UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

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In vitro spermatogenesis in biomimetic natural biomaterial scaffolds.

Anthony Eduardo Jiménez Trujillo

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In vitro spermatogenesis in biomimetic natural biomaterial scaffolds.

Anthony Eduardo Jiménez Trujillo

Nombre del profesor, Título académico Pedro Manuel Aponte, MV, PhD

Quito, 04 de mayo de 2020

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Nombre y apellidos: Anthony Eduardo Jiménez Trujillo

Código: 00131793

Cédula de identidad: 1723473128

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RESUMEN

La infertilidad masculina es un trastorno hormonal y genético que produce baja concentración, anomalías morfológicas y movimiento atípico de los espermatozoides en el proceso de espermatogénesis la cual se basa en la generación de espermatozoides dentro de la gónada masculina o testículo. Este trastorno se presenta comúnmente en mamíferos, asociado en su mayoría a fallas espermatogénicas, disfunciones hormonales y factores de los sistemas endocrinos dentro del proceso natural de la espermatogénesis. La infertilidad afecta gravemente a la población mundial masculina y a medida que transcurren los años se reportan más casos de infertilidad. Existen varios tratamientos para la infertilidad que buscan solucionar este problema usando técnicas de reproducción asistida como la fertilizacion *in vitro*, con el fin de superar los problemas médicos de los pacientes con infertilidad, sin embargo, estos procedimientos no tienen un efecto directo sobre el proceso de espermatogénesis. Por ello el propósito de este estudio se basa en buscar y brindar una posible solución práctica a este problema mediante un modelo de espermatogénesis con el uso de biomateriales naturales biomimeticos. Para la obtención de los biomateriales se obtuvieron muestras del tejido testicular bovino (*Bos primigenius taurus)* lo cuales fueron tratados por un método de descelularización. Se aisló una suspensión de células espermatogoniales de tejido testicular bovino. Se realizó cultivo de tejidos colocando la suspensión celular en la superficie del biomaterial y las muestras se incubaron en diferentes periodos de tiempo. Se realizaron análisis histológicos de las muestras obtenidas del cultivo. En la histología, se evidencia claramente la colonización celular dentro de los biomateriales y la formación de colonias formadas por células germinales. Los resultados obtenidos fueron favorables, por lo que este modelo de ingeniería de tejidos es útil para promover la colonización y proliferación de presuntas células espermatogoniales mediante el uso de biomateriales que mimetizan las condiciones naturales. Sin embargo, se requieren más experimentos para evaluar los tipos celulares que colonizaron el biomaterial y cuantificar la colonización.

Palabras clave: *Bos primigenius taurus,* biomaterial, espermatogénesis, andamios celulares, descelularización

ABSTRACT

Male infertility is a hormonal and genetic disorder that produces low concentration, morphological abnormalities, and atypical movement of sperm in the spermatogenesis process, which is based on the generation of sperm within the male gonad or testis. This disorder occurs commonly in mammals, mostly associated with spermatogenic failures, hormonal dysfunctions, and factors of the endocrine systems within the natural process of spermatogenesis. Infertility severely affects the world's male population, and more infertility cases are reported over the years. There are several infertility treatments that seek to solve this problem using assisted reproduction techniques such as in vitro insemination, in order to overcome the medical problems of infertility patients, however, these procedures do not have a direct effect on the process of spermatogenesis. Therefore, the purpose of this study is based on seeking and providing a possible practical solution to this problem through a spermatogenesis model with the use of natural biomimetic biomaterials. To obtain the biomaterials, samples of bovine testicular tissue (*Bos primigenius taurus*) were obtained, which were treated by a decellularization method. A suspension of bovine testicular tissue spermatogonial cells was isolated. Tissue culture was performed by placing the cell suspension on the surface of the biomaterial and the samples were incubated at different time periods. Histological analyses of the samples obtained from the culture were performed. In histology, cell colonization within biomaterials and the formation of germ cell colonies is clearly evident. The results obtained were favourable, so this tissue engineering model is useful to promote the colonization and proliferation of presumed spermatogonial cells through the use of biomaterials that mimic natural conditions. However, more experiments are required to assess the cell types that colonized the biomaterial and to quantify colonization.

Key words: *Bos primigenius taurus*, biomaterial, spermatogenesis, cell scaffolds, decellularization.

