on the stiffer thiol containing gels. We also observed a slight tendency for group formation. Among the stiffer and softer gel variants, cells showed more healthy morphology on the stiffer gels on both days, and the highest number of cells could be observed on thiol-containing gels.

The adherence and propagation property of the environment created by the gels were also confirmed by quantitative assay by measuring the viability of the seeded cells using WST-1 reagent. After one and three days of incubation, the highest viability was measured on the stiffer thiol-containing gel (CYSE-DAB 1/20). Higher viability rates were also found on the stiffer DAB crosslinked gels and the softer thiol-containing gels. All other gels had lower graft survival rates, which is consistent with the results observed with phase contrast microscopy.

The results of Vybrant DiD staining correlated with previous results. Again, most cells were observed on thiol-containing gels. Stem cells appear on gels arranged in isles of different sizes. Occasionally, there are protrusions between cell groups, suggesting intercellular communication. In addition, the autofluorescence of the gels is clearly visible on the images, which proves that the image was made inside the gel, so that the cells were able to penetrate the gels. Based on these experiments, thiol-containing and stiffer DAB cross-linked gels also support cell adhesion the most effectively, as well as survival and proliferation.

Since no significant increase, but rather minor decrease in viability was observed between the two measurement points, we carried out longer-term experiments to determine the exact cause. We continued to work with the three best-performing gel types (DAB 1/20, CYSE-DAB 1/20 and CYSE-DAB 1/40), which were the most supportive for cell survival, proliferation and viability.

The results of the first three days were consistent with the results of our previous experiments: phase contrast microscopy showed cells with healthy fibroblast morphology on all three gels. After 7 days, phase contrast microscopy images showed healthy, spindle-like

morphology on all the gels, with a fairly large number of PDLSCs in the visual field. The thiol-containing gels also showed a tendency for clustering in this experiment. After 14 days, a similarly large number of tightly clustered cells were observed on the stiffer gels, while the softer gels continued to show distinct spindle-shaped cells.

The viability assay showed significantly higher values for the stiffer thiol-containing gel on the first day compared to the other two gels. At day 3, the decrease of the values was observed again, but the results started to show an upward trend by day 7, suggesting that the decrease was temporary. PDL cultures form a heterogeneous population, and the mechanical properties of the gels support the proliferation of only a particular subpopulation: a temporary decrease in viability and a longer term increase may indicate selective growth of this population.

By day 14, a further increase in viability was observed for the stiffer gels, which was also statistically significant for the DAB-crosslinked gel. By this time, cells cultured on stiffer gels showed higher viability, but the difference was not significant for the three gels.

Cells growing inward the gels were observed after 7 and 14 days on the two-photon microscopic images. By integrating photos taken at the right height, 3D z-stack images were created to give a more accurate picture of the vertical growth of cells. The images showed that the cells did not only proliferate on the surface but also grew vertically (presumably through enzymatic processes) towards the inside of the gels.

According to the experiments, it seems that stiffer versions of the gels and the thiol content promoted more the adhesion and proliferation of the cells. In the literature, many experiments focus primarily on the mechanical properties of gels, since during adherence, cells receive mechanical feedback from their environment through mechanotransduction, to which they react with appropriate changes in the cytoskeleton and morphology. According to some studies, the stiffness of the scaffold may determine the stem cell line specification. When neurogenic differentiation is the target, softer gels (0.1-1 kPa) are recommended, medium stiffness (8 and 17 kPa) is optimal for myogenic differentiation, and rigid matrices (25-40 kPa) are really suitable for osteogenic differentiation In line with this, the gels we used have the following parameters: softer gels (1/40 cross-linking ratio) have a modulus of elasticity of 7.2-10.5 kPa, while stiffer gels (1/20 cross-linking ratio) have values between 55.3 and 66 kPa.

4.2. Gels containing various quantity of thiol-groups

It can be seen from the previous experiments that changing the amount of thiol groups has an effect on cell adhesion and growth, so in the following series of experiments 6 gels with different amounts of thiol groups were tested. In the lowest thiol-containing gel (CYSE 1/80)

contained a thiol group on every 80th monomer, whereas in the case of the highest thiol-containing gel (CYSE 1/2), every second monomer had such groups.

