rijksuniversiteit groningen

IS REMAINING INTERVERTEBRAL DISC TISSUE INTERFERING WITH BONE FORMATION DURING FUSION OF TWO VERTEBRAE?

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Abstract

In the practice of spinal fusion (spondylodesis), the intervertebral disc (IVD) in between the problematic vertebrae is removed, and a cage containing bone filler is inserted. As the bone growth, the structure of problematic vertebrae and bone filler will be integrated.

The spinal fusion is performed using a minimally invasive method so-called laparoscopic surgery. It is less invasive but tends to be more difficult for the surgeon to remove the IVD tissue completely. Therefore, there will be remaining tissues in between the discs.

The aim of this study is to investigate whether the remaining tissue interferes the wound healing in bone formation since an inappropriate bone formation can lead to the requirement of second surgery.

The results obtained confirmed that remaining IVD tissue itself does not produce factors which affect bone formation. The tissue does produce factors that affected the viability of the cells, and this influence was dose dependent. Several previous studies have been done to investigate this issue and confirmed different results due to the pleiotropic character of cytokines presence in the process of wound healing or those are produced by IVD itself. Therefore, the best way to improve this study is by doing an animal *in vivo* study since it represents the mechanism of wound healing in spinal fusion in the presence of cytokines better.

Besides the effects of remaining IVD tissue on bone formation, there might be other possible effects such as bone and matrix degradation of IVD close to the site where the fused spine is. In this case, re-instability could happen, leads to the necessity of re-surgery which brings disadvantages to the patients and more risk to the surgeons.

Keywords : Spinal fusion, IVD, cytokines, bone formation

Ethics Statement (Declaration)

I hereby declare that the work in this master thesis entitles 'Is remaining intervertebral disc tissue interfering with bone formation during fusion of two vertebrae' is my original work, under supervision of the mentioned supervisors and using the reference of the properly cited references. This project was a collaboration among Department Biomedical engineering (BME) University Medical Centrum Groningen (UMCG), Department of orthopaedic surgery UMCG, and Department of orthopaedic surgery Rijnstate Hospital Arnhem.

This study was done *in vitro* using the remaining tissues which were collected from department neurosurgery UMCG, under the consent of the neurosurgeon who performed the surgery. All of details regarding the patients as donors of the tissues were kept as confidential. No animal study was involved, but human Mesenchymal Stem Cells (hMSC) were used in this study under the regulation of BME department at UMCG. All of the experiments were done at the laboratory of BME department UMCG mainly at the cell culture laboratory. All of the results including graphs, observed pictures using phase microscope, and the processed data using statistical software were generated from our own experiments.

The results of the research are important for the clinical application because in the practice of spinal fusion surgery (spondylodesis) using laparoscopic surgery, there is remaining tissue that might affect bone formation. Besides the possibility of interfering bone formation, the remaining tissue might also cause bone and matrix degradation due to the excessive immune responses towards the remaining tissue. Bone and matrix degradation might lead to the necessity of second surgery due to re-instability, which can be a disadvantage for the patients, risk for the surgeons and less cost effective.

One of the pitfalls of this research is we were not able to use the same dilutions concentration of tissue extracts for each experiment due to the limited amount of remaining tissue samples we were able to collect from the neurosurgeon. This fact made the comparison of the results obtained from MTT assay and Alizarin assay was difficult.

All of the data are stored by me as the author and the responsible supervisors. The data will be shared depends on the necessity of supporting data for the related studies inside our research group. The data will be stored until required unlimited time. A future publication is also planned either in the form of independent publication or a collaborative publication by considering the results of another supporting study from our research group.

This study was not funded by any specific organization, and there was no involvement of any of the parts which has an interest in any particular result of the study regarding financial and non-financial matters. Therefore, I hereby declare that this study has no conflict of interest.

List of Abbreviations

AF	: Annulus Fibrosus
BMPs	: Bone Morphogenetic Protein(s)
DBM	: Demineralized Bone Matrix
ECM	: Extra Cellular Matrix
hMSC	: Human Mesenchymal Stem Cell
hOb	: Human Osteoblasts
IFN	: Interferon
IL	: Interleukin
IVD	: Intervertebral Disc
MMP	: Matrix MetalloProteinases
MTT	: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NADPH	: Nicotinamide Adenine Dinucleotide Phosphate
NP	: Nucleus Pulposus
PDGF	: Platelet Derived Growth Factor
PH	: Potential hydrogen
TNF	: Tumour Necrosis Factor
TGF-β	: Transforming Growth Factor-β

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1. Introduction

Spinal Fusion and its Implication

Spinal fusion (spondylodesis) is one of the most performed treatments among all of the surgeries related to spine problems. The number of spinal fusion surgery based on the study which was done from 1992-2003 in the US increased more than 500%, and spent 47% out of the total cost of surgeries related spine. [1]

In spinal fusion, the intervertebral disc (IVD) formed out cartilage structures in between the problematic vertebrae will be removed. Subsequently, a cage containing bone filler material is inserted, to maintain height and for stability. As the bone growth, the structure of problematic vertebrae and bone filler will be integrated into one structure. Therefore, more stability is expected after this treatment.

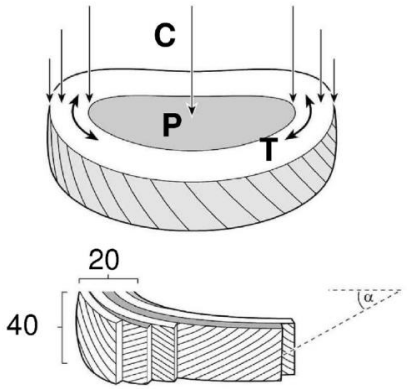


Figure 1. The structure of IVD which is removed during spinal fusion. In the middle is the nucleus pulposus (NP) composed of proteoglycans primarily aggrecan which functions as a shock absorber. The NP is surrounded by the annulus fibrosus (AF) which holds the NP in place when it is loaded. The AF consist of fibro-cartilagenous tissue which is present in a large number of lamellae. The IVD can distribute the received load uniformly throughout the whole structure of the vertebrae. [2]

Nowadays, the spinal fusion surgery is performed using a minimal invasive method so-called laparoscopic surgery. It brings an advantage as it is less invasive. However, in laparoscopic surgery, it tends to be more difficult for the surgeon to remove the IVD tissue completely. Therefore, there will be remaining tissues of NP and AF in between the discs.

