TED ANKARA COLLEGE Foundation High School

Coculturing of Rat Islet Cells with Human Amnion Membrane to Increase Their Cell Viability and Production of Insulin

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Biology Extended Essay

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Abstract

In nowadays, Diabetes Mellitus is a growing threat to humans' health. Unfortunately, there is no permanent and effective cure for it. Usually, transplantations of Langerhans islets or pancreases are not successful in long terms because of immune sytems attack on them. The aim of this research is to find out the potential of usage of human amnion membrane as a protective membrane against the immune system in islet transplantations. No research similar to this one has been done which means that this study is the first one in literature. My research question is "How does human amnion membrane affect rat langerhans islet's cell viability and functionability during coculture by fluorescent microscopy and glucose stimulation test?".

The HAM (Human Amnion Membrane) is the inner layer of the placenta that surrounds the baby during pregnancy. HAM shares its cellular origin with the fetus; together they grow in parallel throughout pregnancy. It supports cell growth and repairing processes. It is cheap and because of its extensive usage in wound clothing, especially in eye wound clothing, it is easy to find. For these reasons, HAM is a good candidate for be used as a protective membrane in langerhans islet transplantations.

During the experiment, 3 experiment group and one control group was taken to coculture with HAM and HAM position in respect to the islets was different in each group. Before the culture, cell viability and insulin indexes of experiment groups were measured by fluorescent microscopy and glucose stimulation test. After 48 hours in coculture, cell viability and glucose stimulation tests were done again to measure the final cell viability and insulin indexes of experiment groups. Iniitial cell viability and insulin indexes are 81.0% and 3.4 respectively G_2 (has no insert solution between islets and HAM) has the lowest final mean cell viability (%64) and index of insulin (2.2) in the experiment groups. Control group's (only islets) final index of insulin and mean cell viability are 3.7 and %82 respectively. G_3 (islets at the top, HAM at the bottom, includes insert) has the second highest final mean cell viability (%86) and index of insulin (4.2). G_1 has the highest final mean cell viability (%93) and index of insulin (5.8). Standard error of control group's final cell viability is 0.63, standard error of final cell viability of G_1 is 0.90, G_2 's standard error of final cell viability is 1.23 and standard error of final cell viability of G_3 is 0.63. Standard error of control group's final insulin index is 0.36, standard error of insulin index of G_1 is 0.33, G_2 's standard error of final insulin index is 0.14 and standard error of final cell viability of G_3 is 0.06.P value of one way ANOVA test of cell viability and glucose stimulation test are ($p= 4.73 \times 10^{-12}$, $p= 1.19 \times 10^{-16}$) respectively. These p values are smaller that alpha value which means HAM can be used as a protective membrane in Langerhans islet transplantations.

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Introduction

The first time that I heard about the diabetes was when I learned that my mother had gestational diabetes during her pregnancy. In order to prevent possible complications that could create severe problems, she followed a strict diet and managed to pass her pregnancy without any problem. Her disease inspired me to make a research on diabetes.

I found an article in internet when I was searching diabetes. The article was about DM (Diabetes Mellitus) type I. While I was reading the article, I noticed for the first time that there was no permanent cure exists for type 1 but some treatments to hide its symptoms. Although patients get permanent cure in some cases by langerhans islets or pancreas transplantation, their success rate is low and patients need immunosuppressive drugs for the rest of their life. This problem attracted my attention to DM Type I and I decided to focus on this subject.

Type I diabetes, once known as juvenile diabetes or insulin-dependent diabetes, is a chronic condition in which the pancreas produces little or no insulin, a hormone needed to allow sugar (glucose) to enter cells to produce energy. Various factors may contribute to type 1diabetes, including genetics and exposure to certain viruses.[1] These factors attack to β cells of Langerhans islets which are responsible for production of insulin.

Human islets of Langerhans are complex micro-organs responsible for maintaining glucose homeostasis. Islets contain five different endocrine cell types, which react to changes in plasma nutrient levels with the release of a carefully balanced mixture of islet hormones into the portal vein.[2] In the human pancreas, 65 to 90 percent of islet cells are beta cells, 15 to 20 percent are alpha-cells, 3 to 10 percent are delta cells, and one percent is PP cells. [3]

Main treatment of DM Type I is the insulin therapy which is the injection of insulin by a syringe, insulin pen or insulin pump to keep the blood sugar level stabile. This therapy continues in the rest of the patient's life. There are some medications except insulin to protect organs from secondary damage of DM Type I. In addition to insulin therapy and medications, Type I patients must pay attention to their diet and they must make physical exercise.

These treatments usually enough to hide the symptoms of the Type 1 but after for a long time, the body may develop resistance to insulin injections. In order to overcome the resistance,

[1] http://www.mayoclinic.com/health/type-1-diabetes/DS00329

[2] In't Veld P, Marichal M, Microscopic anatomy of the human islet of Langerhans., 2010

[3] http://stemcells.nih.gov/info/scireport/pages/chapter7.aspx

higher amounts of insulin and other drugs are used which is harmful and cause kidney failure in some cases which may cause to death.

In order to cure DM Type I for once and for all, several types of transplantations have been tried on Type I patients. First one of them is the pancreas transplantation. Main goal of the pancreas transplantation is to make the body produce its own insulin by its new pancreas' β cells. Pancreas-only transplants require lifelong immunosuppression to prevent rejection of the graft and potential recurrence of the autoimmune process that might again destroy pancreatic islet cells. Immunosuppressive regimens used in transplant patients have side effects whose frequency and severity restrict their use to patients who have serious progressive complications of diabetes or whose quality of life is unacceptable. In addition to the side effects of lifelong immunosuppression, the procedure itself has significant morbidity and carries a small, but not negligible, risk of mortality.[4]

Second one is the islet transplantation. Researchers are experimenting with islet cell transplantation, which provides new insulin-producing cells from a donor pancreas. Unfortunately, islet cell transplantation still requires the use of immune-suppressing medications, and just as it did with its own natural islet cells, the body often destroys transplanted islet cells, making the time off injected insulin short-lived.[5]

Between these two, islet transplantations offer the lowest risk and it is more economic. The problem is despite of usage of immuno-suppressing medications, it may not be possible to protect islets for a long time. Because, the factor that destroyed the patient's own islets (immune system, virus, bacteria or toxics) usually still in the body and destroys the islets again.If they are somehow protected, then the main complication that causes the long term failure of the islet transplantations will be disappear and these transplantations might offer higher success rates to the patients.