Phase contrast microscopy images of the first day showed that cells were able to adhere and proliferate on each thiol-containing gel. PDLSCs had a spindle shape, with only a small amount of circular cells or cell debris present in the visual field. By the third day, the cell count had increased, and the higher thiol-containing gels seemed to be more supportive for cell proliferation, as more cells could be observed on their surface than on the lower thiol-containing gels. By the seventh day, on the highest thiol-containing gel, the cells formed a confluent monolayer, and formed a longer shape. The second highest thiol-containing gel's cells became subconfluent. By day 14, the cells formed confluent monolayers on the two highest thiol-containing gels. Other gels also show high levels of healthy cells, but in a decreasing number with the decreasing levels of thiol content.

In the viability assay, gels with the highest thiol group content showed the highest values, while decreasing the number of thiol groups showed a decreasing tendency in viability as well. CYSE-DAB 1/20 used in previous experiments was used as reference gel. Compared to this gel, cells cultured on the two highest (1/2 and 1/5) thiol-containing gels showed significantly higher viability. On the third day, there was a degression in this experiment, which again was only temporary. By day 7, each gel had a higher viability compared to day three, with a particularly spectacular increase in case of CYSE 1/2. The 14-day increase was significant for almost all gels compared to the previous time point. At day 14, only the highest thiol-containing gel showed significantly higher values than the reference gel.

Two-photon microscope images show large numbers of cells on high thiol-containing gels at each of the three measurement times. The z-stack photographs clearly show the vertical migration of cells into the gels on day 14.

From this result it is clear that gels with an amount of free thiol groups greater than 1/10 exert long-term positive effects on cell proliferation, beside temporary impacts. The above mentioned observations may be due to the ability of the free thiol groups of the polymer matrix to bind to the free thiol groups of L-cysteine in the cell membrane. Many biological processes can be affected through the thiol-containing L-cysteine of the cell membrane, such as gene transcription, translation, and cellular metabolism. The increase in the thiol content is likely to have a positive effect on cell adhesion and proliferation in this way.

4.3. Osteogenic differentation

Numerous studies have demonstrated that PDL cells are capable of osteogenic differentiation, and previous experiments have suggested that certain gel types may support this tendency. For this reason, further experiments were performed to detect osteogenic tendency with 4 gels, which produced outstanding results in previous experiments (DAB 1/20 and 3 thiol containing gels, CYSE 1/2, 1/5 and 1/20 gels).

Phase contrast microscopy images of the seventh day showed large numbers of healthy spindle morphology cells on both of the high-thiol-containing gels. On this day, no significant difference could be observed between the control group and the group receiving the osteogenic medium. However, on day 14, cells in the control group showed a more elongated spindle morphology, and the cells were separated. In contrast, there was a tendency for group formation in the osteogenic group, where cells were densely located and showed more compact morphology.

On the third day, photos from the two-photomicroscope microscope showed clear signs of gel surface cell adherence and proliferation. Similar numbers of cells were observed in the remaining days, but no significant difference was found between the groups. In addition, the z-stack photos showed the migration of cells into gels on day 14.

To quantify the process of osteogenic differentiation, alkaline phosphatase (ALP) activity was also measured. There was a difference between the gels at the first two measurement points (i.e. after 3 and 7 days). Cells cultured on DAB 1/20 gel showed minimal osteogenic susceptibility in each group, whereas all thiol-containing gels showed measurable ALP activity in the osteogenic group. Interestingly, the CYSE 1/2 gel showed higher values in both the control and osteogenic groups, i.e. not only induced but also spontaneous activity was detected on this day.

At day 14, DAB 1/20 had no osteogenic activity in any of the experimental groups. However, in the case of thiol-containing gels, an additional increase in ALP value was observed in the osteogenic groups compared to the seventh day. CYSE 1/2 gel showed the highest ALP activity again, while no significant difference was found between the other two gel types. Thus, in addition to cell viability and adhesion, the thiol group content of gels also appeared to affect osteogenic differentiation.