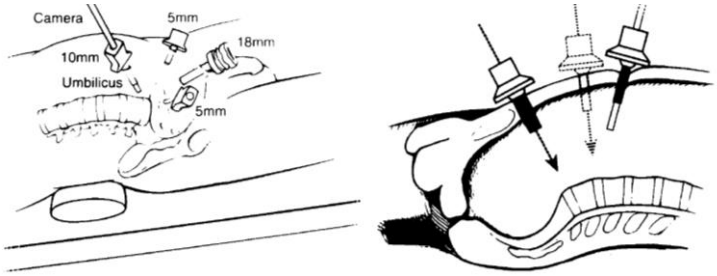


Figure 2. "*Trocars are positioned at the periumbilical site for camera with two 5mm inserted lateral to the inferior epigastric arteries midway between umbilicus and pubis. An 18 mm trocar is placed above the pubis symphisis*" (Left). "*The suprapubic trocar is placed collinier with respect to the disc to be fused*" (Right) [3]

Two examples of spine related problems which are often treated with spinal fusion surgery are scoliosis and spondylolisthesis. Scoliosis is a condition of spine curvature where the angle of curvature is equal or greater than 10º measured from the coronal view [4], whereas spondylolisthesis is a condition where a disc structure of the vertebrae tends to slip more forward (anterior) in comparison to the lower disc. [5]

The clinical symptoms suffered by spondylolisthesis patients are numbness, pain in the lower extremity, pain in the muscle, and vesicorectal disorder. [6] Degenerative scoliosis patients suffer from mild pain to severe pain including neurogenic claudication that can cause a disturbance in walking. [7]

Considering the facts mentioned above, the right treatment indeed is very important. However, the outcomes of the treatment, especially spinal fusion which is one of the most chosen, should be evaluated. Some research had been conducted to evaluate the efficacy of this treatment compared to others such as nerve decompression surgery. [8], [9] Försth et al., stated that nerve decompression takes less time of operation, is simpler, has fewer risks and tends to be more cost effective. [8] However, the exact result is still debatable. There has not been any certainty about the superiority of any treatment over others.

The Fusion Process

In spinal fusion, a cage containing bone filler is inserted. The insertion of the cage is followed by bone formation process. The bone formation is started by inflammation, which is a very essential because it determines fusion rate [10] The sequence is continued by vascularization, when fibrosis tissue is formed. Subsequently, osteoinduction takes part, when differentiation of human Mesenchymal Stem Cell (hMSC) into osteoblast being an important indicator in this sequence. The next sequence is osteoconduction, where the integration between the cage containing bone filler and the host occurs. The process is finalized by remodeling when the

circumference is formed around the fused bone and there is an increase of bone marrow activity. [10]

The material used as bone filler are various such as ceramics which has osteoconductive character, Demineralized Bone Matrix (DBM), which is obtained from allograft and treated to reduce its mineral content, and other materials. Besides the bone filler, synthetic osteoconductive materials were also created to induce a better bone formation. These materials are such as Transforming Growth Factor-β (TGF-β), Bone Morphogenetics Protein (BMPs), and Platelet Derived Growth Factor (PDGF) which enhance the proliferation of hMSC and promote the differentiation of hMSC into osteoblasts. [11]

During the bone formation process, hMSC plays an important role. The formation into bone happens through two pathways, either endochondral or intramembraneous ossification. [12] Through endochondral ossification, hMSC differentiates into chondrocytes, and followed by the secretion of cartilaginous matrix. Through intramembraneous ossification, there is no formation of cartilaginous matrix, thus hMSC can differentiate directly into osteoblast and produce osteoid which has a role in forming the bone. [12]

In intramembraneous ossification, to differentiate into osteoblast, the hMSC accumulate to have a sufficient number of cells, this process is called proliferation. [13] Subsequently, mature hOb produces enzyme alkaline phosphatase and osteoid mainly composed of collagen fiber. By the presence of phosphatase group in alkaline phosphatase, the inorganic phosphate such as β-glicerophosphate is split-off. The split phosphatase reacts with calcium and forms calcium phosphate (hydroxyapatite) at the site where the enzyme works, and cover osteoid. [14] The encapsulation of osteoid in the mineralized matrix induces the formation of osteocytes, which acts as a mechanosensor to give a feedback either to increase production of osteoclast regarding the resorption (breaking the mineralized matrix) or to increase production of osteoblast (continue the production of a mineralized matrix). [15]

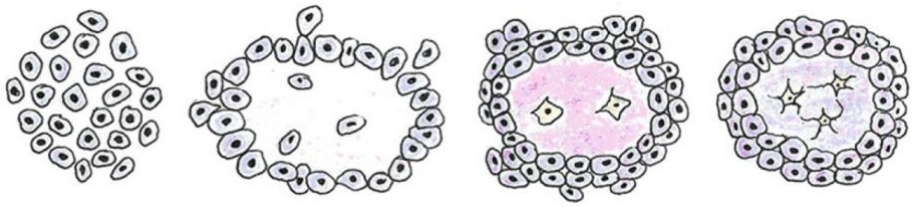


Figure 6. a. The osteoprogenitor (mesenchymal stem cells) aggregate at one site b. Formation of collagen in the middle and in between the cells c. Mesenchymal stem cells differentiate into osteoblasts and produce osteoid in the middle d. mineralized tissue [15]

Since bone is composed of calcium, thus bone formation itself can be observed by using a dye which is named alizarin assay. The dye can bind with calcium or another kind of positively charged ions through its sulfonate and hydroxyl group. [16] The binding depends on the PH. The required PH is in between 4-8. [17] To make a good bonding between stain and calcium, the cells need to be fixed. Alizarin assay is essential to evaluate whether the osteogenic differentiation in the process of bone formation post spinal fusion surgery is affected by the presence of remaining IVD tissue.

The aim of this study is to investigate whether the remaining tissue in the practice of spinal fusion interfere the process of fusion (bone formation), since an inappropriate bone formation can lead to the requirement of second surgery due to the instability in the integrated structure.

Besides bone formation, another aspect which is really important is inflammation. The detail explanation of IVD and Immunological substances is explained below.

IVD and Immunological Substances

Earlier studies have shown that the remnant IVD tissue after surgical removal of the IVD, results in an immunological reaction (wound healing reaction). Some of the research focused on investigating the etiology of IVD degeneration. [18] We focused on the effects of the presence of the IVD tissue post inflammation. The trigger of the inflammation that is being the focus here is the wound healing mechanism resulting from removal of IVD tissue in the process of spinal fusion (surgical intervention).

The wound healing process consists of hemostasis, inflammation, proliferation, and remodeling by the formation of scar tissue (fibrosis). [19]

In the process of tissue repair, cytokines, a protein which is the key of cell signaling, is intensively produced by various cells such as macrophage, lymphocytes, mast cells, and others. The intensive production is needed to control the whole process. Cytokines has been observed in some research, revealing that this substance including several types of proteins belong to this group such as tumour necrosis factor (TNF)-α, Interleukins 1-6-8, are responsible for discogenic pain and induces IVD degeneration due to the production of matrix degrading enzymes. [18], [20]

The creation of new blood vessels (angiogenesis) which belongs to proliferation is very critical in wound healing. [19] The sequences that happen after removal of IVD tissue by surgical intervention follow more or less the order mentioned above, but in slightly different details because NP is an avascular and non-innervated tissue. Therefore, the involvement of angiogenesis in the process of wound healing is different. The site where angiogenesis which is also accompanied by nerve ingrowth, can only happen is the AF. [21] Due to the presence of cytokines in IVD tissue as explained above, it is

become essential to evaluate cell viability in the presence of IVD tissue. Therefore, we also conduct MTT assay evaluation to support osteogenic differentiation evaluation. We expect that the presence of cytokines in IVD tissue either enhance or suppress cell viability.

