IB Biology Extended Essay

Comparison of Dermal Tissue Derived Mesenchymal Stem Cells and Dermal Tissue Derived Epithelium Cells *in-vitro* Wound Healing Model In Terms of Migration Ability and Speed.

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Abstract

In nowadays an increase of wars or accidents around the globe can be observed. Those happenings cause individuals to expose burns, radiation burns or acute wounds. Today those wounds are treated with a method called ‘’skin grafting’’ which is known to lower the patients’ quality of life and costs thousands of dollars. The aim of this research is to find an alternative treatment to dermal wounds and decide which of the two cell types –Mesenchymal Stem Cell (MSCs) and Epithelium Cells (ECs)- would suitable to be used in this alternate method.

My research question is ‘’ How do dermal tissue derived Mesenchymal Stem Cells and dermal tissue derived Epithelium Cells affect the wound healing process in terms of covering the wound area, measured the cell index and percentage wound area coverage by using Real Time Cell Analyzer Migration Experiment and *in-vitro* wound healing model ?’’

The methods used to investigate the research question are Real Time Cell Analyzer Migration Experiment and *in-vitro* wound healing model. Before the both experiments flow-cytometry is done on cells on the third passage in order to identify them as MSCs derived tissues, with respect to surface markers CD11b/c, CD45, CD90, CD49.

After the Real Time Cell Analyzer Migration experiment 48 hour mean values of cell indexes for the cells are as follows; 0.82 for MSCs (from 0.0815 to 0.3451) and 0.71 for ECs (from 0.0819 to 0.2019). In *in-vitro* model it was observed MSCs covered 100 % of the wound gap at 10. Hour, ECs covered 17% of the area at 48. Hour which showed a meaningful difference ($p < 0.05$) in t-test. The conclusion is that MSCs could be used as a cell spray rather than ECs for a batter treatment to dermal wounds or burns. This study merits further investigation for usage of MSCs in wound healing model as a spray.

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Background information

Tissue damages and particularly skin wounds usually heal in a highly regulated sequence of overlapping processes that require coordinated activities of different cell types. Wound healing phases include: vascular and inflammatory phase, a proliferative phase and a remodeling phase.

Human mesenchymal stem cells (MSCs) are a heterogeneous population of fibroblast-like cells, which are present in different locations, including bone marrow, adipose tissue, extra-foetal tissues, gingiva and dermis.

Officially, MSCs have been defined by the International Society of Cellular Therapy as multipotent stromal cells on the basis of three main characteristics:

- Their adhesion to a plastic support;
- Their expression of a specific set of membrane molecules (CD73,CD90 and CD105), combined with a lack of expression of hematopoietic markers (CD14, CD34, and CD45) and human leukocyte antigen-DR;
- Their ability to differentiate along osteoblastic, adipocytic and chondrocytic pathways.

Both for their immunosuppressive effects and their multipotent differentiation potentials, MSCs are more favored than other stem cell types for the cellular treatments.

Even if the first isolation of MSCs has been made in the bone marrow stroma, some researchers have now described MSCs in many others organs, such as adipose tissue, extra-foetal tissues, gingival tissue and dermal tissue (Fig. 1). In some specific situations, these cells have been described to be involved during skin repair processes; in addition, the interest to use these cells to improve wound healing has been studied (Rama P. et al 2010).

Researches of Prof. Dr. Ralf Hass et al. From Medical University of Hannover showed comparison of adult and newborn tissue-derived MSC. Results stated that MSCs represent an important stem cell population with multipotent capabilities which are extremely useful for clinical applications.

Epithelium cells (ECs): Epithelia are formed of cells that line the cavities in the body and also cover flat surfaces. Of the four major tissue types found in the human body, epithelial cells are by far the most prolific.

Avascular • Capillaries do not presented within epithelial cell tissues	**Transitional** • Epithelia are able to stretch • Allows the urinary bladder to distended or contracted without compromising it
Sensory • Axon endings are present within epithelial cell tissues • Comprehend external stimulus	**Tight barrier** • Epithelium is held together more tightly than other cells • Aids cells in withstanding mechanical stress
Gliding surface layer • Epithelial cells glide in order to replace dead cells • This function allows epithelial cells to maintain a closed barrier to the external environment	**Different from endothelial cells** • Endothelial cells line the insides of structures that aren't exposed to the "outside"

Table 1. Differences of Epithelial Cells from Other Cells

Inability of regeneration of third degree burns and cultured otology keratinocytes' capacity to save thousands of people's lives (Green 2008), bring up a possibility about threating third degree burns with other cell types apart from the epithelium cells. Some medical experiments which use MSCs for wound treatments showed that MSCs' grafts ease the regeneration for both chronic and acute wounds. Some researchers found same results (Falanga V. at al.2007, Yoshikawa T. et al. 2008, Lu D. et al. 2011) by using bone-marrow derived MSCs, adipose tissue derived MSCs (Kim M. et al. 2011) and Wharton jelly derived MSCs (Fong CY. Et al. 2014). It is proved that fat tissue derived MSCs with decellularizated dermal matrix, could differentiate into endothelial and epithelium cells and excrete angiogenic factors in ischemic wound model on suitable conditions.

In 1975, Rheinwald and Green suggested, cultured cell which are taken from German Swiss 3T3-J2 rats beamed in high doses, as treatment for massive dermal losses (Rheinwald and Green 1975). Green et al. studied on cultured otology Epithelium cells' keratinocyte for years, before transferring them into the humans. Since then this technology was used on a lot of patients around the globe (Gallico et al. 1984; Kumagai et al. 1988; Teepe et al. 1990; Haith et al. 1992; Rue et al. 1993; Paddle-Ledinek et al. 1997; Ronfard et al. 2000; Chester et al. 2004; De Luca et al. 2006; Cirodde et al. 2011, Can A. 2014). Rheinwald's and Green's method about transferring keratinocyte culture is highly effective and enables forming departure culture from very tiny dermal biopsy (Barrandon and Green 1987; Rochat et al. 1994). The keratinocytes with fibroblasts which are taken form the particular patient could form a new epidermis-dermis structure like the normal skin in order to increase the formation rate of the dermis when they are buried into the collagen gels, fibrin matrix or polymers in order to constitute a composite graft (Boyce and Warden 2002).

With respect to the this previous researches I came up with conclusion that; We can create a new treatment method for dermal tissue healing which can be stored without any deterioration and used on all patients without using a suitable donor to prevent tissue rejection by using MSCs or ECs in a form of spray.