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INTRODUCTION

Tissue Engineering has developed new tendencies in science by creating functional tissues from cells at laboratory scale. By that meaning, tissue engineering is used in regenerative medicine, for the treatment and repairment of damaged organs and tissues. Damages are caused by many factors: genetic mutations, injuries, and congenital abnormalities. There are important issues involved in tissue regeneration: the right type of cells, an extracellular matrix support (known as scaffold) and the use of mechanical forces (Patnaik et al., 2014).

Cells commonly used in regenerative medicine are stem cells, but they can be used simultaneously with other derived cellular types. Spermatogonial stem cells in suspension with other testicular cell types, are commonly used in studies of spermatogenesis *in vitro*. The second factor, a scaffold, acts as physical support for cells generating a propitious environment. Finally, mechanical forces ensure the insertion of cells into the scaffolds by injection (Kargar-Abarghouei et al., 2018).

Scaffolds produced from extracellular matrix have been extensively used in regenerative medicine and tissue engineering. They are represented by non-cellular components of the tissue environment and play an important role in the regulation of cell behaviour by giving cells a structural support and biochemical interactions, thereby stimulating cell to cell signalling, migration, proliferation and differentiation (Toshio Takahashi, 2016).

Within the body, cells normally attach to an extracellular matrix, but with components generating a propitious environment for cells. The composition of the extracellular

matrix depends specifically on the tissue, but generally is composed of structural proteins like collagen and elastin, adhesive proteins such as fibronectin or laminin, and protein-polysaccharide complexes known as glycosaminoglycans (GAGs) (Pina et al., n.d.). Scaffolds can be classified into two important groups, (i) synthetic scaffolding biomaterials and (ii) natural scaffolding structures that mimic the biological extracellular matrix of the tissue (Kanungo & Gibson, 2010). The latter is commonly used, typically involving cells arranged in a three-dimensional (3D) porous scaffold. The porosity of these structures is strongly linked to their functionality, high porosity usually allowing cell colonization and intercellular interactions/connections. A wide variety of synthetic and natural based biomaterials have been utilised for scaffold processing and likewise are continuously giving rise to advanced approaches for tissue regeneration (Sackett et al., 2018).

In vitro spermatogenesis provides a perspective for preservation and treatment of infertility. Likewise, it represents a tool for the understanding of the biology and biological processes involving spermatogonial stem cells and their derived cell types. It is also useful to study factors involved in the regulation of male fertility. In order to promote those studies a good *in vitro* culture system providing the right communication between somatic and germ cells and the extracellular matrix (ECM) should be developed. In a biological system whether human or mammal, mature spermatogenesis occurs in the seminiferous tubules of the post pubertal males' testes, where spermatogonial stem cells are the germ stem cell. These cells enter into the initial mitotic phase where they divide and transform into diploid primary spermatocytes, and consequently they pass upward in the epithelium towards the lumen and start meiosis I, resulting in diploid secondary spermatocytes, thereafter they start meiosis II producing haploid spermatids and through spermiogenesis they transform into spermatozoa (Ibtisham et al., 2017).

The most utilised culture method for in vitro spermatogenesis studies is the 2D monolayer culture. However, other promising methods developed in the last years involving the use of scaffolds, are known in general as 3D cell culture. Traditional 2D monolayer cell culture systems provide a proper environment for cells to expand to a limited extent side to side, but in contrast, in 3D cell culture, cells are free to stretch out, interact with each other, interact with the scaffold and simulate the real biological conditions and environment (Ishikura & Saitou, 2018).

The main problems of 2D cell culture versus 3D cell culture are mainly due to the limited expansion of cells into a 3rd dimension network and the fact that cells cannot experience gravity, an important factor for the development of cells because it provides mechanical cues and enables cell capacity to migrate and proliferate. Thereby the lack of gravity interferes with communication among cells and for that reason 2D cell culture systems loose relevance for physiological studies generating issues during drug/treatments halting the progress from one phase to another in preclinical and clinical trials (Gharenaz et al., 2020).

A common disorder in human populations known as male infertility, is typically associated with spermatogenic failures, hypogonadism, as well as the lack of functionality of hormones and factors associated with the endocrine process. Male infertility is one of the critical problems of our society and it affects almost 20-30% of the worldwide male population (Agarwal et al., 2015).