2. Materials and Methods

Materials

Extract of Nucleus Pulposus (NP) and Annulus Fibrosus (AF)

Tissue samples containing nucleus pulposus (NP) and annulus fibrosus (AF) were collected during spinal surgeries by neurosurgeons of the University Medical Centrum Groningen (UMCG). The tissues were collected from 7 patients (2 male and 5 female) ranging in ages between 30 - 70-years-old. The samples for experiments were chosen randomly. To collect tissue and establish the amount (weight) of the tissue sterile vials were prepared, filled with sterile transport medium consisting of Dulbecco's modified eagle medium-high glucose (DMEM-HG) (Gibco-Life Technology) supplemented with antibiotics, ascorbic acid and fetal bovine serum (FBS) and individually marked. The vials were weighed before transportation to the operating center of the UMCG.

The surgeon placed the tissue in a vial, the vial was collected from the operating center and weighed again. The netto weight was considered to be the weight of the tissue. In the laboratory the tissue was washed one time with PBS and two times with the culture medium consisting of 90% α-MEM (α-MEM (Gibco-Life Technology), 10% of FBS, 1% of antibiotics, and 0.2 mM of ascorbic acid(α-MEM complete medium). Subsequently α-MEM complete medium was added to each of the vial with tissue, 1 mL for each 20 mg of tissue. The vials were incubated in a shaker incubator (Shaker Heidolph Titramax 101) for 24 hours. Then, the extracts were collected in sterile conical tubes and centrifuged (IEC Centra CL2) with the speed of 1500 rpm for 15 minutes. The supernatant was stored in a sterile tube at -23ºC until further use.

Methods

Human Mesenchymal Stem Cell (hMSC) Passage and Seeding

Human mesenchymal stem cells were obtained from bone marrow from patients at total hip or total knee surgery and characterized according to the guidelines of the International Society of Cellular Therapy (Buizer et al. 2014). hMSC passage 3 (P-3) was taken from the nitrogen storage, thawed and pipetted gently into a sterile conical tube containing α-MEM complete medium. Subsequently, the cell suspension was transferred into a T75 flask and incubated in a cell culture incubator (Thermo Forma Hepafilter) at 37°C, 5% CO_2, and 100% humidity. Cultures of hMSC's reached a 50%-60% confluence in approximately 2-3 weeks. The the cells were passaged using trypsin-EDTA. The passage was done by aspirating the medium, rinsing the cells with PBS solution, and detaching of the cells with 3 ml of trypsin-EDTA (Life Technologies, Rijswijk). After the cells had detached, fresh α-MEM complete medium was added and the cell suspension was centrifuged at 1200 rpm for 5 minutes. Subsequently, the

supernatant was aspirated, and the pelleted cells were resuspensded in 5 ml of α-MEM complete medium.

The hMSC were counted using a Bürker-Türk haemocytometer using a Leica inverted phase-contrast microscope (Leica, DMIL LED). For MTT assay assays, 2000 cells/well were seeded in a 96 well plate (Greiner Bio One-Cell Star). hMSC were allowed to adhere for 24 hours in the cell culture incubator. The same procedure of passage applied for osteogenic differentiation test (Alizarin assay). For osteogenic differentiation, measured using an Alizarin Red Assay kit (ScienCell, Inc) 50.000 cells/well were seeded in 6 well plate (Greiner Bio One-Cell Star). Cells were allowed to adhere for 24 hours before extracts of IVD tissues were added.

Human Osteoblast (hOb) Passage and Seeding

Primary. human osteoblasts (hOb) passage 3 (P-3) were also obtained from nitrogen storage and thawed carefully using a water bath. The culture medium consisted of 88% of DMEM F12 (Gibco-Life Technology), 10% of FBS, 2% of anti-anti, and 0,2 mM of AA2P. hOB were cultured in T75 flasks until 60% confluent, when the cells were passaged as described for hMSC. For MTT assays cells were seeded and adhered for 24 hours in 96 well plates.

For osteogenic assays hOb (positive control) were seeded in 6 well plates (50000/well), adhered for 24 hours after which extracts of IVD tissue were added.

MTT Assay Evaluation

MTT assay procedure was done according to the protocol of Department Biomedical Engineering, University Medical Centrum Groningen (BME-I-R-002). The detail of the protocol is provided in Appendix 1.

Osteogenic Differentiation using Alizarin Assay

The kit of alizarin assay (40 mM Alizarin red staining, 10% Acetic acid, 10% Ammonium Hydroxide, and standard dilution solution) was purchased from Sciencell research laboratory. The procedure for alizarin assay to evaluate osteogenic differentiation was done according to the protocol provided by Sciencell (ARed-Q, Catalog #8678, 100 Tests). The detail of the protocol is provided in Appendix 2.

Statistical Analysis

Differences between samples in both the Alizarin assay and the MTT assay were tested for significance using Sigmaplot 13 software. First the data were tested for normal distribution with a Shapiro test. Normally distributed data were analyzed with an ANOVA; differences between groups with either a Tukey-Kramer test of a Newman-Keuls test. Not normally distributed data were analyzed with a Kruskall Wallis ANOVA on ranks; followed by a Dunn's or Newman-Keuls post hoc test.

3. Results and Discussion

Results

Effects of extracts of IVD tissue on Osteogenic Differentiation

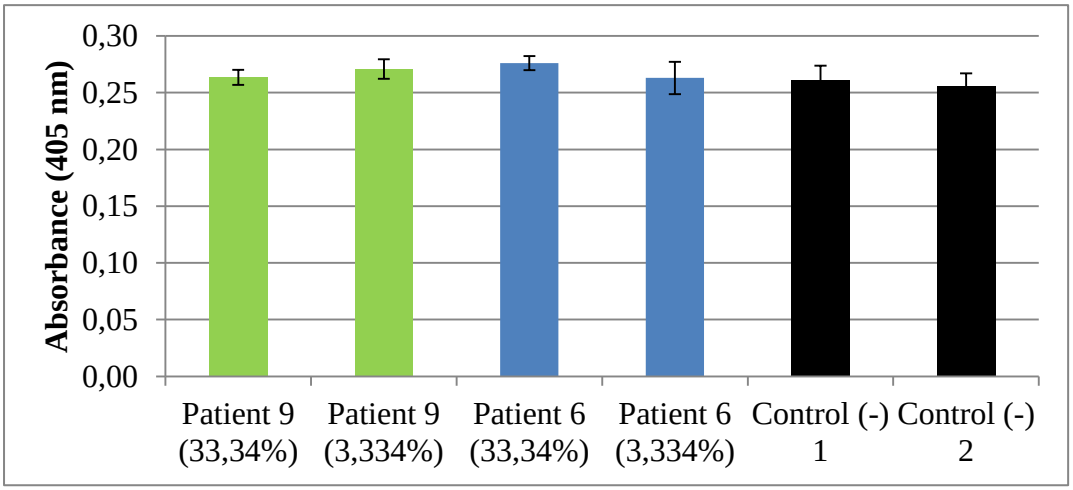


Figure 3.1 Absorbance measurement of the Alizarin Red assay indicating the amount of calcium deposited by human osteoblasts on the bottom of the well in the samples with- and without extracts of IVD tissue.