Hypothesis

There is a chance that the body will reject the new tissue after ‘’skin grafting’’. To prevent this, the patient usually must be treated with long-term immunosuppressant drugs. So in my project I aimed to develop a practical and easy method that will not include a suitable donor or use of immunosuppressant drugs for the wound healing process.

As past researches shows MSCs have ability to synthesizing many of growth factors (GM-CSF, G-CSF, M-CSF) and their receptors, various Interleukins (IL-6,7,8 etc.) and also their receptors, proteins of the extracellular matrix (Collogen,Fibronectin, Laminin) and finally Cell adhesion molecules (CAMs). So with those properties MSCs will have a significantly better migration speed and datively better effect on the wound healing process.

Can we create a new treatment method for dermal tissue healing which can be stored without any deterioration and used on all patients without using a suitable donor to prevent tissue rejection by using MSCs or ECs in a form of spray?

Hypothesis: *Usage of Dermal tissue-derived Mesenchymal stem cells (MSCs) will be more effective than usage of Dermal tissue-derived Epithelium cells(ECs) in wound healing process by using in-vitro wound healing model in terms of cell migration ability, speed and percentage cover.*

Method development

When I was first planning my method for my experiment I had a plan to made animal testing, perhaps a *pre-Clinic* experiment, as the end product -cell spray- have a potential to be used on humans. This medical product must pass *in-vitro*, *in-vivo*, *pre-Clinic* and *Clinic* experiments and tests. However by the unethical nature and strict restrictions as being a high-school student, I decided to conduct animal testing *–pre-Clinic-* when I get my license degree on Molecular Biology and Genetics and testing this method *in-vivo* and *in-vitro* conditions.

My researches on wounds and their treatments showed me that today's methods for wound healing are ineffective, long-term and expensive. Further research gave me an idea to use stem-cells. Stem cells are considered ''alchemy of the Modern Age''. Scientists are trying to produce personal organs from the person's stem cells and end the need of the organ donation. Same problem occurs in skin grafts. Suitable donor must be found. However, some stems cells are cannot be recognized by the immune system as ''alien'' or as a ''threat''. This prevents tissue rejection problem. So I found that MSCs can differentiate into dermal tissue and have a lack of expression to leukocyte antigen-DR which is responsible to detect ''alien'' cells or tissues. So making a cell spray from MSCs were logical. However, when I share this opinion, people always ask why I need a Stem Cell when we could use our own Dermal Cells (Epithelium Cells). This made me to think and reconsider my experimental set-up. So I decided to compare this to cell types for their availability for a possible medical treatment.

Before starting my experiment I came up with a hypothesis in consideration of previous researches and available data about both cell types; Usage of Dermal tissue-derived Mesenchymal stem cells (MSCs) will be more effective than usage Dermal tissue-derived Epithelium cells (ECs) in terms of wound healing process in-vitro wound healing model.

In order to test the hypothesis, the differences between Dermal tissue-derived Mesenchymal stem cells (MSCs) and Dermal tissue-derived Epithelium cells (ECs) must be outlined in terms of migration ability and time. Apart from that Enzymatic digestion of the tissue taken from the tissue bank and MSC and EC isolation from that tissue must be designed and performed.

Presence of microorganisms or any foreign chemicals can harm all of the cultures during the experiment. In order to avoid such harms, the experiment was conducted in the lab of Dışkapı Training and Research Hospital and Gazi University Faculty of Pharmacy's Pharmacology Cell Research Laboratories. In this experiment I used Wistar-Albino rat's femur area dermis-epidermis skin pieces which are taken from, Dışkapı Training and Research Hospital's Stem Cell Centre Tissue Bank, as cell provider. As Dışkapı Hospital's Stem Cell Centre Tissue Bank was best in the Ankara in terms of sample types I decided to conduct my research there. Also Gazi University Faculty of Pharmacy's Pharmacology Cell Research Laboratories have the JuLI device which I used on my wound healing experiment. The labs was hygienic and any instrument was washed with %70 alcohol disinfectants before using it in order to kill all of the

microorganisms and prevent any contamination. All tubes, wells, chemical and biological material have not been used before to eliminate the possibility of contamination of toxic materials and microorganisms. Also all precautions were taken to prevent accidents by using proper lab instruments and protective gear like gloves, full body lab suit were used.

Our initial step on designing the method was designing the enzymatic digestion conditions for cell isolation. We have to arrange the enzyme rations in the correct way. So Collagenase Type 1 produced in ratio that 3 mg collagenase Type 1(Sigma, USA) dissolved with 3ml HBSS (Lonza, Belgium) and used as 1mg/ml. L-glutamine is commonly added to the media in cell culture because Glutamine supports the growth of cells that have high energy demands and synthesize large amounts of proteins and nucleic acids. It is an alternative energy source for rapidly dividing cells and cells that use glucose inefficiently. So we used it in our experiment. However, the high level of glutamine in the culture media may inhibit other amino acid transport activities.

Next step was deciding which kind of culture medium do I use during the culture of MSCs and ECs. Dulbecco/Vogt modified Eagle's minimal essential medium (DMEM), contains approximately four times as much of the vitamins and amino acids and glucose present in the original formula. DMEM is suitable for most types of cells, including human, monkey, hamster, rat, mouse, and fish cells. These properties make DMEM a very suitable medium. Also Keratinocyte Medium is used as it enables the cultivation of epidermal keratinocytes without feeder cells. Moreover the medium is optimized for primary human cells but can also be used for horse and porcine keratinocytes, as well as mouse and rat keratinocytes after adapting the $CaCl_2$ concentration which was needed for the experiment. Standard temperature, moisture and CO_2 concentration of a mammalian cell culture are 37 °C , %95 moisture and %5 CO_2 and because of these are the optimum conditions for the culture of cells; I set these conditions in the incubator. For the growth mediums different types of serums can be added to medium to supply the medium with necessary nutrients, like Fetal bovine serum (FBS). Fetal bovine serum is the most widely used serum-supplement for the in vitro cell culture of eukaryotic cells.

After the design of the cell culture conditions, we designed the Flow cytometry. We had two options for data analysis of process; gating manually and by Computational analysis. We choose computational analysis because recent progress on automated population identification using computational methods has offered an alternative to traditional gating strategies. Automated identification systems could potentially help findings of rare and hidden populations.