While I was searching DM Type I, I read that a protective membrane that encloses langerhans islets after the langerhans islets transplantation might be able to protect them from external threats such as bacterial or viral infection, toxics and immune system. Unfortunately, there are many important characteristics that a protective membrane must have. First of all, it must allow the diffusion of respiratory gases, cellular wastes and nutritients in order to allow the islets to make the necessary matter exchange. Secondly, this membrane must be antinflamatuar because any immunologic reaction against the membrane can harm both membrane and islets. In addition to these characteristics, this membrane should be cheap, easy to produce and avaible if it will be used in general treatment of DM Type I.

. The HAM (Human Amnion Membrane) is the inner layer of the placenta that surrounds the baby during pregnancy. HAM shares its cellular origin with the fetus; together they grow in parallel throughout pregnancy. The Amniotic Membrane is the only tissue designed by nature specifically as a universal transplant, protecting the baby and ensuring acceptance by the

[4] R. Paul Robertson, MD; Connie Davis, MD; Jennifer Larsen, MD; Robert Stratta, MD; and David E.R. Sutherland, MD, Pancreas Transplantation for Patients With Type 1 Diabetes, DIABETES CARE, VOLUME 26, SUPPLEMENT 1, JANUARY 2003

[5] http://www.mayoclinic.com/health/type-1-diabetes/DS00329/DSECTION=treatments-and-drugs

mother's immune system. Human Amniotic Membrane has been used in medicine for more than 100 years. Thanks to its usage for a very long time it is definitely known that HAM has no side effects.[5] Previous researches show that HAM allows diffusion of glucose. It supports cell growth and repairing processes. It is cheap and because of its extensive usage in wound clothing, especially in eye wound clothing, it is easy to find. For these reasons, HAM is a good candidate for be used as a protective membrane in langerhans islet transplantations.

The aim of this research is to find out the potential of usage of human amnion membrane as a protective membrane in islet transplantations. No research similar to this one has been done which means that this study is the first one in literature.

My research question is "How does human amnion membrane affect rat langerhans islet's cell viability and functionability during coculture by fluorescent microscopy and glucose stimulation test?".

Hypothesis

Human amnion membrane has been used for wound dressing for a century. It supports cell growth. Most importantly, it does not allow leukocytes to pass through which is very important to protect islets after the transplantation. Human amnion membrane has no antigen which makes it antinflammatory. It is derived from placenta after birth which makes it easy to find. It is easy to store and very basic and small number of procedures and required to use it in transplantation. [6] Thanks to these characteristics, it is very economic. Previous researches showed that HAM allows diffusion of glucose and insulin. I believe that all of these characteristics make HAM a very good candidate to be used as a protective membrane in islet transplantations. It is hypothesized that staying in coculture of islet cells &HAM increases the viability and insulin production (insulin index) of islet cells indicated by fluorescent microscopy and glucose stimulation test. Null hypothesis of this research is that presence of HAM creates no difference in cell viability and insulin index of langerhans islets.

[6] Tseng and ark., 1998; Meller and ark., 2000

Method Development

Research question of this research is "How does human amnion membrane affect rat langerhans islet's cell viability and functionability during coculture by fluorescent microscopy and glucose stimulation test?". My first step on designing the method was designing the culture conditions. I had two option of coculture: indirect coculture and direct coculture. The difference between them is that there is a physical contact between human amnion membrane and islets in direct coculture but there is a chemical interaction between HAM and islets rather than physical contact in indirect coculture. I decided to use both of them because both of these coculture methods can be applied to islet transplantation and presence or lack of physical contact may affect the experiment results. Next step was deciding what kind of medium do I use during the culture of HAM and islets. RPMI 1640 medium is preferred in mammalian cell and tissue cultures and it does not contain animal serum that may intergene with the characteristics of islets of langerhans cells. These properties make RPMI 1640 medium a very suitable medium for my research and my reasons for choosing RPMI 1640 medium. Standard temperature, moisture and CO_2 concentration of a mammalian cell culture are 36 ^{0}C, %95 moisture and %5 CO_2 and because of these are the optimum conditions for the culture of islet cells; I set these conditions in the incubator.

After the design of the culture conditions, I designed the experiment groups. In order to evaluate and analyze the effects of HAM by comparing results of HAM-included cultures with not HAM-included cultures, I decided to create a controll group (group C). There are two groups of indirect coculture, in group 1 (G_1), langerhans islets are at the bottom of the well and HAM is on the topside of the well. They are seperated by insert solution. The second group with indirect coculture (G_3) is same but positions of HAM and islets are different in respect to position of each others. In G_3, islets are at the top and HAM is at the bottom. My reason for using two experiment groups with indirect coculture is to find out does the position of HAM in respect to the islets have any effect on islets. Because if there are any negative effects caused by position of HAM in respect to the islets, HAM might partially damage islets during the transplantation and makes the transplantation inefficient because although body starts to produce its own insulin, it won't be enough and exogenous insulin injection would be still required.

There is one experiment group of direct coculture (G_2). The reason of why there is only one experiment group of direct coculture is that effect of position of HAM in respect to the islets will be determined by indirect experiment groups, so I did not need two experiment groups of direct coculture.