Many causes of infertility are treatable if advanced techniques are developed to this end. Thereby, the objective of this research is the development of scaffolds made by natural

and synthetic biomaterials for optimal cell culture systems to promote *in vitro* spermatogenesis, to understand and treat male infertility potentially better. Possible applications derived from this study include the production of biomedical devices, biopharmaceuticals, germ stem cell lines at industry scale and regenerative therapies like fertility preservation of oncogenic patients (Yu et al., 2016).

METHODS

Collagen extraction procedure

Collagen was extracted by chemical hydrolysis that is often used in industry, first treated with an acid hydrolysis using a 2,5 L acid solution prepared with 75 ml of Glacial Acetic Acid in distilled water, followed by an alkaline hydrolysis with 2 L of an alkaline solution, prepared with 8g of sodium hydroxide in distilled water.

The testis samples were collected from slaughterhouse adult bulls weighing 390g of testicular tissue. Tissues were hydrolysed in the acid solution for 3 days. The remnant liquid was retrieved and washed with tap water for 10 minutes, then tissues passed to alkaline hydrolysis for 3 days and finally 40ml of liquid phase of the treatment was placed in 50ml falcon tubes and centrifuged at 4000 rpm for 10 minutes.

Decellularization of bull testes

The testis samples were cut into circular disks and placed into 50ml falcon tubes (total weight 160 – 200g) . Thereafter, the testes were washed in Phosphate Buffered Saline (PBS)

two times, each one per 15 minutes in agitation. The tissue was immersed into a solution of 1% Triton X for 4h, immersed into a solution of 1% SDS solution for 24h, and finally placed in 1% SDS for 24h. Tissues were washed with PBS and lyophilized to obtain the biomaterial.

Spermatogonial stem cells isolation from bovine model

Testes were obtained from the Metropolitan Slaughterhouse of Quito by excision from the animal. Then, the testes were transported to the laboratory in a box, covered with ice (~4°C). This procedure provides a preservation of most of the germ cells for 3-4 hours. The testes were rinsed in tap water to remove contaminating materials.

Enzymatic spermatogonial isolation

Testes were rinsed with sterile saline 0.9%. and cut. into about 20g of testis parenchyma for cell isolation procedures. A sample was taken for histological examination and was located in a tube containing Bouin´s fluid. The testis material was transferred into a sterile Petri dish and cut in two similar sized pieces. Each piece of testis was placed in separate Petri dishes and 12,5ml of MEM+DNAse were added on top. Tunica albuginea of the testis was removed with the use of a pincet and a surgical blade, and then the samples of testis were minced with 2 surgical blades, removing the connective tissue and separating the seminiferous tubules as much as possible. The minced samples were transferred into two 50ml conical tubes, approximately 10g on each tube, then 12,5ml of an enzyme mix: (collagenase I 2mg/mL, hyaluronidase 2mg/mL and trypsin 2mg/mL) was added to each tube, obtaining 25ml in each tube, containing the material and the enzyme mix.

Tubes were closed and then transferred into a shaking water bath, 140 cycles/minute for 60 min at 32°C. After the water bath, the tubes were centrifuged at 500rpm for 1 minute. The supernatant was removed and then the pellet was rinsed with MEM+DNAse. The process was repeated 3 times. The final supernatant was removed with an electronic pipette until 12,5ml and then a 12,5ml volume of a mixture of collagenase I 2 mg/mL and hyaluronidase 2 mg/mL was added to each tube. The tubes were transferred to a shaking water bath, at the same conditions, but in this step, time was changed for 45min. Then tubes were centrifuged at 400rpm for 2 min. The supernatant was collected from both tubes with a membrane filter into a 50ml tube and centrifuged at 900rpm for 5 min. The supernatant was aspirated, and the pellet was re-suspended in 4ml MEM+DNAse+BSA and then was maintained on refrigeration or ice. The final cell suspension was diluted in 10% FBS in MEM 1X in a 50ml conical tube.