For MSCs differentiation steps AD Basal Medium, CD Basal Medium and OD Basal Mediums are used to differentiate MSCs to Osteocyte, Chondrocyte and Adipocyte cell types. Flow cytometry executed on the differentiated cells in order to prove them they are MSCs. CD11b/c, CD45, CD90, CD49 surface markers checked as MSCs have lack of expression to the CD11b/c, CD45, but have an expression to CD90 and CD49.

Maybe, the most important step was deciding how to measure the migration ability of both MSCs and ECs. We used xCELLigience DP CIM Plate as it can draw graphs which shows individual Cell Index curves for all 16 wells and its usage for broad range of applications for cellular assays and cell invasion migration assays. By having 16 wells CIM Plate enabled me to execute different experimental trials at the same time and decreased the randomness.

I preferred using JuLI systems for the observation of migration speeds of the samples because of the system's ability to scent live-cell images from various cell culture dishes which are directly captured by a tissue-culture hood. This compact design prevents contamination that can occur during classical measurement experiments, providing better experiment results. Also JuLI enables to record and store the speeded-up migration of the cells. Make it more easily to see differences between MSTs and ECs speed to cover the tissue model. Moreover with its high-sensitivity monochrome CCD (Sony sensor 2/3") 1,936 x 1,456 pixels (2.8 M) cameras the recording are in the most high quality in terms of visuals.

JuLI experiment had been conducted for 48 hours. I consider a variety factors while making this decision. My priority was to conduct the experiment long enough to see the full potential of both cell types. Because of nutrients in the culture is limited, if I choose too long time, cells might increase their competition between each other and slow down the covering of the wound area. I also thought that concentration of cellular waste can reach to dangerous levels for cells if they stay in the culture for a long time. Lastly these cell types never compared in an in-vitro experiment so there was no literature value for their migration speeds. So time for cells to cover wound area could be up-to 2-3 days. I decided 48 Hour would be enough to observe a meaningful difference.

4 trials for each experiment and cell type done in order to increase accuracy and decrease the effect of errors on the results.

Method

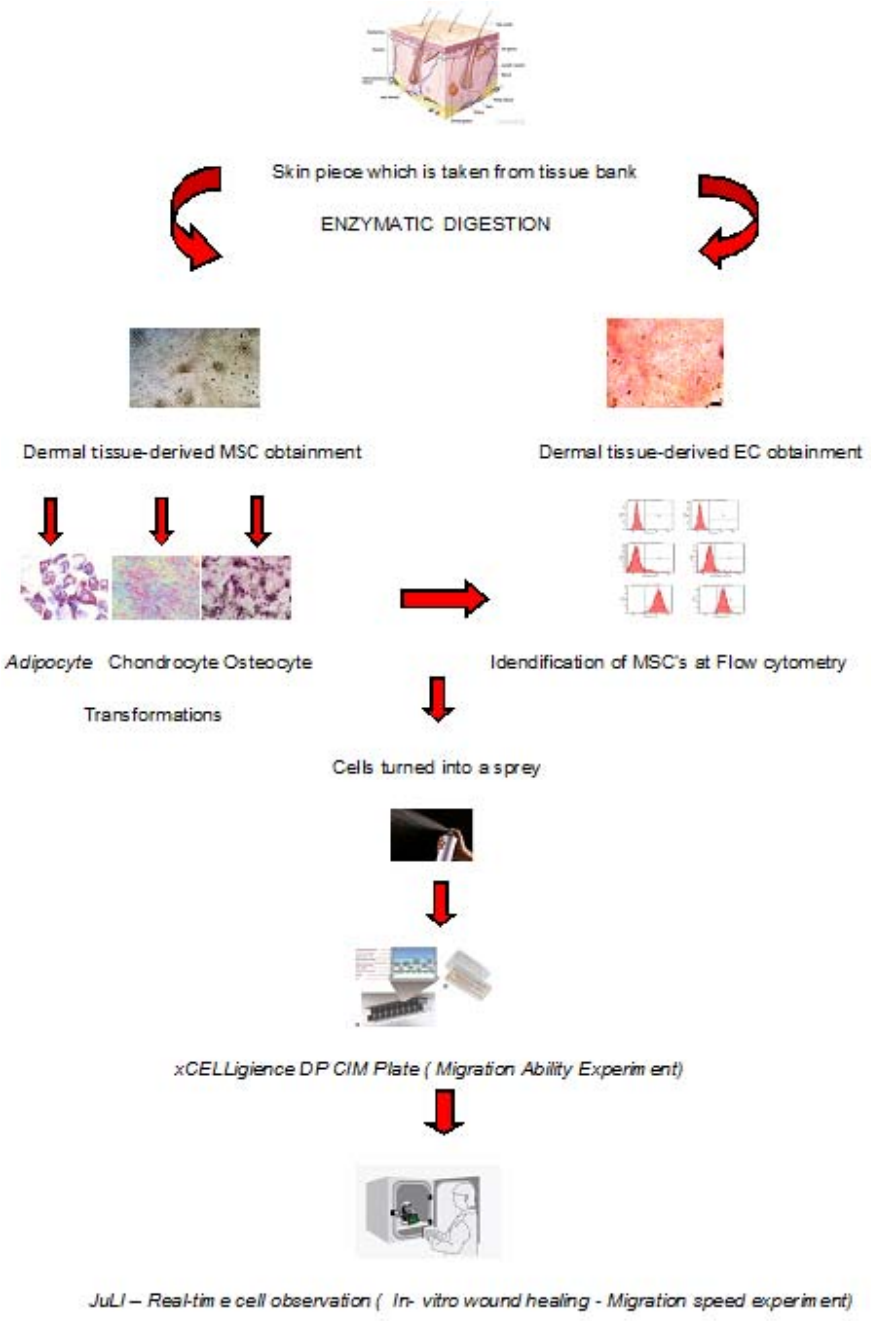


Figure 1. Work Flow Diagram (Flow diagram was drawn by myself)

Material list:

- *MSC Growth Medium (Ingredients are given in Appendix 1)*
- *Keratinocyte Medium*
- *Collagenase Type 1 (Preparation are given in Appendix 1)*
- Flow cytometry device(FACSAria III,USA)
- Real-Time Cell Analyzer (RTCA) (Roche, Germany)
- JuLI(Nanotek- Germany)
- Leica invert microscope(Leica, Germany)

A. Dermal-Tissue Derived MSC and EC Isolation:

Small pieces of dermal tissue incubated in 5 ml Mesenchymal Stem Cell growth medium under appropriate conditions. Differentiation of the Adipocyte, Chondrocyte and Osteocyte tissues made according to the previous studies (Pınarlı et al).