Perhaps, the most important step was deciding how to measure the effects of human amnion membrane on islet cells because choosing wrong parameters may not show the true effects of HAM or show no effect at all. I think that knowing HAM's effect on cell viability is crucial because if it harms islets, there is no sense to use it as a protective membrane. Also, it is very important to know the impacts of presence of HAM in coculture on production of insulin of β cells of islets because the aim of islet transplantations is to give the abilitiy of producing

its own insulin to body and any negative effect of HAM on insulin production of β cells makes the transplantation useless.

After deciding the parameters of effects of HAM, I chose the test to measure those parameters. There are many tests for measuring cell viability but fluorescent microscopy as it is very precise and it does not only measure cell viability but also differentiate the dead and alive cells. The location of the dead cells may indicate presence/absence of external damage or lack of oxygen and such causes of damage on islets. In order to measure the effects of HAM on insulin production of islets, I decided to use glucose stimulation test. My reason was that glucose stimulation test is precise, accurate and easy to do. The hardest step of the designing the method for me was designing the preparation of HAM for the experiment. I made a lot of research and spend many hours to design the method. You can find it at Appendix B.

Presence of microorganisms or any toxic material can harm or destroy all of the islets during the culture. In order to avoid such harms, the experiment was conducted in the rat langerhans islet lab of Dışkapı Training and Research Hospital. With Dr. Ferda Alparslan's permission, I chose Dışkapı Training and Research Hospital's Rat Langerhans Islet Lab to conduct my research because they have the every instrument that I need and demand no money for the expenses of my research.

The lab was hygienic and any instrument was washed with %70 alcohol disinfentents before using it in order to kill all of the microorganisms and prevent any contamination. All ltubes, wells, chemical and biological material have not been used before to eliminate the possibility of contamination of toxic materials and microorganisms. As I mentioned before, the lab was hygienic to prevent contamination.

This experiment had been conducted for 48 hours. I consider several factors while making this decision. My priority was to conduct the experiment long enough to see the significant effects of HAM. Because of nutrients in the culture is limited, if I choose too long time, islets might starve and die which may hide the positive effects of HAM. I also considered that concentration of cellular waste can reach to dangerous levels for islets if they stay in the culture for a long time, and again hide the positive effects of HAM.

The impact of deviation due strictly to chance is diminshed as the sample size increases.[7] I thought that measuring cell viability and production of insulin for 5 times diminshes the impact of deviation due strictly to chance enough. Although it did not create a significant problem, due to the malfunction of instruments, glucose stimulation test was performed for 3 times for every experiment group.

After the experiment, in order to evaluate the results, it is essential to know that the difference between results of the experiment groups are significant or not. I decided to use One-Way ANOVA test to calculate that the difference is significant or not because it is used for calculation the mean difference between two or more samples.

[7] S. Klug William, Concepts of Genetics, Prentice Hall,1997

Materials used in Experiment

L-Glutamin,

Penisilin/ Streptomisin/ Ampisilin,

FBS (Fetal Bovine Serum),

Roswell Park Memorial Medium,

Propidium Iodide,

PBS,

Fluorescein diacetate,

Aceton,

Glucose

Indirect coculture dishes

Rat Langerhans Islets

Human Amnion Membrane

DMEM- LG with % 50 glicerol

Tripsin-ETDA C

Pasteur pipettes

Method

Steps of the Method

A.Fluorescent microscopy before the experiment

Cell viabilities of islets of experiment groups before the culture are measured by fluorescent microscopy. (Procedure is described in Appendix A)

B. Glucose stimulation test before the experiment

This research's main goal to find a possible cure to DM Type 1 and because of that, effect of HAM on insulin production capacity of β cells of Langerhans islets was determined. In order to do this, insulin production capacity of β cells was measured at both before and after the experiment by glucose stimulation test. (Procedure is described in Appendix C)

C. Preparation of Human Amnion Membrane

HAM was provided by Dışkapı Training and Research Hospital. To prepare it for the experiment, it was washed by different solutions and to separate amnion epithel, it was treated with EDTA solution. Detailed protocol is included in Appendix B.

D. Preparing the culture

Next step is was preparing cocultures of HAM and Langerhans islets. There are 4 experiment groups. These are control group, G_1,G_2,G_3. (The procedure is described in Appendix D).

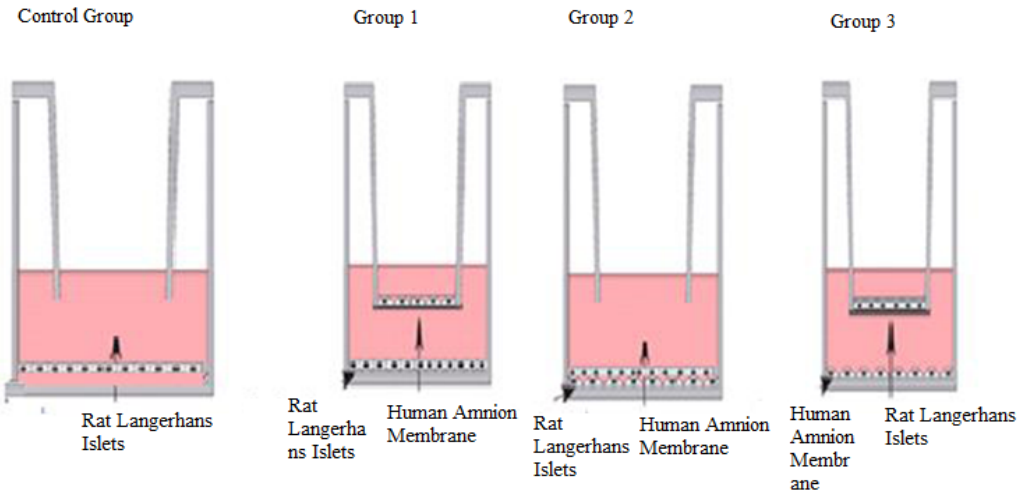


Figure 1: Figure shows experimental groups in culturing HAM and islet cells. Totally 4 groups tested for viability assays: Control group (no HAM), G_1 (HAM at the top and islets are at the bottom, no physical contact), G_2 (HAM at the top and islets are at the bottom, physical contact is present), G_3 (HAM at the bottom and islets are at the top, no physical contact)