Cell Culture

The viability and the concentration of spermatogonial cells was estimated by the formula (total number of viable spermatogonial cells = volume x concentration in cells/ µL). In this experiment a low and a high concentration of cells were used. The low concentration corresponds to 15 cells/µL and the high concentration corresponds to 25 cells/µL. A 500ml stock basic culture media was prepared adding MEM 10X + 7,5% of sodium bicarbonate + 200mM L-glutamine + 100X Non-essential amino acids + 10mg/ml gentamicin + 5ml penicillin-streptomycin and 1M Hepes Buffer. The culture media was prepared in two 50ml conical tubes adding 45ml of the stock basic culture media + 5ml of FBS (Fetal Bovine Serum 10%). Two biomaterials thickness types were used, a low level that correspond to a thin biomaterial and a high level that correspond to a thick biomaterial. Cells were seeded in 4 6-well cell culture plates by gravitational drop in the surface of the biomaterials and then

incubated in a humidified atmosphere with 5% CO_2. The media was refreshed every three or four days. A plate was collected at 3 days, 7 days, 11 days and 14 days and the biomaterials were fixed in a Bouin's fluid solution for further histological procedures.

Cell fixation

A 70% saturated picric acid, 25% formaldehyde (37%), 5% Acetic acid (glacial) / (Bouin's fluid) solution was prepared. Samples of cell culture were rinsed with 1X PBS (Phosphate Buffered Saline) and then the Bouin's fluid solution was added to each well covering the biomaterial. Then Bouin's fluid was withdrawn after 1 day and 70% ethanol was added for storage at 4°C.

Histology

Samples were treated with ethanol at 90, 95 and 100%, Xylene and then embedded in paraffin at 60°C, then were cut into 5μm slices using a Leica microtome and placed on microscope slides. The samples were next de-paraffinized rehydrating with 90, 95 and 100% aqueous ethanol. Then samples were washed with distilled water and stained with haematoxylin and then put in a tray with a continuous water flow (10 min), and finally cleared with xylene. Slides were then set with mounting fluid and let dry overnight to analyse by microscope.

RESULTS

Influence of the type of biomaterial on the growth capacity of spermatogonial cells in a three-dimensional culture

As the results presented in Table 1, there is evidence of a significant effect on the response by the two factors. The type of biomaterial has a statistically significant effect on the response with a p-value of 0,002 that is smaller than the significance level of $\alpha = 0{,}05$. The graphic of principal effects (Fig. 3A) reveals an effect from the low-level biomaterial that corresponds to a thin layer biomaterial to the high-level biomaterial that corresponds to a thick layer biomaterial. The highest effect in cell colonization and proliferation is obtained at a high-level biomaterial obtaining a mean of 20 cells/tissue fraction.

From the results obtained in Table1, regarded to the general model, it is revealed some important data. The average of the p value is much less than $\alpha = 0{,}05$ that is the significance level used in the design of the experiment's analysis. The results related to the general model indicate that the established conditions of the model have a high significance.

Influence of the initial concentration of cells on the growth capacity of spermatogonial cells in a three-dimensional culture

From the results presented in Table 1, there is evidence of a significant positive effect on the response by the initial concentration of living cells (CLC) that was seeded. This is based in the fact that if the levels of the CLC factor are changed from low (-1) to high (+1), there is a significant change in the percentage of cells into the biomaterial. From the graphic of principal effect on the response variable (Fig. 3A) the evidence is consistent since using a high level of CLC results in a percentage higher than 20% , the highest percentage of cells obtained from

cell culture using 25 cells/uL in the initial seeding. The factor with positive effect and values lead to an increase in the percentage of cells colonizing and growing into the biomaterial.

The coefficient of determination known as R^2 (Table 2) which corresponds to the proportional variation in the response explained by the independent variables in the linear regression model is 0.907, and the adjusted coefficient of determination (R^2_{adj}) is 0.872. The values obtained from the R^2 test (0.907) demonstrates that the change in the response can be explained with the variables of the model with more than 90% of confidence. Therefore, the model used for the experiment is the most appropriate one to determine a significant effect in the response variable using the two factors of thickness of biomaterial and initial concentration of living cells. There is a slightly difference between the values of R^2 and $R^2_{adj.}$ That difference shows that some insignificant experimental conditions were included in the model so that the values differ in a small percentage.

In order to determine which is the interaction between the factors analysed in this experiment, a graphic of interaction between factors was made. It also helps to understand if there is interaction or not between the factors, and what is the direction of the interaction. Interaction is defined as inability of one factor to cause the same effect on the response with different levels of another factor. Thus, there is evidence of interaction only when the change in the values of either, the thickness of biomaterial or initial concentration of living cells alters the effect on the other one.

In figure 3B it is shown an apparently no interaction between factors, but regarding to the results from the Table 1 with values of p on the interaction between the two factors smaller than 0.05 there is sufficient evidence of a significant effect of the interaction between factors, that is not exactly visible in the graphic because it relates to the perspective of the investigator.