Epithelium cells undergo similar processes which differ from MSCs as; pieces of dermal tissue added to collagenase enzyme solution which was prepared as 1mg/ml. Incubated at 37 C° in water bath for 1,5 hours. After the incubation, cells passed through 425 micrometer sieve.

Details are given in Appendix 2.

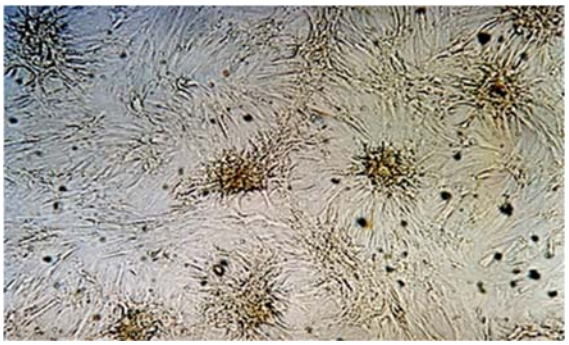


Figure 2. MSCs under light microscope, day 18 (Leica 1200, 10X20).

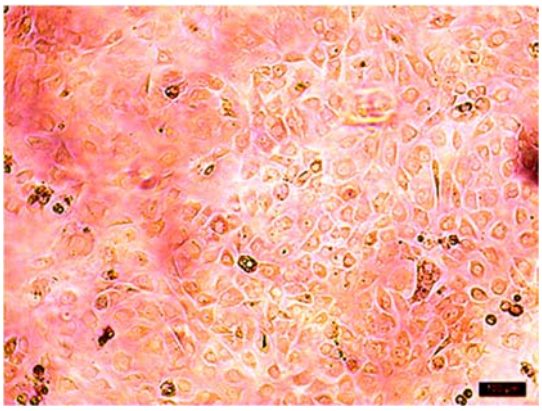


Figure 3. Dermal Tissue Derived ECs under light microscope, day 7 (10X20,Leica 1200).

B. MSC Adipocyte, MSC Chondrocyte and MSC Osteocyte Differentiation:

For Adipocyte differentiation, AD Basal Medium and its supplement are used. Fat cells observed with Leica invert microscope (Figure 1A).

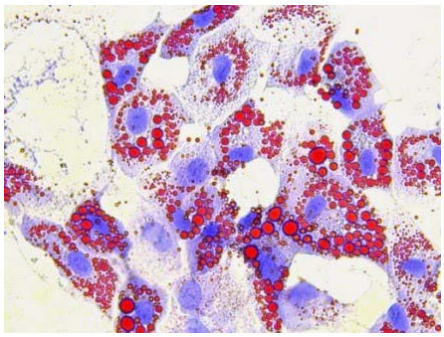


Figure 1A, Fat cells observed with Leica invert microscope

For Chondrocyte differentiation, CD Basal Medium and its supplement medium are used. At the end of the second week, cells are colored with Alcian Blue. Chondrocyte cells observed with Leica invert microscope. (Figure 1B).

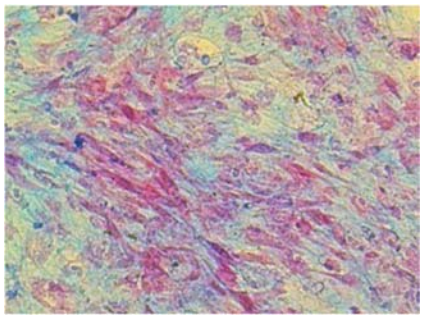


Figure 1B. Chondrocyte cells observed with Leica invert microscope.

For Osteocyte differentiation, OD Basal Medium and supplement medium are used. Osteocyte cells observed with Leica invert microscope. (Figure 1C).

Cells on the third passage are identified on Flow cytometry device with respect to surface markers CD11b/c, CD45, CD90, CD49. Details about flow cytometry is given in appendix 7.

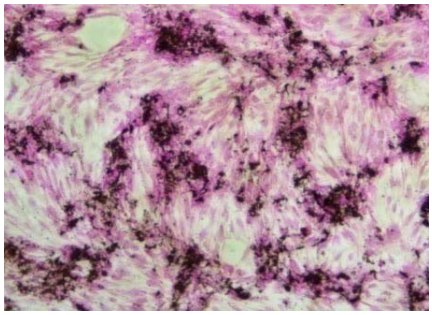


Figure 1C, Osteocyte cells observed with Leica invert microscope.

C. Real Time Cell Analyzer Migration Experiment:

In migration experiment Real-Time Cell Analyzer (RTCA) and CIM-plate -which is compatible with it- are used. Both MSC and EC culture mediums is added to the base of the CIM-plate. CIM-plate's upper part is placed gently. 50 µl Keratinocyte-SFM medium added to upper part. Plate incubated for 1 hour at 37º in a 5% CO_2 incubator. Details to this experiment is given in the Appendix 3 and 6.

D. Preparation of Dermal-Tissue Derived MSCs and ECs:

Conditioned medium which was taken from the isolation step of MSCs and ECs are placed in to a flask. After detachment process of the cells they are transferred into 10 cc sterile tube in order to centrifuge them. The process repeated one more time. Supernatant part thrown away again and Keratinocyte-SFM added.

MSCs and ECs which are inside the Keratinocyte-SFM are turned into suspension transferred into two different sterile tubes with a spray apparatus. It was checked that each spray has approximately 20.000/100µl cells, with spraying trials. Than cells are planted into wells of plate by means that each well have 20.000 cells in it with 100µl Keratinocyte-SFM. After that plate placed to Real-Time Cell Analyzer (RTCA) device. Experiment started. After 48 hours, experiment ended and data are taken. Details are given in Appendix 4.

E. Wound Healing Experiment:

Cells which are inside the Keratinocyte-SFM are turned into suspension and transferred into a sterile tube with a spray apparatus. It was checked that each spray has approximately 20.000/100µl cells, with spraying trials. Than 20.000 cells planted into a petri plate which was coated with fibrin-lamin. When the cells reached confluency scratch was formed and MSC growth medium is added to MSCs and Keratinocyte-SFM is added to ECs. Scratch area observed with Leica invert microscope and photograph of area taken. JuLI device placed as it can observe the starch area, observation continued for 48 hours. At the end of the 0. hour and 48. Hour scratch area's photographs are taken with invert microscope. The pre-experiment and after experiment photographs' area calculated with Image J program. Details of the experiment are given in the Appendix 5.