E. Fluorescent microscopy after the experiment

Cell viabilities of islets of experiment groups after the culture are measured by fluorescent microscopy. (Procedure is described in Appendix A)

F. Glucose stimulation test after the experiment

Insulin indexes of islets of experiment groups after the culture are measured by glucose stimulation test. (Procedure is described in Appendix B)

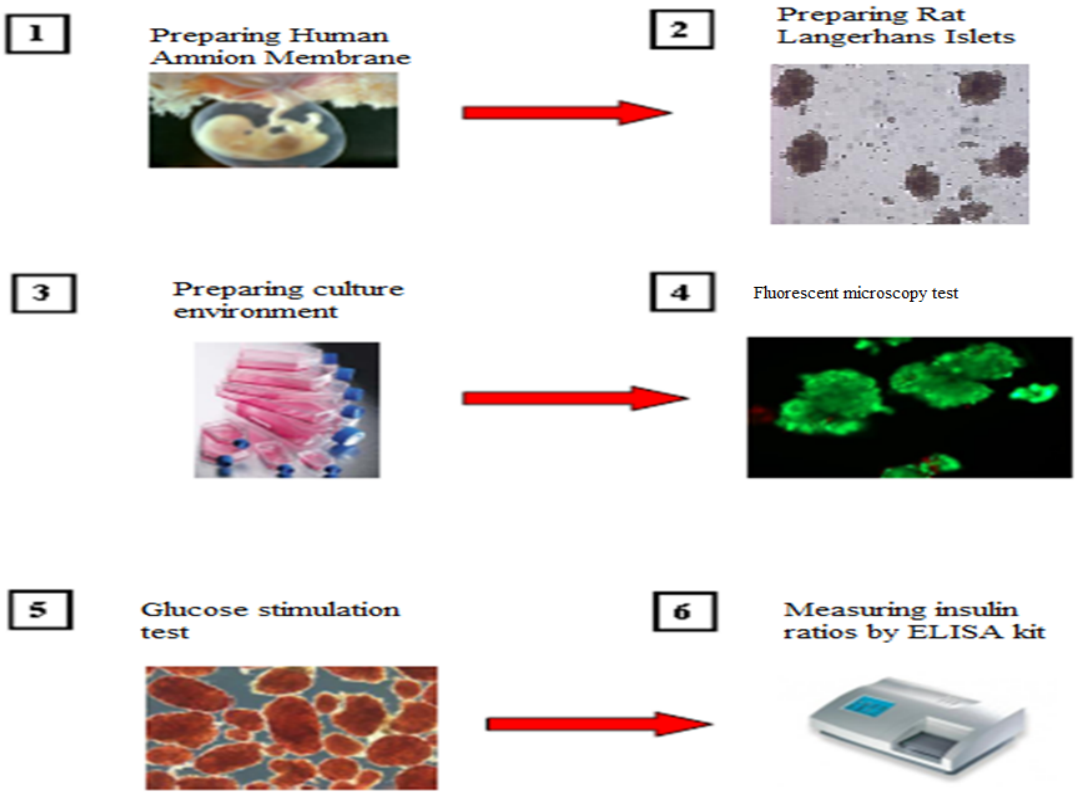


Figure 2: Main steps of the experiment

5.Results

Time	Cell viability of Control Group	Cell viability of Group 1	Cell viability of Group 2	Cell viability of Group 3
Hour 0	%83.5	%80.0	%84.0	%78.0
	%81.0	%82.0	%81.0	%80.0
	%82.0	%83.0	%78.0	%86.0
	%81.0	%79.0	%83.0	%80.0
	%77.5	%81.0	%79.0	%81.0

Table 1:This table shows the cell viability of islets of experiment groups before the culture (Hour 0)

Time	Control Group	Group 1	Group 2	Group 3
Number of terms	5	5	5	5
Mean	81.0	81.0	81.0	81.0
Mode	81.0	none	none	80
Median	81.0	81.0	81.0	80.0
Range	6	4	6	8
Variance	3.9	2.0	5.2	7.2
Standart deviation	1.97	1.41	2.28	2.68
95% confidence interval	78.628-83.372	79.249-82.751	78.169-83.831	77.672-84.328
Standard Error	0.88	0.63	1.00	1.20

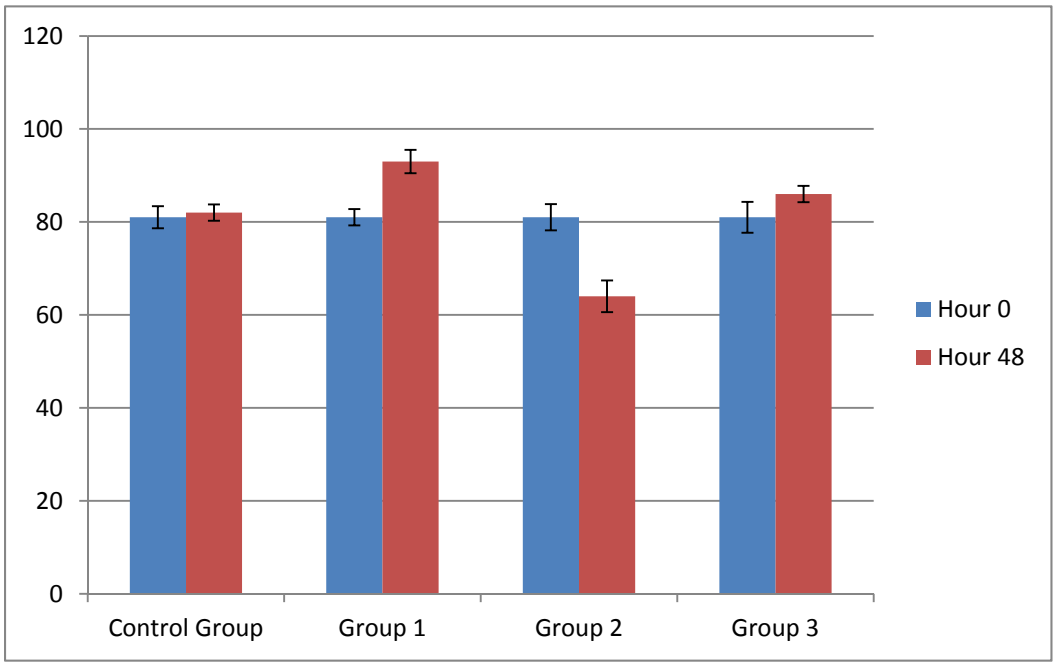
Table 2: Statistics of values of cell viability of each group before the culture