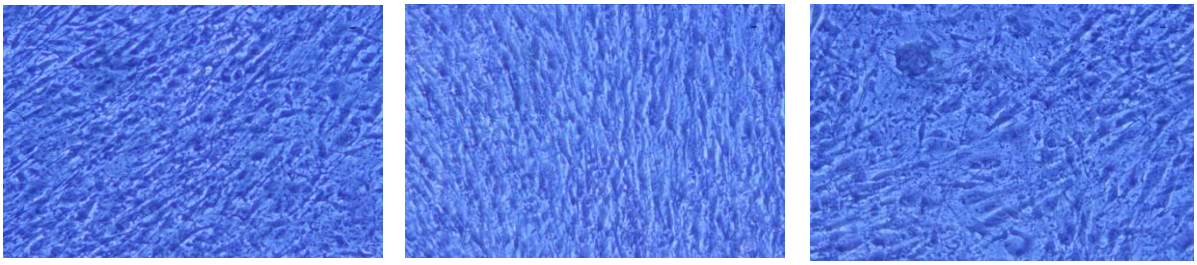
Figure 3.1 shows that there were no differences in amount of deposited calcium by human osteoblasts between the samples incubated with IVD tissue extract and the untreated samples. This was confirmed with statitical analysis..

A human Osteoblast (hOb) is a finally differentiated cell, responsible for the formation of osteoid and the subsequent mineralisation of this immature bone tissue. One way to distinguish hOb from another kind of cells is its ability to form a mineralized extracellular matrix (ECM). [22] The Alizarin assay measures the amount of mineral deposited in the extracellular matrix produced by the cells.

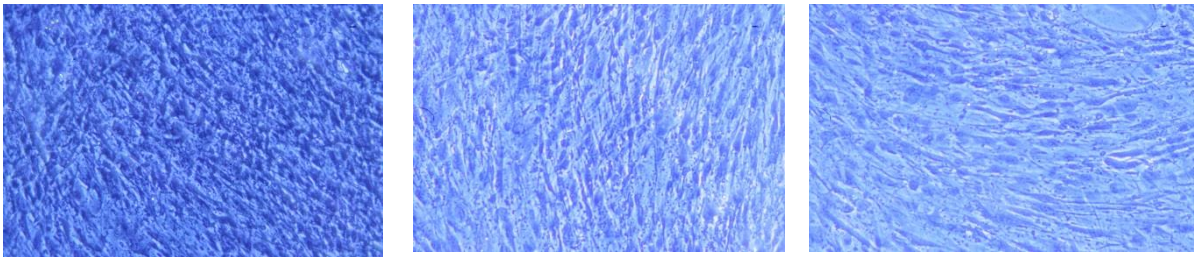
The calcium of the mineralized ECM reacts with the phosphate structure of hydrolized beta-glycerophosphate in the osteogenic medium. [23] Together, both of these substances form calcium phosphate crystals of which the amount is measured. Even though hOb is a differentiated form of MSC, it is possible that its capability to mineralize ECM could be affected by the presence of IVD extract. However, in this experiment neither enhancement nor inhibition was found. hOb which was incubated in the absence of IVD extract plays a role as a positive control in this assay.

Besides measuring the amount of calcium deposited in the ECM, the observation of the confluence of the cultures of hOB was also done using a phase contrast microscope to ensure that the density of the osteoblast cultures was sufficient to produce mineralized

ECM. The pictures are provided in Figure 3.2 and show that all cultures had a similar, high density of cells.



a. Patient 6 (3,334%) *b. Patient 6 (33,34%)* *c. Patient 9 (3,334%)*



d. Patient 9 (33,34%) *e. Negative Control 1* *f. Negative Control*

Figure 3.2 (a, b, c, d, e and f) show that in 3 weeks the confluence of hOb has reached between 90%-100%.

Next we tested whether extracts of IVD tissue were able to influence the osteogenic differentiation of hMSC. hMSC were cultured in osteogenic differentiation medium without or with IVD tissue culture extracts. As control samples hMSC grown in proliferation medium were included (non osteo) as well as empty wells (background of the essay).

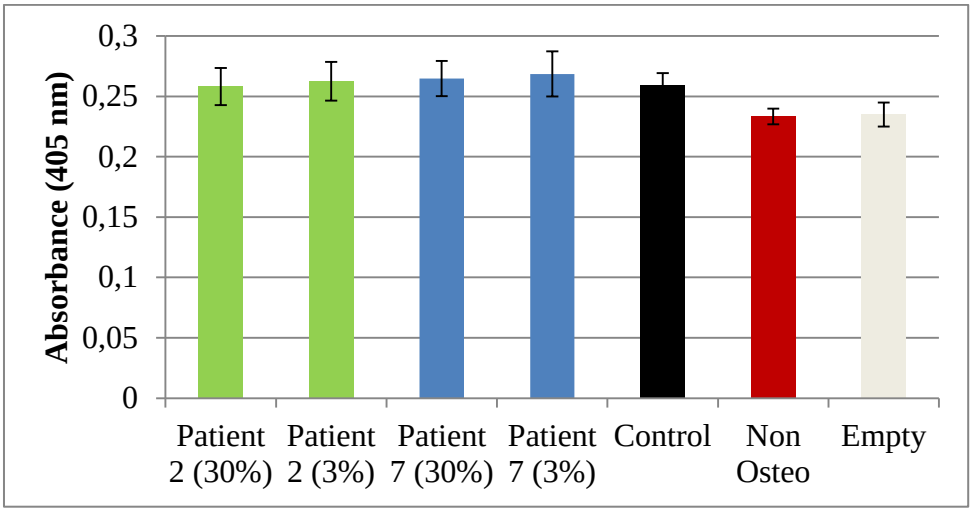
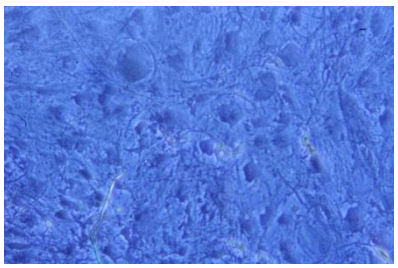