Data Analysis

Table 2. *xCELLingence Migration Cell Index Real Time Migration Experiment*

		Cell Index	
Hours	Trials	MSC	EC
0	1	0	0
	2	0	0
	3	0	0
	4	0	0
4	1	0.0815	0.0819
	2	0.0567	0.0921
	3	0.0829	0.0978
	4	0.0736	0.0997
8	1	0.0711	0.1023
	2	0.0875	0.1038
	3	0.1061	0.1085
	4	0.0987	0.1091
12	1	0.0815	0.1102
	2	0.1071	0.1124
	3	0.1174	0.1151
	4	0.1112	0.1164
16	1	0.0917	0.1201
	2	0.129	0.1265
	3	0.1277	0.1279
	4	0.1189	0.1294
20	1	0.108	0.1324
	2	0.1493	0.1332
	3	0.1389	0.1379
	4	0.127	0.1387
24	1	0.1159	0.1467
	2	0.1516	0.1444
	3	0.1446	0.1484
	4	0.1347	0.1488
28	1	0.1274	0.1526
	2	0.1624	0.1557
	3	0.1556	0.1565
	4	0.1378	0.1584
32	1	0.1366	0.1507
	2	0.1675	0.1511
	3	0.1644	0.1524
	4	0.1484	0.1537
36	1	0.1585	0.1618
	2	0.1811	0.1625
	3	0.1993	0.1635
	4	0.2077	0.1639
44	1	0.2276	0.1651
	2	0.2377	0.1652
	3	0.2454	0.1674
	4	0.2547	0.1684
48	1	0.2819	0.1765
	2	0.2903	0.1854
	3	0.3248	0.1954
	4	0.3451	0.2019

Details about cell indexes and Real Time Migration experiment are given in Appendix 3 and 6.

Table 3. *Percentage values of cell covered area in JuLI Experiment.*

	MSC	EC
1. hour	10%	3%
2. hour	18%	8%
3. hour	27%	10%
4. hour	39%	12%
5. hour	52%	13%
6. hour	63%	14%
7. hour	82%	14%
8. hour	89%	16%
9. hour	93%	17%
10. hour	100%	17%

Graph contains percentage values of wound area covered by both cell types till the 10 Hour as MSCs were able to cover up all wound area and ECs are unable to cover more than 17% of the wound area until the 48. Hour, end of the experiment.

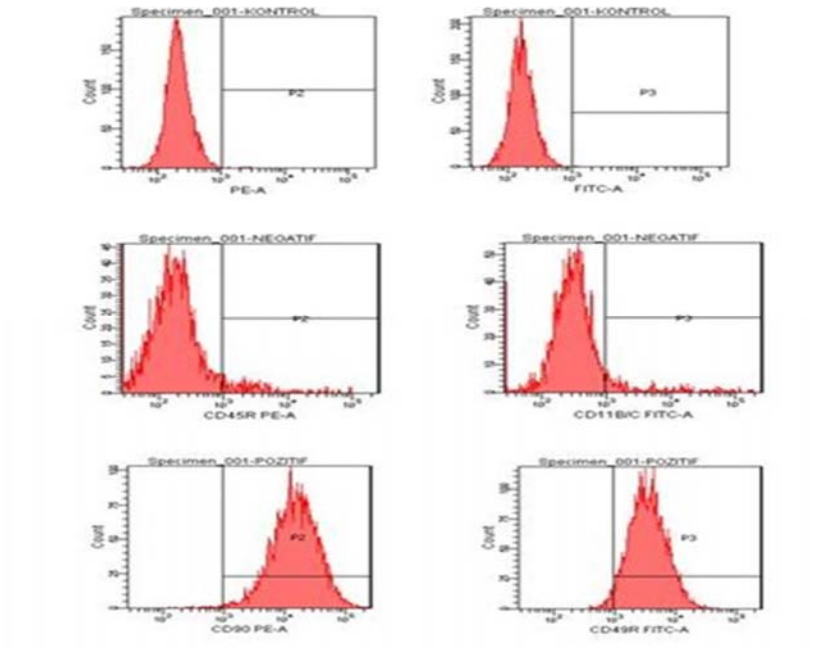


Figure 4. MSCs histogram results in flow-cytometry device (FACSAria III,USA) by surface markers; CD11b/c(-),CD45(-) CD90(+), CD49(+). Details are given in Appendix 7.

Data Processing:

F. Statistical Analysis

1. Mean

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

n = number of trials

x_i = number of viable cells per mL of culture

2. Variance:

$$s^2 = \frac{1}{(N-1)} \sum_{i=1}^{N} (x_i - \bar{x})^2$$

n = number of trials

xi = number of viable cells per mL of culture

$\bar{x}$ = mean

3. Standard Deviation:

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2}$$

n = number of trials

xi = number of viable cells per mL of culture

$\bar{x}$ = mean

4. Standard Error:

$$SE_{\bar{x}} = \frac{s}{\sqrt{n}}$$

s = standard deviation

n = number of observations of the sample

Sample Calculation:

Trial 1 of Cell Migration Experiment, MSC;

$$\overline{x} = \frac{0 + 0.0815 + 0.0711 + 0.0815 + 0.017 + 0.0917 + 0.108 + 0.01159 + 0.1274 + 0.1366 + 0.1585 + 0.2276 + 0.2819}{12}$$
$$= \mathbf{0.651345}$$

$s^2 = \frac{1}{12}\sum_{n=1}^{12}[(0.0815 - 0.651345)^2 + (0.0711 - 0.651345)^2 + (0.0815 - 0.651345)^2 + (0.017 - 0.651345)^2 + (0.0917 - 0.651345)^2 + (0.108 - 0.651345)^2 + (0.01159 - 0.651345)^2 + (0.1274 - 0.651345)^2 + (0.1366 - 0.651345)^2 + (0.1585 - 0.651345)^2 + (0.2276 - 0.651345)^2 + (0.2819 - 0.651345)^2]$ = **0.0067128**

s = $\mathbf{0.0819321}$

$SE_{\overline{x}} = \frac{0.0819321}{\sqrt{13}} = \mathbf{0.0227238895095}$

Cell Type	Mean	Standart Error	Variance	Standart Deviation	Confidence Level (95.0 %)
ECs	0.71	0.01	0.002	0.04	0.02
MSCs	0.82	0.02	0.005	0.07	0.04

Table 4: Descriptive statistics of calculated values of cell index in Real Time Cell Analyzer Migration Experiment taken from Table 1.