	Cell viability of Control Group (%)	Cell viability of Group 1(%)	Cell viability of Group 2(%)	Cell viability of Group (%)
Sample 1	82.0	95.0	68.0	86.0
Sample2	81.0	93.0	64.0	84.0
Sample 3	83.0	91.0	61.0	88.0
Sample 4	84.0	95.5	61.0	85.0
Sample 5	80.0	90.5	66.0	87.0

Table 3: Table 1:This table shows the cell viability of islets of experiment groups after the culture (Hour 48)

	Control Group	Group 1	Group 2	Group 3
Number of terms	5	5	5	5
mean	82.0	93.0	64.0	86.0
Mode	none	none	61	none
Median	82.0	93.0	64.0	86.0
Range	4	5	7	4
Variance	2.0	4.1	7.6	2.0
Standart deviation	1.41	2.02	2.75	1.41
95% confidence interval	80.249-83.751	90.492-95.508	60.585-67.415	84.249-87.751
Standard Error	0.63	0.90	1.23	0.63

Table 4: Statistics of values of cell viability for each group after culture.



Graph 1: Results of florescent microscopy test ,which shows the cell viabilities of islet cells for each group, before and after the experiment.

	Control Group	G_1	G_2	G_3
Sample 1	5.9	6.1	5.7	6.1
Sample 2	6.8	9.7	6.3	6.8
Sample 3	5.9	5.8	5.8	5.7
Sample 4	6.1	6.6	6.0	6.2
Sample 5	6.3	6.8	6.2	6.5

Table 5: This table demonstrates the ng of insulin per ml in each groups' 3.3 Mm glucose solution after one hour of incubation of islets in those glucose solutions before the coculture.

Time	Control Group	Group 1	Group 2	Group 3
Number of terms	5	5	5	5
Mean	6.2	7.0	6.0	6.26
Mode	5.9	-	-	-
Median	6.1	6.6	6.0	6.2
Range	0.9	3.9	0.6	1.1
Variance	0.11	1.95	0.05	0.14
Standart deviation	0.33	1.40	0.23	0.37
95% confidence interval	5.91-6.49	5.77-8.23	5.71	6.29
Standard error	0.14	0.63	0.10	0.17

Table 6: Statistics of values of insulin per ml in 3.3 Mm glucose solution for each group before culture.

	Control Group	G_1	G_2	G_3
Sample 1	20.5	24.1	20.1	19.6
Sample 2	24.7	23.2	18.2	21.7
Sample 3	18.5	27.1	24.1	22.9
Sample 4	19.9	25.1	19.2	23.4
Sample 5	22.5	24.6	22.6	19.9

Table 7: This table demonstrates the ng of insulin per ml in each groups' 16.7 Mm glucose solution after one hour of incubation of islets in those glucose solutions before the coculture.

Time	Control Group	Group 1	Group 2	Group 3
Number of terms	5	5	5	5
Mean	21.2	24.8	20.8	21.5
Mode	-	-	-	-
Median	20.5	24.6	20.1	21.7
Range	6.2	3.9	5.9	3.8
Variance	4.68	1.69	4.79	2.36
Standart deviation	2.16	1.30	2.19	1.53
95% confidence interval	18.52-23.88	23.19-26.41	18.08	16.60-23.40
Standard error	0.97	0.58	0.98	0.68

Table 8: Statistics of values of insulin per ml in 16.7 Mm glucose solution for each group before culture.

	Control Group	G_1	G_2	G_3
Sample 1	7.6	9.8	3.4	8.2
Sample 2	6.1	11.3	4.1	8.1
Sample 3	8.5	12.1	3.2	8.3
Sample 4	7.9	11.2	3.3	8.4
Sample 5	7.2	11.1	3.5	8.0

Table 9: This table demonstrates the ng of insulin per ml in each groups' 3.3 Mm glucose solution after one hour of incubation of islets in those glucose solutions after the coculture.

Time	Control Group	Group 1	Group 2	Group 3
Number of terms	5	5	5	5
Mean	7.5	11.1	3.5	8.2
Mode	-	-	-	-
Median	7.6	11.2	3.4	8.2
Range	2.4	2.3	0.9	0.4
Variance	0.64	0.55	0.10	0.02
Standart deviation	0.80	0.74	0.32	0.14
95% confidence interval	6.50-8.49	10.18-12.02	3.10-3.90	8.03-8.37
Standard Error	0.36	0.33	0.14	0.06

Table 10: Statistics of values of insulin per ml in 3.3 Mm glucose solution for each group after culture

	Control Group	G_1	G_2	G_3
Sample 1	27.3	67.6	7.8	34.4
Sample 2	26.8	59.8	7.1	39.2
Sample 3	26.1	65.9	8.3	31.3
Sample 4	27.2	66.2	8.2	34.3
Sample 5	26.8	63.7	8.4	36.7