The thiol content of the gels promotes osteogenic differentiation based on the attachment of free thiol groups to cell membrane proteins. As mentioned, the cell membrane contains many proteins that contain L-cysteine. Through its ability to form disulfide bonds, L-cysteine initiates a redox-driven conformational change in membrane proteins, affecting

signaling cascades leading to cell proliferation and differentiation. There are several studies on exactly which signaling pathway leads to osteogenic differentiation in PDL stem cells. Based on these, it is believed that the endogenous hydrogen sulfide produced by the cystathionine-g-lyase enzyme, the Wnt / β-catenin and the p38-MAPK (mitogen-activated protein kinase) pathways play an important role in this effect.

4.4. Gels crosslinked with cystamine and lysine

Instead of DAB, lysine was used as a cross-linker in this series of experiments for its better biodegradability properties as a natural amino acid. The gels in this series of experiments always contained as many crosslinkers as the stiffer gels of the previous experiments, but different proportions of lysine and cystamine. The percentage of lysine cross-linker is indicated in the name of the gels.

Most of the gels showed similar morphology on all the three days: they had a spherical shape that is typical of the beginning of the adhesion process, with only a small amount of cells showing spindle shape or developing smaller protrusions. Most cells showing fibroblast morphology were found on gels containing less lysine.

The viability assay showed that cells cultured on the CYS 100% -LYS gel on day 1 had the highest viability index, consistent with the results of phase contrast microscopic analysis: this gel did not contain lysine, only redox sensitive cystamine. Most gel types showed minor increase in viability between day one and day three. Based on viability values, it seems likely that a 60-80% LYS ratio leads to the highest proliferation activity in PDL cells.

Photographs taken on the third day using a two-photon microscope showed that although only a few cells with non-specific morphology could be observed in the visual field, they appeared to be able to penetrate the gel matrix (due to the green autofluorescence of the gel). The number of cells with healthy morphology varies with the CYS / LYS ratio, the higher the CYS ratio is, the more cells are visible. The highest amount of cells were seen on the CYS 100% -LYS gel, which correlates with our previous results.

Although there are examples of successful in vitro use of lysine-scaffolds in the literature, in summary it was found that the cells were only able to adhere and proliferate on the lysine-containing gels to a limited extent, therefore further their investigation was discarded. This is probably due to the lower mechanical properties and lower stability of the gels, which has a major effect on the cell adhesion, as I have already explained in the previous chapters.

4.5. Gels containing dopamine

In this series of experiments, two different CYSE and DAB crosslinked gels containing dopamine were investigated for 14 days. CYSE-DAB 1/20 gel, which does not contain dopamine, was used as a control.

Phase contrast microscope images show that after one day the cells completely overgrowed the lower dopamine-containing gel surface. The cells showed healthy fibroblast morphology and their proliferation was highly accelerated. The higher dopamine-containing gel had adverse mechanical properties, its rigidity and fragility made it difficult to treat, and cells did not adhere after one day. After 3 days, the culture became confluent with the lower dopamine-containing gel (DOPA 1/20), while very few cells remained on the high-dopamine-containing gel (DOPA 1/10). After 7 and 14 days, the trend described above continued: the highest cell count was observed on the lower dopamine-containing gel, which showed slightly better results than the control, dopamine-free gel.

The highest viability score on the first day was measured for the lower dopamine-containing gel, which was significantly higher than both the control and DOPA 1/10 gels. On the third day, the value decreased for all three gels. Even at this day, DOPA 1/20 showed the highest viability ratio. A similar trend could be seen at day 7 and day 14, however, at the last measurement point, there was no significant difference in viability between the different gels. Both phase-contrast microscopy images and viability assays concluded that dopamine has a concentration-dependent support on cell survival: while lower concentrations had a marked positive effect on cell adhesion and proliferation, higher concentrations had an inhibitory effect which is in accordance with the data in the literature.