But it is evidence of an interaction giving a similar effect to the response variable but increasing the values of it.

The change on the percentage of colonization by spermatogonial cells with a change in the levels of the two factors simultaneously are presented as lines in the fig. 3B and the values are represented in the Y axis , if lines are not parallel, the graphic presents an interaction between the factors. The highest the degree of deviation, the deeper is the effect of the two factors interacting with each other. The ANOVA analysis give a statistically reliable result that is already shown in the graphic of interaction in the fig. 3B, that result show an observed significant interaction at both levels and the combinations of the factors of the study.

Figure 3D shows all possible combinations between the two factors that are significant to the study. The X and Y axis show the two studied factors and the Z axis shows the variable of response. The use of this type of plots in this experiment are very useful for visualizing the expected response when random values of the two factors are admitted in the study range from the low level characterized by (-1) to the high level characterized by (+1).

Histology evaluation of cellular colonization into biomaterials

Histological evaluation revealed cell colonization and colony formation in the three-dimensional cell culture with the use of decellularized testis biomaterial as a scaffold. The principal evaluation was performed for the samples obtained at 14 days of incubation (Figure 4A). The samples were also evaluated at 3, 7 and 11 days. In day 3 there was no evidence of colonization or growth into the scaffold. Therefore, at 3 days of incubation is not a propitious time for cell colonization (Figure 4B). Samples obtained at day 7 revealed colonization of a small percentage of spermatogonial cells. The evaluation at 11 and 14 days of incubation

revealed a considerable percentage of spermatogonial cells colonization and agglomeration forming colonies (Figure 4C).

DISCUSSION

This study showed a statistical significance of the initial concentration of live cells and the use of decellularized biomaterial with different thicknesses. The combination between the two factors was assessed to evaluate the statistical significance of the interaction on the variable of response. We found an extended significant effect of the factors and the interaction, with P-values much less than 0.05 and high values of F-ratio. Currently, in biomedical studies the use of a significance level of 0.05 is effective because an $\alpha = 0.05$ indicates that the investigator is willing to accept a 5% probability of being wrong by rejecting the null hypothesis, and that is effective to reduce the error produced by the systematic choice of the investigator and not from the factors or the conditions of the experiment (Montgomery, n.d.). It is also recommended to use a lower value of α to reduce this type of risk, but using a lower value means that it will be much difficult to detect a difference when it truly exists.

One of the main assumptions for the experimental model is that the values of error are normally distributed, and they have constant variance. To evaluate the established assumption, normal probability plot of residuals was done. The residuals correspond to the difference between the observed and the predicted values of the response. If the distribution of the residuals forms a straight line, it is considered that the data are normally distributed and the model was adequate to explain the experiment (Montgomery, n.d.).

The graphics of interaction on fig. 3B demonstrates nonparallel lines. That means that the factors can interact with each other and that interaction can make a different effect on the variable of response. The graphic of interaction between initial concentration of living cells (CLC) and thickness of biomaterial (Biomaterial) show that the percentage of colonization was the lowest when the two factors were at low level with a value less than 20%. Nevertheless, when both factors were at high level that means the use of a thick biomaterial and an initial concentration of cells of 25 cells/μL that produced a large effect on the variable of response obtaining the highest value on the response of more than 30% of the cellular colonization into the biomaterials. This values are consistent with similar studies that recommend a thickness of biomaterials around 150-200 mm (Bhaskar & Gupta, 2020). Regarding the initial concentration of cells seeded in cell culture, there are several reports that suggest an initial concentration of 10^6 cells / ml, however, this study shows that with lower concentrations, there is a favourable evidence of colonization and proliferation (Reda et al., 2014). It can be inferred that the initial concentration of cells and the thickness of biomaterial seem to be dependent to obtain the highest possible percentage of colonization of spermatogonial cells.

The response surface plot shown in fig. 3D helps to establish the values of the response that were optimal or desired in the experimental conditions. The plot is represented in a 3D surface of response graphic, and that plot explains the mutual impact of the factors analysed in the experiment on the colonization of spermatogonial cells and the colony forming during the conducting of the experiment.