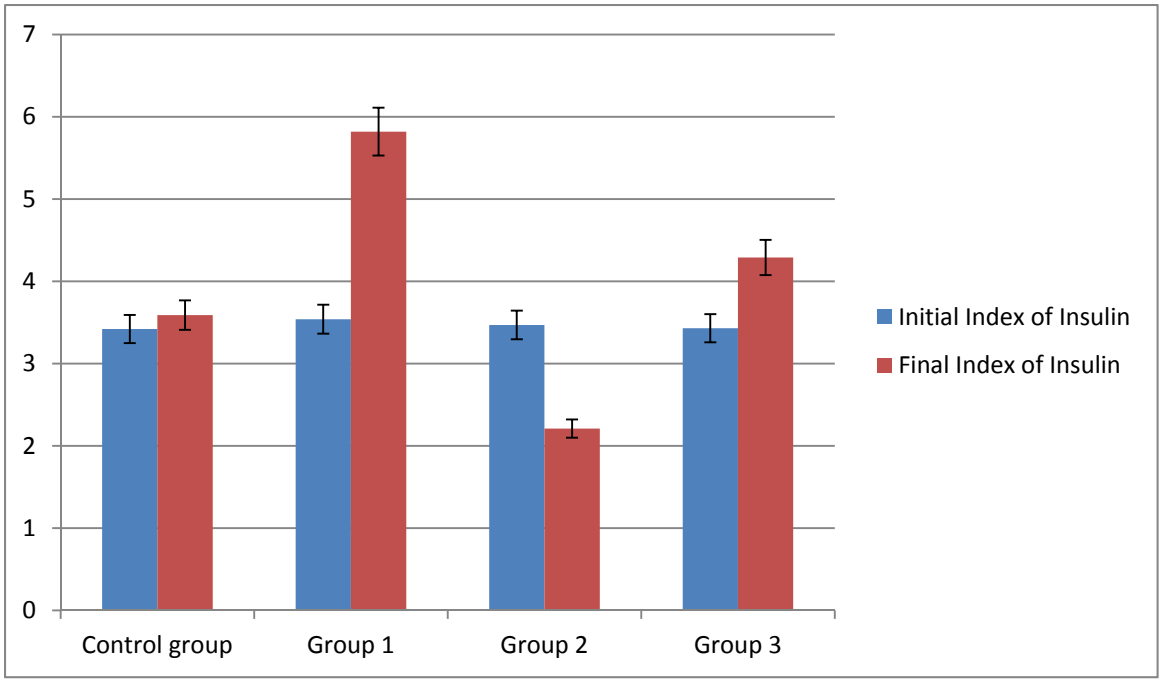
Table 11: This table demonstrates the ng of insulin per ml in each groups' 16.7 Mm glucose solution after one hour of incubation of islets in those glucose solutions after the coculture.

Time	Control Group	Group 1	Group 2	Group 3
Number of terms	5	5	5	5
Mean	26.8	64.6	8.0	35.2
Mode	26.8	-	-	-
Median	26.8	65.9	8.2	34.4
Range	1.2	7.8	1.3	7.9
Variance	0.18	7.41	0.23	6.98
Standart deviation	0.42	2.72	0.48	2.64
95% confidence interval	26.28-27.32	61.22-67.98	7.40-8.60	31.92-38.478
Standard Error	0.19	1.22	0.21	1.18

Table 12: Statistics of values of insulin per ml in 16.7 Mm glucose solution for each group after culture.

	Control Group	G_1	G_2	G_3
Initial Index of Insulin	3.42	3.54	3.47	3.43
Final Index of Insulin	3.59	5.82	2.21	4.29

Table 13: Initial and final index of insulin of experiment groups



Graph 3: Initial and final index of insulin of experiment groups

Anova

Variance source	*SS*	*df*	*MS*	*F*	*P-value*	*F critical*
Between Groups	2293,75	3	764,5833	155,8386	4,73E-12	3,238872
Within Groups	78,5	16	4,90625			
Total	2372,25	19				

Table 14: One-way ANOVA analysis of fluorescent microscopy ,which shows the cell viabilities of islet cells for each group, results

Variance source	*SS*	*df*	*MS*	*F*	*P-value*	*F critical*
Between Groups	8345.366	3	2781.789	601.2728	$1.19X10^{-16}$	3.238872
Within Groups	74.024	16	4.6265			
Total	8419,39	19				

Table 15: One-way ANOVA analysis of glucose stimulation results

6.Evaluation

Main goal of this research was to find out can HAM be used in Langerhans islet transplantations for DM Type 1 patients and if it can, with or without a barrier that seperates islets and HAM. It was hypothesized that there would be a slightly difference between cell viability and capacity of producing insulin of groups that contains insert solution because area of the bottom of a well is slightly larger than area of the top of the well. It was also hypothesized that there might be a significant difference between cell viability and capacity of insulin production of insert-included groups and group that contains HAM but no insert (G_2) because a physical contact between islets and HAM can decrease the access of islets to oxygen and damage structure of islets. No significant change on cell viability and capacity of insulin production of control group was hypothesized.

G_2 (has no insert solution between islets and HAM) has the lowest final mean cell viability (%64) and index of insulin (2.2) in the experiment groups. Control group's (only islets) final index of insulin and mean cell viability are 3.7 and %82 respectively. G_3 (islets at the top, HAM at the bottom, includes insert) has the second highest final mean cell viability (%86) and index of insulin (4.2). G_1 has the highest final mean cell viability (%93) and index of insulin (5.8). Standard error of control group's final cell viability is 0.63, standard error of final cell viability of G_1 is 0.90, G_2 's standard error of final cell viability is 1.23 and standard error of final cell viability of G_3 is 0.63. Standard error of control group's final insulin index is 0.36, standard error of insulin index of G_1 is 0.33, G_2 's standard error of final insulin index is 0.14 and standard error of final cell viability of G_3 is 0.06.