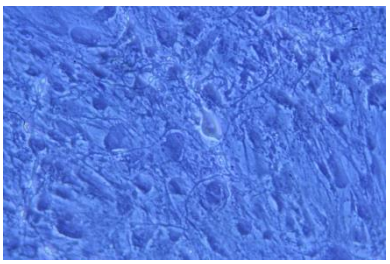
Figure 3.3 Absorbance measurement using fluoro-star plate reader indicated the amount of calcium deposit on the bottom of the well in samples of human mesenchymal stem cell (hMSC)with NP-AF extracts, without extract, samples treated with non-osteogenic medium and the empty well

The results are shown in Figure 3.3 and indicate that IVD tissue extracts do not affect osteogenic differentiation. Neither inhibition nor stimulation was found in samples incubated with IVD extract, compared to the not incubated control sample. All samples cultured in osteogenic medium had significantly higher absorbance values, than the samples in non-osteogenic medium or the empty wells.

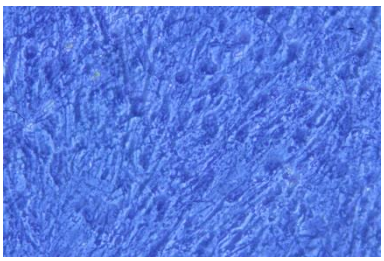
The cell densities of the samples cultured in osteogenic medium were high, and similar to each other as observed by phase contrast microscopy (Figure 3.4)**.**



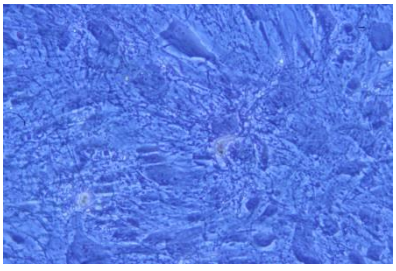
a. P 2 (30%)



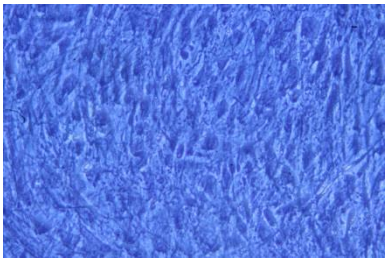
b. P 2 (3%)



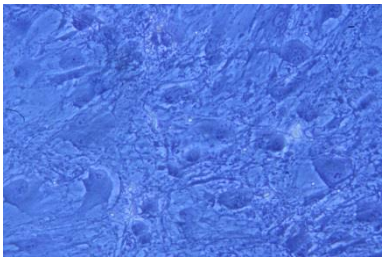
c. P 7 (30%)



d. P 7 (3%)



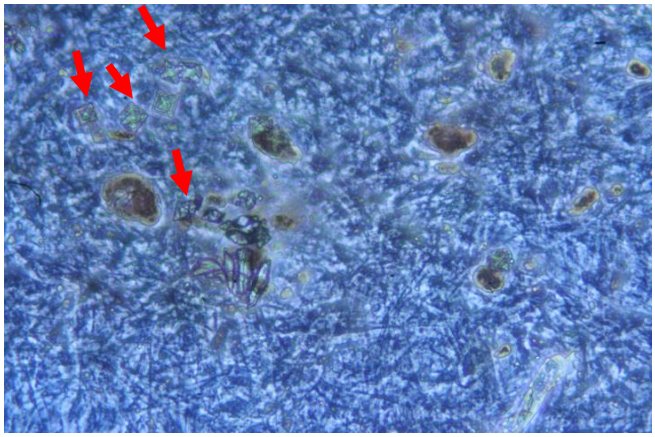
e. C 1



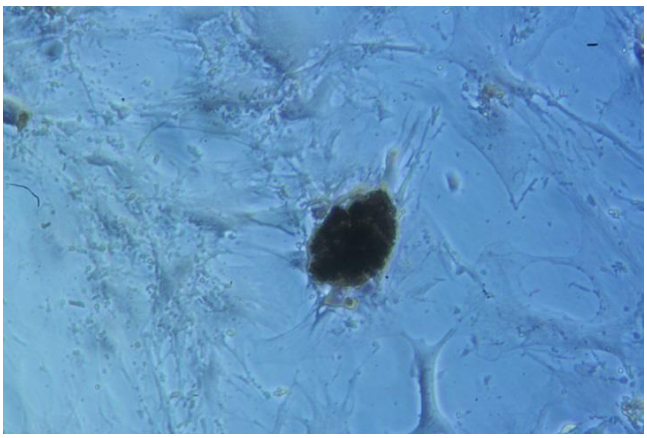
f. C 2

Figure 3.4 Phase contrast images of hMSC cultured for 3 weeks in 6 well plates in osteogenic medium with and without(C1, C2) 3% or 30% extract of IVD tissues of patients 2 and 7 (P2, P7).

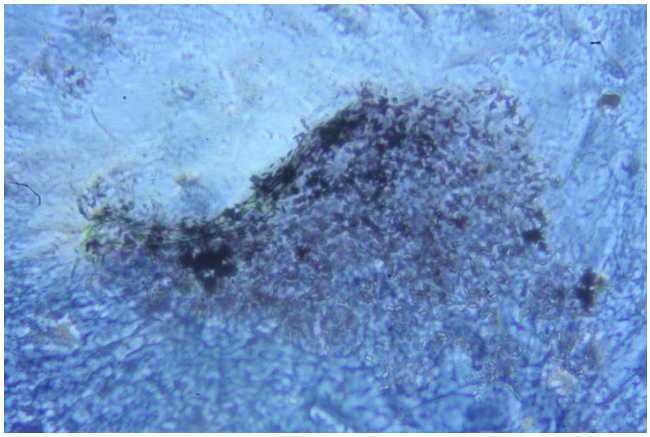
Also during the execution of the Alizarin red assay pictures with the phase contrast microscope were made to visualize mineral in the cell cultures. The red color indicates the presence of calcium. In all samples cultured in osteogenic medium crystals were visible. In samples cultured in non-osteogenic medium we did not observe any crystals. We cannot exclude that this was caused by the low cell density in the cultures in non-osteogenic medium.