	ECs	*MSCs*
Mean	0.71	0.82
Variance	0.002	0.005
Observations	48	48
Hypothesized Mean Difference	0	
df	78	
t Stat	7.633198	
P(T<=t) one tail	0.000791	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.001582	
t Critical two-tail	2.776445	

Table 5: Independent t-Test for cell indexes of MSCs and ECs in Real Time Cell Analyzer Migration Experiment. Data taken from Table 1.

	MSC	*EC*
Mean	100	17
Variance	0.25	0.25
Observations	4	4
Hypothesized Mean Difference	0	
df	6	
t Stat	234.75945	
P(T<=t) one tail	2.016E-13	
t Critical one-tail	1.9431803	
P(T<=t) two-tail	4.031E-13	
t Critical two-tail	2.4469119	

Table 6: Independent t-Test for percentage covered area of scratch by MSCs and ECs in-vitro Wound Healing Model. Data taken from Table 2.

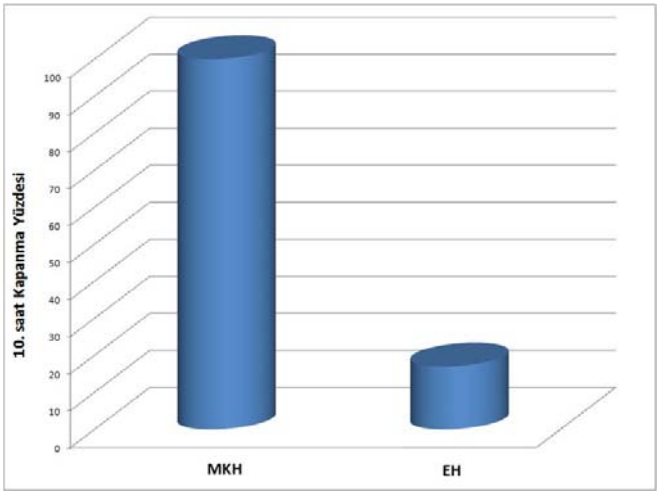


Figure 3*: Bar graph of MSCs' and ECs' migration speeds in-vitro wound healing model at 10. hour. Error bars are not shown as SE is less than 0.001.*

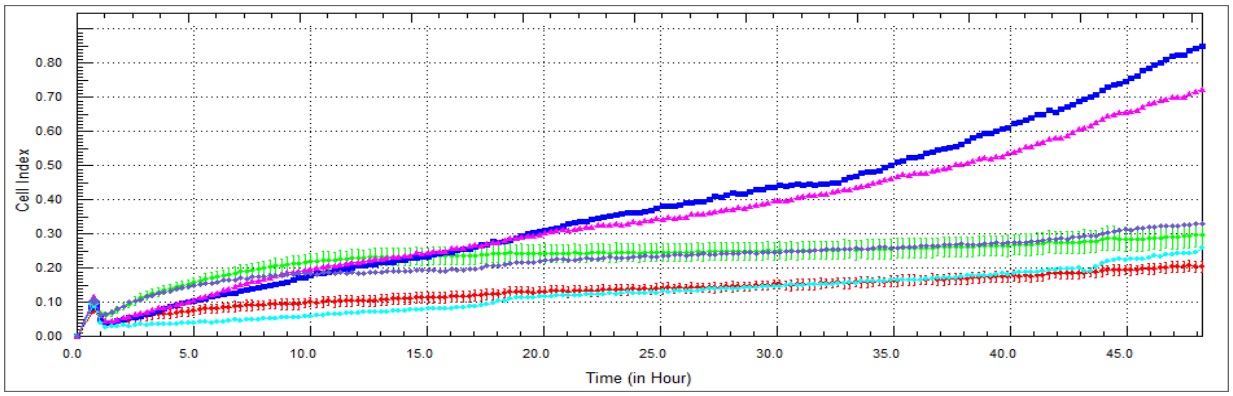


Figure 4*: xCELLingence DP device, 48 hour cell index graph: MSC (Blue), EC (Pink), controls (Green and Red).*

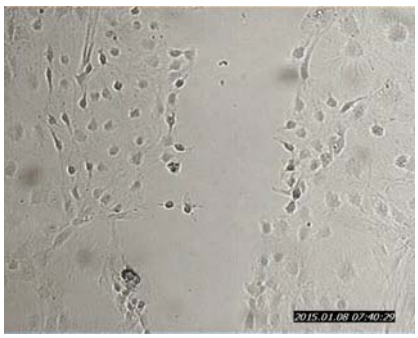
Evaluation

This study deals with the comparison of Dermal Tissue derived MSCs and Dermal Tissue derived ECs in Real Time Cell Analyzer Migration Experiment and *in-vitro* Wound Healing Model in 48 Hour time in terms of cell migration speed, ability and percentage area coverage.

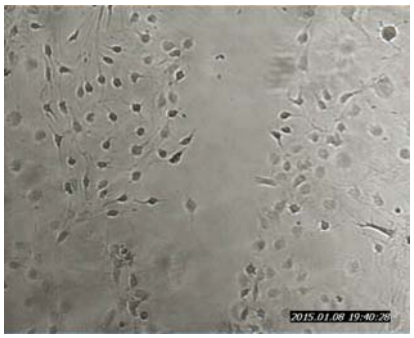
The experimental results from both of the experiments were analyzed statistically using the SPSS 20 software. Limit of significance taken as ($p<0.05$).

After 48 hour long comparing the migration skills of Dermal tissue derived MSCs and ECs on the CIM plate of xCELLingence DP (Rhoche) device, it was observed that mean values MSCs' cell index is 0.82 (from 0.0815 to 0.3451), ECs' index was 0.71 (from 0.0819 to 0.2019). This result showed that MSCs are more favorable in wound healing treatment than ECs in terms of cell migration speed and ability. As P(T<=t) one tail value found as 0.000791 which is lower than the limit of significance, the results of differences are meaningful for the Real Time Cell Analyzer Migration Experiment.

In *in-vitro* model which Real-time camera system –achieved with JuLI- used, it was observed that MSCs covered the gap at the 10th Hour 100%, but ECs could only covered up 17% of the area. The experiment conducted 3 times, no significant difference between trials (SE is less than 0.001). Statistical analyzes showed that MSCs' migration speed is meaningfully more than ECs' ($p < 0.05$) as P(T<=t) one tail value calculated as 2.016E-13. This study carried out by comparing the 0. Hour and 10. Hour data of the area with the help of the image J software (Figure 5, Figure 6, Figure 7).