In order to determine the difference between cellof viability of experiment groups after the coculture is significant or not, I used one-way ANOVA test. As a results of ANOVA test which you can see at table 14, p value is smaller than alpha value ($p= 4.73 \times 10^{-12} < \alpha = 0,05$), the difference is significant in terms of statistics. So, ANOVA test rejects H_0 hypothesis (null hypothesis) which is there is no significant difference between means of cell viability of experiment groups after the coculture and supports the hypothesis which is there is a significant difference between means of viability of experiment groups after the coculture. Also, ANOVA test of glucose stimulation rejects H_0 hypothesis ($p= 1.19 \times 10^{-16} < \alpha = 0,05$) which suggests that there is no significant difference between glucose index of insulin. Standard error of experiment results was low which indicates that number of trials is enough. More trials can be done for performing statistical analysis more deeply to improve the investigation.

My background information, my hypothesis and results of the experiment are compatible. But I believe that method of this experiment can be improved by the changes below:

1. Roswell Park Memorial Institue Medium (RPMI 1640) created no problem during my research. However to obtain better results, Dubecco's Modified Eagle's Medium (DMEM), or HamF12 medium can be used in further researches to evaluate the effect of other growthmediums. It is essential that these mediums must be serum free.

2. I believe that smaller area of the top of the wells in respect to their bottom created the difference between cell viability and insulin indexes of G_1 (amnion membrane is at the top and islets are at the bottom of wells) and G_3 (islets are at the top and amnion membrane is at the bottom). Usage of wells that their area of the top is equal to their bottom's area may prevent that difference in further researches.
3. In order to see the difference between insulin indexes of different experiment groups, glucose molarity of solutions can be changed in glucose stimulation tests. This change would increase the insulin indexes of experiment groups therefore shows the difference between experiment groups better.

It was expected that physical contact of Langerhans islets and HAM would cause a significant decrease in cell viability and index of insulin, but its significance surprises me because such damage to islets by the physical contact was not expected. Under these circumstances, I believe it is safe to presume that HAM damages islets are deadly affected in the presence of a physical contact.

7.Conclusion

My research question is "How does human amnion membrane affect rat langerhans islet's cell viability and functionability during coculture by fluorescent microscopy and glucose stimulation test?" was answered by the experimental results of my investigation. There is a significant relationship between presence of human amnion membrane in coculture and cell viability, index of insulin of rat langerhans islets. In the absence of a physical contact, human amnion membrane improves both cell viability and index of insulin of rat langerhans but in the presence of physical contact, it decreases cell viability and index of insulin.

Reason of why I chose this subject was suggesting a new way of treatment of Diabetes Mellitus Type I with lower price and higher success rate. Results of this experiment shows that islets transplantations with HAM as a protective membrane might be the cure of DM Type I. Therefore, my research created a new hope for DM Type I patients.

However, before the trials of the islet transplantation with HAM, there are many questions that has to be answered. Perhaps, the most important one is how to use HAM as a protective membrane. My investigation answered the question "can HAM be used as a protective membrane in islet transplantations?" but it does not answer "how to use HAM as a protective membrane?". Fortunately, it gives a very important clue which is there should not be a physical contact between islets and HAM. It is possible to prevent any physical contact by using cell encapsulation. Cell microencapsulation technology involves immobilization of the cells within a polymeric semi-permeable membrane that permits the bidirectional diffusion of molecules such as the influx of oxygen, nutrients, growth factors etc. essential for cell metabolism and the outward diffusion of waste products and therapeutic proteins. At the same time, the semi-permeable nature of the membrane prevents immune cells and antibodies from destroying the encapsulated cells regarding them as foreign invaders.[8] These unique characteristics of cell encapsulation makes it very possible to be used in future islet transplantations. This research is admitted to Biotechnology Congress of European Union 2013 in Bratislava and I have presented it in Commenius University in Bratislava.

[8] http://en.wikipedia.org/wiki/Cell_encapsulation

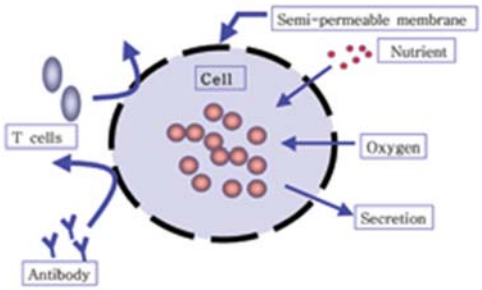


Figure 2: A diagram of cell encapsulation

Between the membrane and cells, there is a matrix. The further researches should focus on which substance can form this matrix because its characteristics are crucial on the life time of islets. Then, it is important to decide how to create the semi permable membrane from amnion membrane and later produce.

Another important question is where to transplant the Langerhans islets. This question has a very high significance because without the correct location of transplantation, no matter how effective the islet transplantation, islets would not provide insulin, cannot get nutrients and die and eventually the therapy would fail. Therefore, further researches also should focus on this question. If all these goals achieved, then islet transplantations with HAM on animal subjects can be tried.

Appendix A: Fluorescent microscopy

At the very beginning of the experiment, islets were separated to 4 groups which are G_0, G_1, G_2 and G_3.In order to measure initial cell viability of langerhans islets of these groups, florescent microscopy test was used. First, 918 µL PBS (Fetal Bovine Serum) (Ph 7,4) was added to 35 mm petri dishes in order to create a suitable medium for islets to keep their cell viability constant during the test. If their cell viability would not be constant, then there would be significant error in the test results. Second step was adding 90 µL from islet cultures to petri dish. After that 20 µL FDA solution and 20 µL PI solution was added to dish. Next step was keeping the petri dish in a dark room for 5 minutes. Then, it was observed under 40X floruscent microscope. FDA can only pass through cell membrane of alive cells and under light that has 490 nm wavelength, it emits green florescent light. Because of these characteristics, under 490 nm light, FDA mark alive cells. PI connects itself to the DNA of autolysed cells and under 536nm light, it emits red florescent light. These characteristics make PI a useful tool for marking dead cells by a florescent microscope.

During florescent microscopy, 5 islets photos were taken twice, one under 490 nm light, other one was under 536 nm light. Then, red and green light ratios of the photos were analysed separately. Islets green light (alive cell) and red light (dead cell) ratios were calculated by colour analysed code of Matlab.