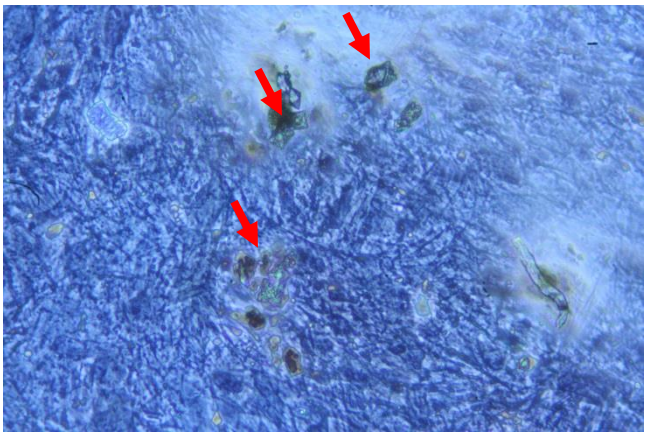
a. Patient 3 (12%)



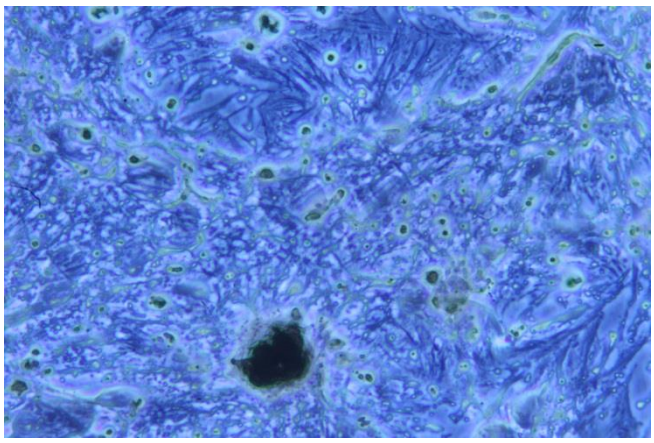
b. Patient 3 (1,2%)



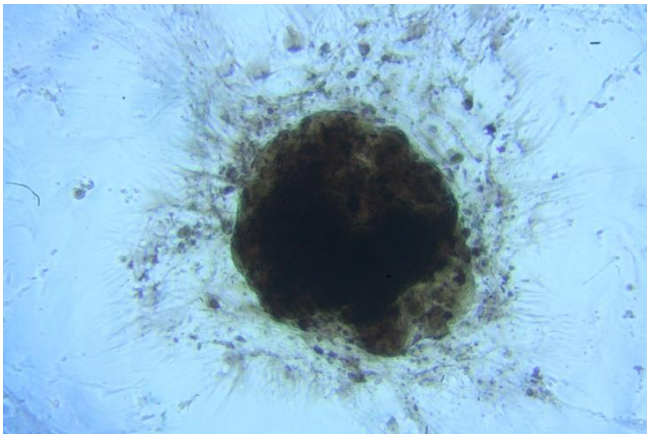
c. Patient 4 (12%)



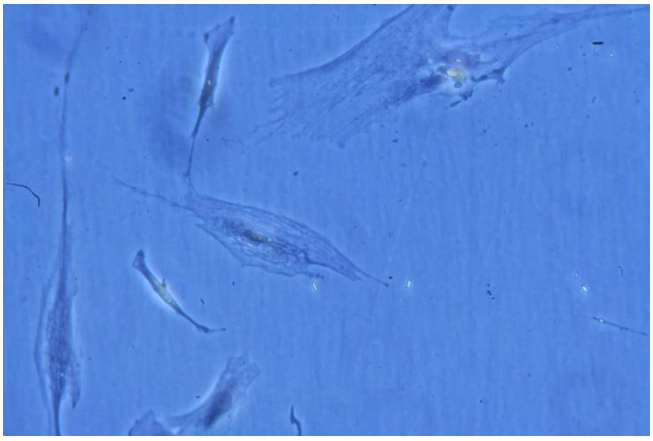
d. Patient 4 (1,2%)



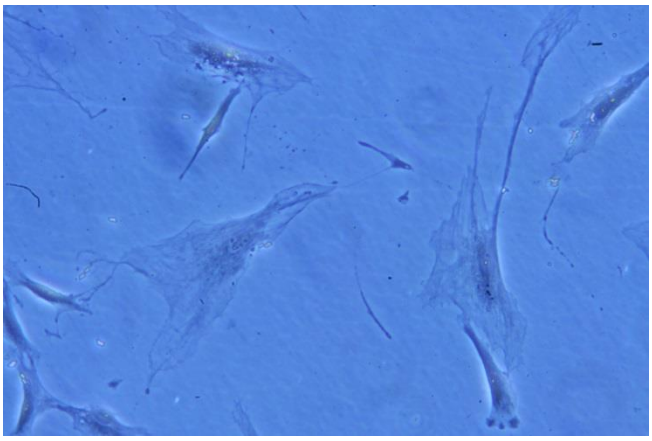
e. Negative Control 1



f. Negative Control 2



g. Non-Osteogenic 1



h. Non-Osteogenic 2

Figure 3.5 (a-h) shows differences in deposited mineral in individual samples of cultured hMSC in osteogenic medium and non-osteogenic medium (α-MEM). Alizarin staining was performed on all these samples. Crystals are visible in the samples treated with osteogenic medium (3.5 (a,b,c,d,e,f)), indicated by the dark red color. In some cases, crystals were clearly visible indicated by red arrows. In samples cultured in proliferation medium (non-osteogenic) no crystals were observed (figure (3.5 (g,h)).

Effects of extracts of IVD tissue on the viability of hMSC

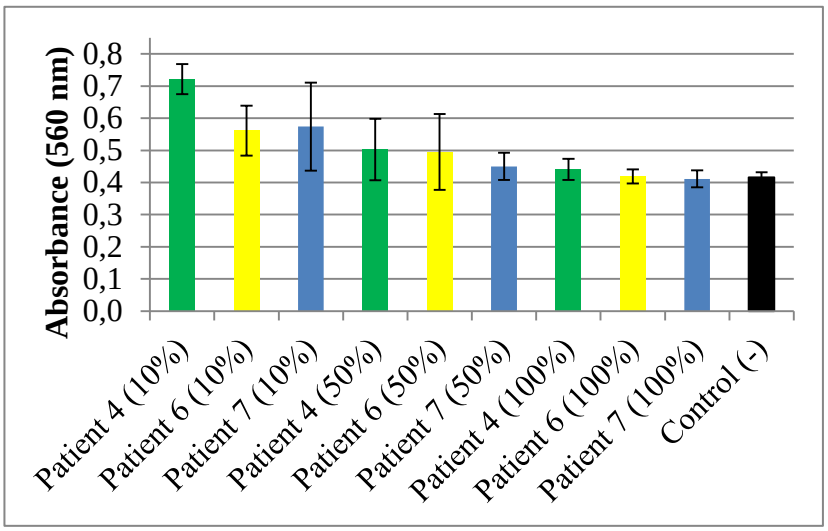


Figure 3.6 Absorbance measurement using fluoro-star plate reader showed hMSC viability after the adding of the extracts of nucleus pulposus (NP) and annulus fibrosus (AF), first measurement