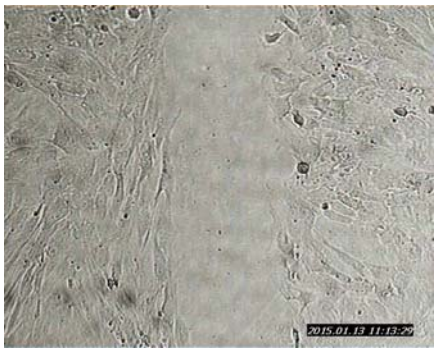


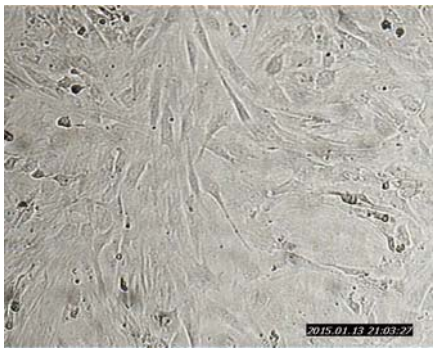
(A)



(B)

Figure 6: *Dermal tissue derived ECs' images on Real-time cell observation device (JuLI) in-vitro wound healing model at 0. hour(A) and 12. hour (B).*



(A)


(B)

Figure 7: *Dermal-tissue derived MSCs' images on Real-time cell observation device (JuLI) in-vitro wound healing model at 0. hour(A) and 12. hour (B).*

- Pre-clinic study: Before testing the spray on humans (Clinic studies), animal testing must be done in order to prevent possible or unexpected health issues. However, as I am a High-school student I am not allowed to execute experiments on animals. So I plan to do pre-Clinic experiments when I attend to Molecular Biology and Genetics License Program. By those pre-Clinic testes the product could show its potential in living organisms better than *in-vitro* experiments
- Stability of cells must be controlled. Stability tests are not done as cells stayed in a spray form for a short period of time. If this spray will be used as a medical product the cells must be stable in spray form. Different buffer solutions containing different stability agents could be used to increase the stability of cells. Also different protective proteins could be used to provide stability.
- Different culture media and excipients can be used for a better and accelerated growth for cells. Both for MSCs and ECs.

Conclusion

In this project, I found that mean values of cell indexes of Dermal Tissue Derived MSCs and ECs respectively, 0.82 and 0.71 which showed a meaningful difference. I calculated resulting values from cell indexes of both cell types by data taken from xCELLingence CIM plate device in in-vitro wound healing model after cells were isolated from the skin and turned into a spray for the first time. Those cell types weren't compared in in-vitro wound healing model ever before.

When migration abilities compared with JuLI, MSCs' covered 100% of wound area however ECs could only covered 17% of the area on the 10. hour which showed a meaningful difference ($p < 0.05$). Even if there should be special conditions must be fulfilled for production and reproduction of ECs, in this experiment I used specific medium for ECs. However, usage of MSCs' as cell spray proved itself superiority to ECs and capability to be a good method of treatment.

In means of expenses, this new cell spray product would be significantly more cheaper than existing ways of treatments to dermal wounds. Even if skin transfer have its own advantages, specifically produced otology epidermis grafts' formation to an effective product is seems difficult as it would be a high-cost project. For example, a commercial epidermis graft which have approval by American Food and Drug Administration (FDA) costs approximately 26 $ per centimeter square, including all steps of production. According to this graft costs 260.000 $ per meter square (Can A. 2014). Even if it is possible to produce otology graft in emergency situations, the situation should be responded in a short time and it would cost very high. Those are the main problems with otology grafts.

When all those values evaluated and after necessary in-vivo experiments are carried out, ECs which are used in treatments like this however, have a high expense and obligation to produce for specific for the patients created an idea to me to use MSCs as way of treatment which could easily produced in laboratories with GMP(Good manufacturing practice) conditions and without an obligation to finding a suitable donor. This treatment would be easily stored and accessible to everyone and most importantly without high expenses.

So with the both experimental results from Real Time Cell Analyzer Migration Experiment and Wound Healing Model my hypothesis; ''*Usage of Dermal tissue-derived Mesenchymal stem cells (MSCs) will be more effective than usage Dermal tissue-derived Epithelium cells(ECs) in terms of wound healing process by using in-vitro wound healing model in terms of migration ability and speed*.'' was proved.

Appendices

Appendix 1:

Material list:

- *MSC Growth Medium*

%20 Fetal Bovine Serum (Lonza, Belgium),

%2 L-Glutamine (Lonza, Belgium),

%1 Penicillin Streptomycin Amphotericin (Biological Industries, Israel)

%77 Dulbecco's Modified Eagle Medium (DMEM)(Lonza, Belgium)

- *Keratinocyte Medium:*

%20 Fetal Bovine Serum (Lonza, Belgium),

%2 L-Glutamine (Lonza, Belgium),

%1 Penicillin Streptomycin Amphotericin (Biological Industries, Israel)

%77 Keratinocyte-SFM (Gibco, USA)

- *Collagenase Type 1 Preparation: 3 mg collagenase Type 1(Sigma, USA) dissolved with 3ml HBSS (Lonza, Belgium) and used as 1mg/ml.*
- Flow cytometry device(FACSAria III,USA)
- Real-Time Cell Analyzer (RTCA) (Roche, Germany)
- JuLI(Nanotek- Germany)
- Leica invert microscope(Leica, Germany)

Appendix 2:
Dermal-Tissue Derived MSC and EC Isolation

In this experiment I Wistar-Albino rat's femur area dermis-epidermis skin pieces are used which are taken from, Dışkapı Training and Research Hospital's Stem Cell Centre Tissue Bank, as cell provider.

Dermal tissue material bathed in mediums which i filled with antibiotics consecutively. It separated into small pieces with the help of nester. Those small pieces of dermal tissue put into T25 flasks. Incubated in%5CO2 and 37 ° incubator for 30 minutes. After the incubation 5 ml medium which contains; %20 Fetal Bovine Serum(Lonza, Belgium), %2 L-Glutamine(Lonza, Belgium), %1 Penicillin Streptomycin Amphotericin (Biological Industries, Israel) and %77 Dulbecco's Modified Eagle Medium (DMEM)(Lonza, Belgium) added and put into the incubator. After two days MSC medium changed by taking consideration of the growth of the cells. The production of cells obtained by changing medium per 2-3 days. After flask reach to %70-80 confluency sub-culturing has been done. 1-2 cc Phosphate Buffer-Saline (PBS) (Lonza,Belgium) added to medium. After 2 minutes, by draining PBS, 2 cc %0,05 Trypsin EDTA-C(Lonza,Belgium), solution added.. Left 2 minutes in incubator. When separation of cells and disconnection from flask could be observed in order to reduce the effects of Trypsin DMEM solution added. Cells taken into a 10 cc sterile tube and centrifuged for 10 minutes at 1200 RPM. After the centrifuge supernatant part (The part which is in the surface of the solution) has been separated and pellet was broken. Cells centrifuged again. Supernatant part separated from the cells. Than MSC medium added and cells separated into 2 different flasks. Mediums changed per 2-3 days. After observing the development of cells, the cells which are on the third passage differentiated into the Adipocyte, Chondrocyte and Osteocyte tissues.