Two-photomicroscope images showed similar results to the previous two examinations. On the third day, large numbers of cells were seen both on the lower dopamine-containing and control gel, immersed in the gel matrix, whereas far fewer cells were observed on the higher dopamine-containing sample. In the z-stack images, it was clear that on the DOPA 1/20 gel, cells were able to penetrate into the gel matrix to a depth of about 170 µm (corresponding to about 4 cell layers) while on the control gels they were only able to penetrate to a depth of 60 µm.

Thus, cells have penetrated deeper into dopamine-containing gels compared to previously observed gel variants making this type of gel extremely promising for the cultivation

of dental stem cells. This correlates with literature data: dopamine promotes the migration of MSCs via the D2 receptor and the alternative phosphoinositide-3-kinase / Akt pathway.

The experiment thus suggests that the appropriate concentration is crucial for dopamine usage, which is also consistent with data from the literature (144, 145). Adequate amounts of dopamine have a remarkably positive effect on cell viability and proliferation, making this type of gel extremely promising for stem cell culture. However, further studies are required to optimize dopamine content and to explore the cellular proliferation and differentiation potential of gels.

5. CONCLUSIONS

The novel scientific results of my research work can be summarized as follows:

1. I have shown that mesenchymal human periodontal ligament stem cells adhere and multiply on polyaspartic acid based hydrogels containing cystamine and diaminobutane crosslinkers. Gels containing diaminobutane and thiol groups showed the highest viability of the cells, and cell penetration into the gels was also high. Cystamine and diaminobutane cross-linked gels are thus biocompatible and biodegradable.

2. I have also shown that stem cells derived from periodontal ligament adhere and multiply on polyaspartic acid-based hydrogels containing different amounts of thiol groups. The increase in thiol content implies an increase in cell viability and proliferation, therefore, high thiol-containing gels are particularly suitable for cell culture.

3. I found that the high thiol-containing hydrogels support the osteogenic differentiation potential of the PDLSCs. These gels are also capable of inducing spontaneous osteogenic activity in cells in vitro.

4. I have shown that dental stem cells have only limited ability to adhere and proliferate on polyaspartic acid-based hydrogels containing lysine and cystamine cross-linkers. The extent of proliferation is strongly dependent on lysine concentration.

5. I have established that dopamine-containing hydrogels may be suitable for culturing dental stem cells. Using an appropriate concentration of dopamine, PDL cells exhibit outstanding proliferation and penetration activity.

Our team has developed and tested several novel poly (aspartic acid) based hydrogels in vitro, which have been shown to be biocompatible and suitable for culturing PDL-derived mesenchymal stem cells. In the future, these gels, due to their ability to support osteogenic differentiation, may be useful in regenerative therapy, particularly for bone defects.

6. LIST OF PUBLICATIONS

6.1. Publications related to the subject of the thesis

1. **Orsolya Hegedűs**, Dávid Juriga, Evelin Sipos, Constantinos Voniatis, Ákos Juhász, Abdenaccer Idrissi, Miklós Zrínyi, Gábor Varga, Angéla Jedlovszky-Hajdú, Krisztina S. Nagy: Free thiol groups on poly(aspartamide) based hydrogels facilitate tooth-derived progenitor cell proliferation and differentiation. *PLoS One*. 2019;14(12):e0226363. **IF 2,776**
2. Dávid Juriga, Evelin Sipos, **Orsolya Hegedűs**, Gábor Varga, Miklós Zrínyi, Krisztina S. Nagy, Angéla Jedlovszky-Hajdú: Fully amino acid-based hydrogel as potential scaffold for cell culturing and drug delivery. *Beilstein J Nanotechnol*. 2019;10:2579-93. **IF 2.269**

6.2. Publications independent of the subject of the thesis

1. Perczel-Kovách Katalin, Farkasdi Sándor, Kálló Karola, **Hegedűs Orsolya**, Kerémi Beáta, Cuisinier Frederic, Blazsek József, Varga Gábor: Fogbél eredetű őssejtek hatása a titánimplantátumok osszeointegrálódására patkány farokcsigolya-modellben *Fogorvosi Szemle 110:(1) pp. 7-14. (2017)*