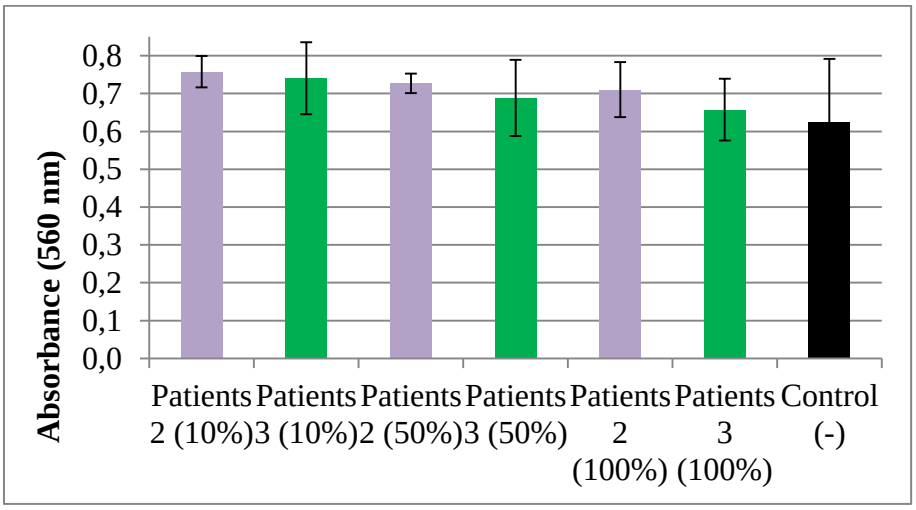


Figure 3.7 Absorbance measurement using fluoro-star plate reader showed hMSC viability after the adding of the extracts of nucleus pulposus (NP) and annulus fibrosus (AF), second measurement

Results of the MTT assays show that the extracts of nucleus pulposus (NP) and annulus fibrosus (AF) tisues of patients 2, 3, 4 and 7 enhanced the metabolic activity of the cells. This is indicated by the increase in the value of absorbance compared to the control sample, that was not incubated. The differences were significant.

The two figures above also show that samples from different patients vary in their effect on metabolic activity of hMSC. The other important thing to be noted is samples

incubated with the lowest concentration of NP-AF extracts (10%) give higher values than those incubated with the more concentrated extracts.

Discussion

This study was performed to investigate whether the presence of remaining IVD tissue in spinal fusion surgery affects bone formation (osteogenic differentiation). Additionally, due to the intensive production of cytokines in the IVD during the healing of the surgically inflicted wound the effect of IVD tissue to cell viability was also evaluated.

Figure 3.3 confirmed that the presence of NP-AF extracts does not influence bone formation, indicated by no significant difference found between the treated samples and the non-treated controls. An additional experiment using hMSC in non-osteogenic medium was also done to check if the assay worked properly. The results showed that MSC cultured in non-osteogenic medium did not form calcium phosphate deposits (figure 3.3 and 3.5 (g-h)).

However, a further interpretation of the results above should be done by considering the results of MTT assay which was also done in this study. Both results of the MTT assay evaluation showed that the presence of IVD extracts enhanced the viability of cells with a significant difference between the treated and untreated samples. The lowest concentration of IVD tissue extract (10%) showed the highest enhancement in cell viability compared to the moderate concentration (50%) and the highest concentration (100%). This phenomenon can be explained by the fact that at higher concentrations, inhibiting factors overrule the growth factors, while at lower concentrations inhibiting factors might be less effective.

The viability is represented by the amount of formazan produced by the cells. The enhancement in viability might be caused either due to higher proliferation (more cells) or a higher enzymatic activity of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) -oxidase which forms formazan. We believe that a higher enzymatic activity is the reason in this case, and the content of cytokines in the IVD tissue is the cause of this elevation. Data from the literature confirm the influence of the cytokines TNF-α, interleukins, IFN-γ, in increasing NADPH-oxidase production by mitochondria. [24]

Cytokines, which are believed to be the reason behind the elevation of cell viability in this study, are continuously produced by different cells such as macrophage, lymphocytes, endothelial cells, fibroblast, and others. These factors are key in cell signaling. However, as mentioned in the introduction, in wound healing, the production of these substances is enhanced to orchestrate the whole process. A small example of the function of the cytokines, TNF and interleukins (ILs) is the role as a chemotactic agent for white blood cells and connective tissue cells. The stimulated cells produce the

degrading enzymes Matrix Metalloproteinases (MMP) to remove the damaged tissue. [25]

During the wound healing reaction following the surgical intervention in the IVD, several types of cytokines are found such as Tumour Necrosis Factor (TNF)-α, IL-4, -6, -12 and interferon-γ. [26] Besides the presence of cytokines due to post-surgical inflammation, basal NP itself also can be a source of cytokines such as IL-1, and the cytokines mentioned before. [27] The samples we collected at the operating center in this study probably varied in degree of inflammation. Next to that, during the extraction period many cells in the tissues underwent necrosis and released cytokines and chemokines. [27] Excess amount of cytokines are lead to unwanted effects, such as recruitment of other inflammatory substances and MMP enzymes, matrix degradation, and phagocytosis *in vivo*. [21] The number of cytokines which affected the results of this study is considered to be small compared to the number present in the body, which means that the triggered effects in the body could be much greater compared to those observed in this study.

Successful wound healing also depends on the formation of new blood vessels (angiogenesis) since they are the source of oxygen and nutrients. However, as mentioned before, a normal NP is avascular and not innervated. Thus, there are differences in the mechanism of wound healing. A previous study stated that there was new formation of nerves and blood vessels found in the AF of herniated IVDs. [28] The displaced NP placed in contact with nerve and blood vessels of the AF can mediate the entry of macrophages, a process which is considered as source of discogenic pain. [28], [27]

When the results of the Alizarin Red assay and the MTT assay are combined, it can be concluded, that although MSCs in the presence of IVD tissue extracts will obtain a higher metabolic activity, their osteogenic capacity is not affected.

Croes et. al found TNF-α was able to enhance osteogenic differentiation of hMSC. [29] So, in our tissue extracts either no TNF-α was present, or its concentration was too low to exert a measurable effect. Also other cytokines present in the tissue extracts could have had antagonistic effects on osteogenic differentiation of MSCs. Nevertheless, the difference in the results of studies which investigated the influence of cytokines on osteogenic differentiation of hMSC is not the first one. Deshpande et.al, in 2013 summarized many of the studies which had been done in this field. These authors tried to find the causes of the differences. The first Cause was considered to be the dose since certain cytokines like TNF has several thresholds to give a significant effect. Dependent on the dose, the effect could be pro-osteogenic, anti-osteogenic, or neutral. The second reason was cytokines come in many different types. These work together instead of working separately. The final effect is the sum of of the influences of all cytokines, as

mentioned in the previous paragraph. The third reason was the targeted cell type. Effects of cytokines vary dependent on the type of cell. [30]

The collected samples in this study are considered to be a good representation of the wound healing happening in spinal fusion conditions since they were obtained directly from patient.