Epithelium cells undergo similar processes which differ MSCs as; 3 mg collagenase type 1 (Sigma, USA) enzyme weighted and dissolved in 3 Ml HBSS (Lonza. Belgium). Small pieces of dermal tissue added to enzyme solution which was prepared as 1 mg/ml. Incubated at 37 C° water bath for 1,5 hours. After the incubation cells passed through 425 micrometer sieve.

Appendix 3

Real Time Cell Analyzer Migration Experiment

In migration periment Real-Time Cell Analyzer (RTCA) and CIM-plate -which is compatible with it- are used. A culture which includes; 160 µl %20 Fetal Bovine Serum (Lonza, Belgium), %2 L-Glutamine(Lonza,Belgium), %1 Penicillin Streptomycin Amphotericin (Biological Industries, Israel), %77 Keratinocyte-SFM(Gibco, USA), is added to the base of the CIM-plate. CIM-plate's upper part is placed gently. 50 pi Keratinocyte-SFM (Gibco, USA) medium added to upper part. Plate left incubated in 37 degree Celsius and %5CO_2 surrounding conditions for 1 hour.

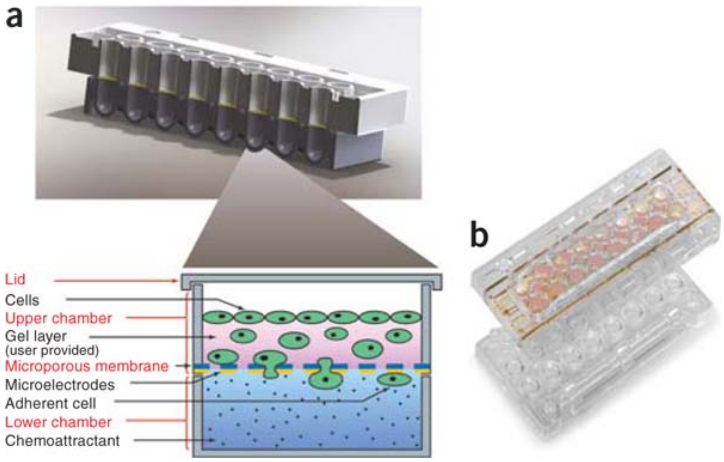


Figure 1: Analyze invasion/migration in real time with the CIM-Plate 16. The plate features two separable sections for ease of experimental setup. Cells seeded in the upper chamber move through the microporous membrane into the lower chamber that contains a chemoattractant. Cells adhering to the microelectrode sensors lead to an increase in impedance, which is measured in real time by the RTCA DP Instrument.

Appendix 4

Preparation of Dermal-Tissue Derived MSCs

Medium is placed in to a flask. 1-2 cc Phosphate Buffer Saline (PBS)(Lonza,Belgium) added. After 2 minutes PBS drained away and 2 cc %0,05 Trypsin EDTA-C(Lonza,Belgium), solution added. Flask waited in incubator for another 2 minutes. Keratinocyte-SFM (Gibco, USA) solution added in order to decrease the effect of trypsin when it can be observed that cells draw apart from each other and rise from the bottom of the flask. All cells are transferred into 10 cc sterile tube in order to centrifuge them on 1200 RPM for 10 minutes. After the centrifuge, supernatant part is thrown away and pallet is shattered. Cells undergo centrifuge again. Supernatant part thrown away again and Keratinocyte-SFM (Gibco, USA) added. Cells' number and viability are observed with Countess® Automated Cell Counter (Invitrogen,USA).

Cells which are inside the Keratinocyte-SFM (Gibco, USA) are turned into suspension transferred into a sterile tube with a spray apparatus. It was checked that each spray has approximately 20.000/100µI cells, with spraying trials. Than cells are planted into wells of plate by means that each well have 20.000 cells in it with 100p1Keratinocyte-SFM (Gibco, USA). After that plate placed to Real-Time Cell Analyzer (RTCA) (Roche, Germany) device. Experiment started. After 48 hours, experiment ended and data are taken.

Appendix 5

Wound Healing Experiment:

Cells which are inside the Keratinocyte-SFM (Gibco, USA) are turned into suspension are transferred into a sterile tube with a spray apparatus. It was checked that each spray has approximately 20.000/100µI cells, with spraying trials. Than 20.000 cells planted into a petri plate which was coated with fibrin-lamin. When the cells covered 90% of petri plate's surface and form colonies, a scratch formed in the middle of the plate with a pipe. After the scratch was formed, a medium which is formed by %1 Fetal Bovine Serum(Lonza, Belgium), %2 L-Glutamine(Lonza,Belgium), %1 Penicillin Streptomycin Amphotericin (Biological Industries, Israel), %77 Keratinocyte-SFM(Gibco, USA) added. Scratch area observed with Leica invert microscope (Leica, Germany) and photograph of area taken. JuLI (Nanotek- Germany) device placed as it can observe the starch area, observation continued for 48 hours. At the end of the 0. hour and 48. Hour scratch area's photographs are taken with invert microscope (Leica, Germany). The pre-experiment and after experiment photographs' area calculated with Image J program.

Appendix 6

Cell Based Assays For Cell Adhesion & Cell Morphology

The presence of the cells on top of the electrodes will affect the local ionic medium at the electrode/solution interface, leading to an increase in the electrode resistance. The more cells are attached on the electrodes, the larger the increases in electrode resistance. In addition, the resistance depends on the quality of the cell interaction with the electrodes. Thus, electrode impedance, which is displayed as cell index (CI) values, can be used to monitor cell viability, number, morphology, and adhesion degree.

Appendix 7

Flow cytometry is technique that quantifies and then analyzes a variety physical and chemical characteristics of single particles as they flow in a viscose stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity. These characteristics are determined using an optical-to-electronic match-up system that records how the cell or particle disperse incident laser light and emits fluorescence.

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