Appendix B: Preparation of Human Amnion Membrane

Amnion membrane was provided by Dışkapı Research and Training Hospital Stem Cell Center Amnion Membrane Bank. Before the experiment, membrane was washed in 100 µg/ml Penicillin and 100 µg/ml streptomisin PBS (phosphate buffer saline) solution. After separating epitel and basal membrane from other tissues, membrane was divided to 10 parts and stored in a low glucose (1g/L) DMEM-LG solution with %50 glycerol. Before the experiment, in order to separate amnion epithel, membrane pieces were kept in a %0.02 EDTA (Tripsin-EDTAC) which has %0.25 tripsin for 30 minutes. After this process, membrane was ready for the experiment.

Appendix C: Glucose Stimulation Test

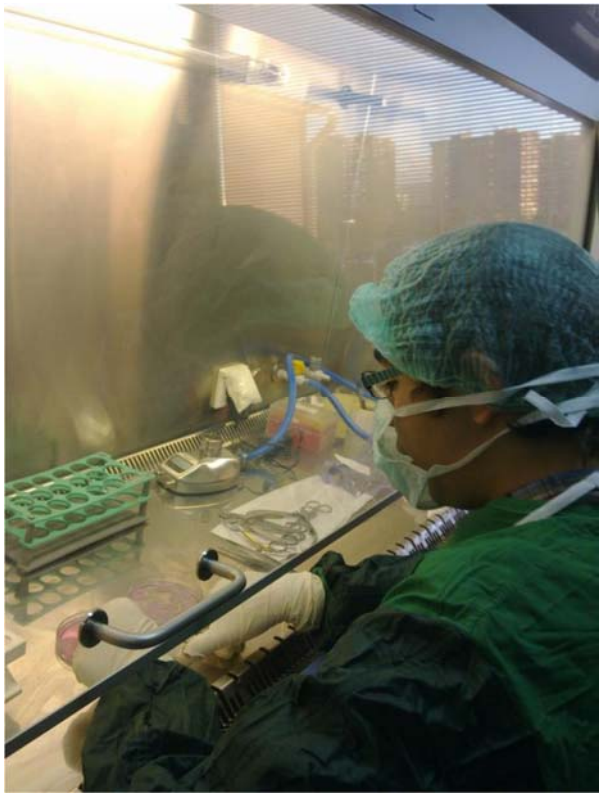
At the beginning of the glucose stimulation test, 150µL islet and 100 µL insert was added to a well for 13 times. Solution was checked for any islets that was dropped from the insert and put back to there by handpick if there was any. Then, 3.3 mM or 16.7Mm glucose solution was added to islets till volume of the new solution (islets+insert+glucose) became 5 ml. After this step, islets were incubated at 37^0 C in a 95% CO_2 incubator for an hour and solutions were put to ependorf tubes from wells after noting their experiment group and molarity of glucose solution. These samples were centrifuged at 1800 rpm for 1 minutes and 400 µL samples were taken from all tubes. These samples were stored at -20^0 C for Elisa kit. Insulin index is calculated by using the formula below:

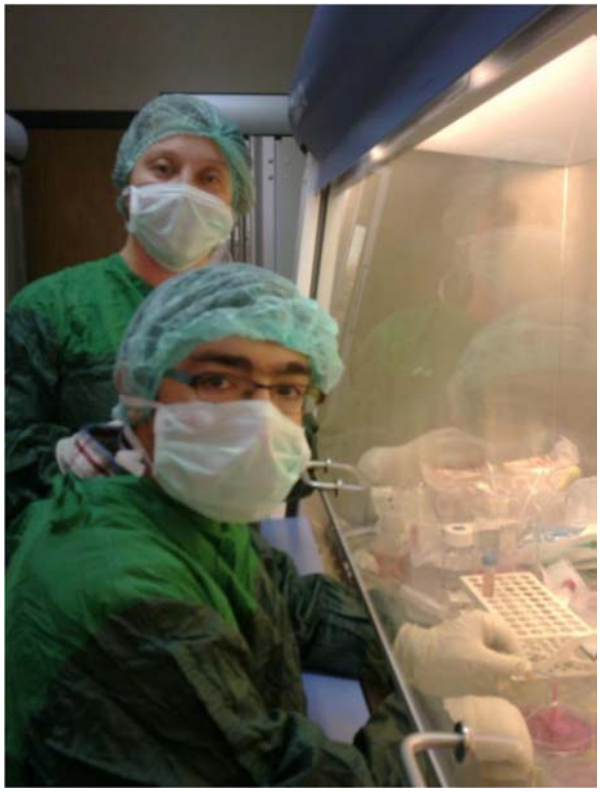
$$mean\ ng\ of\ insulin\ per\ ml\ in\ 16.7\ mM\ Glucose\ Solution / mean\ ng\ of\ insulin\ per\ ml\ in\ 3.3\ mM\ Glucose\ Solution$$

Appendix D: Preparing the Cultures

There are two different possible methods for langerhans islet transplantations with HAM based on interaction of HAM with islets. At the first method, there is no barrier between islets and HAM. In order to simulate conditions of islets in this method, islets were at the bottom of the well and HAM was placed on islets without any insert to separate them(G2). Second method (incapsulation) includes a barrier between islets and HAM to separate them and prevent possible damage to islets because of results of physical contact. Insert solution separated islets and HAM in two experiment groups. At one of these groups, islets were at the bottom and membrane was on the top(G1), islets were at the top and HAM was on the bottom at the other group(G3). In order to decide the effect of HAM on islets of HAM-included experiment groups, a control group was prepared which did not have HAM but only islets(Control Group). RPMI (+) solution was added to all experiment groups. Islets were incubated at 37^0C, %5 CO_2 and %95 moisture for 48 hours. After incubation, florescent microscopy and glucose stimulation tests were repeated.

Appendix E: Photos from experiment





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