Both the types of and the concentrations of the cytokines present in the tissues will have differed. Cytokine profiles were not determined nor were concentrations measured.

Besides that, in the *in vivo* environment there can have been more diverse effects compared to a better controlled *in vitro* model. For future work, an *in vivo* study will be a potential choice. The presence of cytokines also can be observed through an intensive histological study and ELISA measurement.

Besides the investigation of the effect of remaining IVD tissue to bone formation, there could be several other effects of remaining IVD tissue which are important to be investigated, such as further matrix degradation, [21] or bone resorption. [31] Matrix degradation and bone resorption can be caused by the immune responses towards the remaining IVD tissue. NP pieces cause a greater recruitment of immune substances and MMP because it is recognized as non-self. [21] In this case, we think of the possibility that re-degeneration of IVD on the site close to the bones which are fused could happen. Re-degeneration causes re-instability and leads to the requirement of re-surgery which will bring disadvantages for the patients. One of the limitations of this study is that we were not able to use the same dilutions of tissue extracts in all experiments. This makes it difficult to compare and combine the results of the Alizarin red assays and the MTT assays.

4. Conclusion

In this study we show that remaining IVD tissue itself does not produce factors which affect bone formation. The tissue does produce factors that affected the viability of the cells, and this influence was dose dependent. Several previous studies have been done to investigate this issue as well and confirmed different results due to the pleiotropic character of cytokines. Therefore, the best way to improve this study is by doing an animal *in vivo* study since it represents the mechanism of wound healing in spinal fusion in the presence of cytokines better.

Besides the effects of remaining IVD tissue on bone formation, there might be other possible effects such as bone and matrix degradation of IVD close to the site where the fused spine is. In this case, re-instability could happen, leads to the necessity of re-surgery which brings disadvantages to the patients and more risk to the surgeons.

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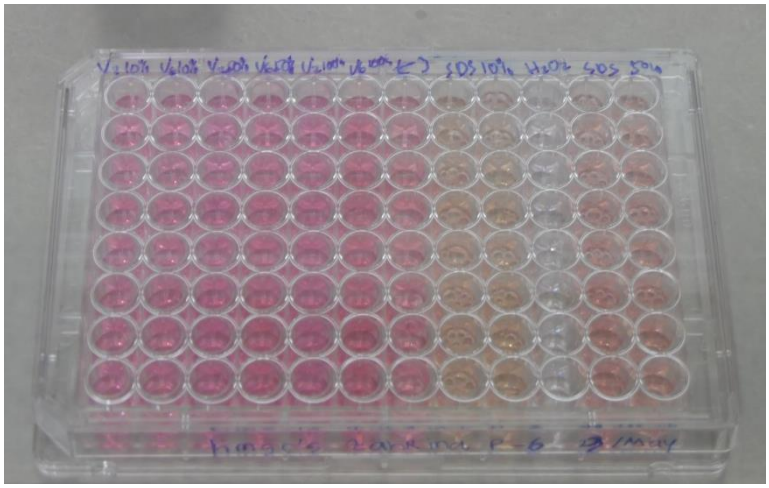
6. Appendix

Appendix 1.

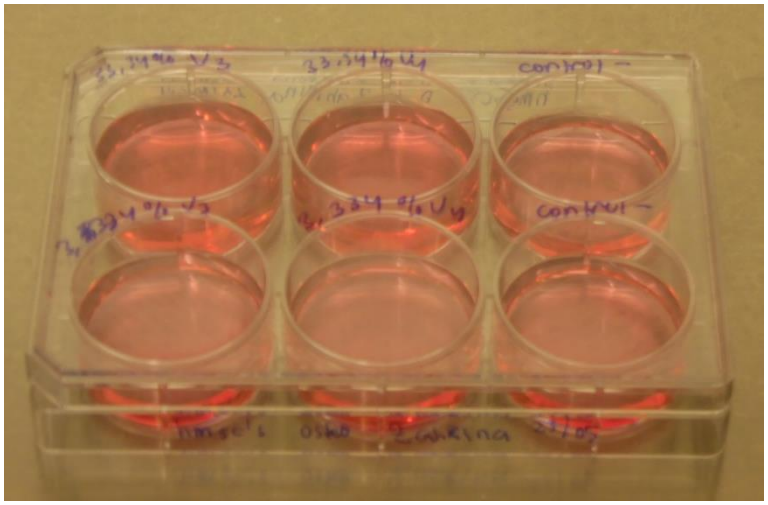
MTT Assay Evaluation Procedure

The cell seeding in 96 well plate was followed by 24 hours of incubation. Subsequently, the extracts of NP-AF were added to the wells with several variations in concentration. 10% of extracts (10% extract-90% culture medium), 50% of extracts (50% extract-50% culture medium), and 100% of extracts (100% extract-0% culture medium) were made in 8 times multiplication for each. The last two columns were used as negative control (no extract was added). Subsequently, the plate had to be incubated for 48 hours.

Following 48 hours of incubation, the medium was aspirated from each well and MTT powder (thiazolyl blue tetrazolium bromide) purchased from Sigma Life Science was added to each well with the concentration 0,5 mg/mL. Latterly, another incubation for 3 hours was done and followed by the adding of 2-propanol (Merck, EMD Millipore Corporation). Afterwards, the well plate containing 2-propanol had to be placed on a shaker for 15 minutes. Finally, measurement was done using the fluorostar Optima plate reader (BMG Labtech) with wavelength of 560 nm.



The 96 well plate after the adding of the extracts



Seeding of hMSC in 6 well plate

Appendix 2.

Alizarin Assay Procedure

After the plate containing hOb had been incubated for 1 x 24 hours, the extracts were added with the concentration of 33,34% and 3,334%. The extracts were obtained from two different patients. Whereas the rest two wells were used as controls (no extract was added). For hMSC, the extracts were also obtained from two different patients but with the concentration 12% and 1,2%. Afterwards, the wells containing extracts were further incubated for 48 hours. Finally, alizarin assay was done by aspiration of the medium, followed by rinsing of the cells, fixing the cells, and staining of the cells using alizarin red. Cells were observed using a phase microscope (Leica DMIL LED) after the staining.

Following the observation, 10% acetic acid was added to each well, and the cell suspensions were collected in 1,5ml micro centrifuge tubes. The tubes had to be processed using a vortex, heating, cooling and centrifugation treatments. Subsequently, the supernatants were collected, and 10% of ammonium hydroxide was added to neutralize the acid. Finally, 150µl/well of aliquot obtained from the suspension was created in 96 well in quadruplicate, and the absorbance was read using fluorostar Optima plate reader with a wavelength of 405nm.

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