In this study the use of Haematoxylin was useful for analyse by observation the spermatogonial cells colonization by counting cells in the slides obtained from samples of cell culture with the use of biomaterials as scaffolds. There is evidence of colonization due to fact that in the graphic

of histological evaluation, cellular nucleuses can be clearly identified in some parts of the layers and the tissue of the biomaterials. It is also clear the evidence of accumulation of the spermatogonial cells in the tissue, that phenomena are explained since cells make colonies (Mintz & Palumbo, n.d.).

Cells can form colonies since they need cellular interactions to grow and differentiate, also it is due to the environmental conditions, the interaction with the substrate and their ability to proliferate (Reda et al., 2014). The ability to proliferate depends on the proliferation index of every cell type (Brinster & Kubota, 2017). The interaction with the substrate depends on the conditions such as type of media or amount of serum available (Reda et al., 2014). It depends also on the time that they need for conditioning to the media, for example, in this experiment, the effect of time is evident with the results obtained from the experiment, which can be observed at 3, 7, 11 and 14 days. At 3 days of incubation there is no evidence of colonization, in contrast, at 14 days of incubation there is a significant evidence of colonization. The environmental conditions optimal for cell growth and proliferation is due to the density of the biomaterial, that can permit the colony formation.

CONCLUSION

The present study demonstrates that the use of a thick biomaterial in conjunction with an initial concentration of living cells of 25 cells/µL result in colonization and colony forming. It also evidences that the use of three-dimensional cultures does influence the functionality of spermatogonial cells. The decellularized biomaterials can provide cells with a support for their natural functions. However, further analysis is required to determine cells differentiation and the spermatogenesis stages of mitosis and meiosis to produce spermatozoids. Thereby, the use

of immunohistochemical, fluorescent and morphological evaluations are required to determine different phases of differentiation and detect endocrine/ paracrine pathways involved in the process of spermatogenesis in vitro. In conclusion, the natural function of spermatogonial cells in such a complex three-dimensional culture with the use of biomaterials depends on various factors, but the principal one is that this kind of cell cultures require a diversity of testicular cells and a complex combination of in vitro conditions.

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APPENDIX

APPENDIX A. Table 1. ANOVA Analysis of Variance for the factors of Biomaterial and CLC and the interaction between factors.

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	3	2152,2	717,42	26,01	0,000
Linear	2	1918,2	959,08	34,77	0,000
Biomaterial	1	574,1	574,08	20,81	0,002
CLC	1	1344,1	1344,08	48,73	0,000
2-Way Interactions	1	234,1	234,08	8,49	0,019
Biomaterial*CLC	1	234,1	234,08	8,49	0,019
Error	8	220,7	27,58		
Total	11	2372,9			

APPENDIX B. Table 2. R^2 Analysis for model summary

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
5,25198	90,70%	87,21%	79,08%

APPENDIX C. Probability Plot

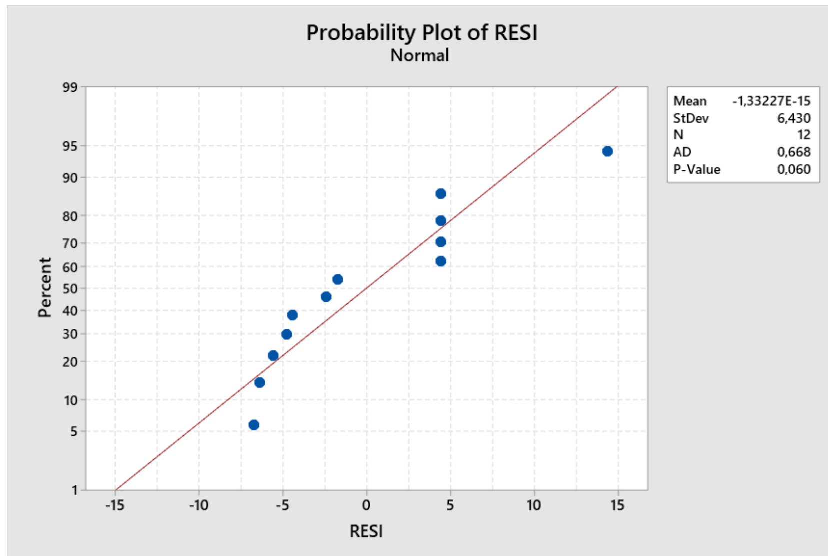

Figure 1. Normal probability plot of residuals

APPENDIX D. Equal Variances Plot

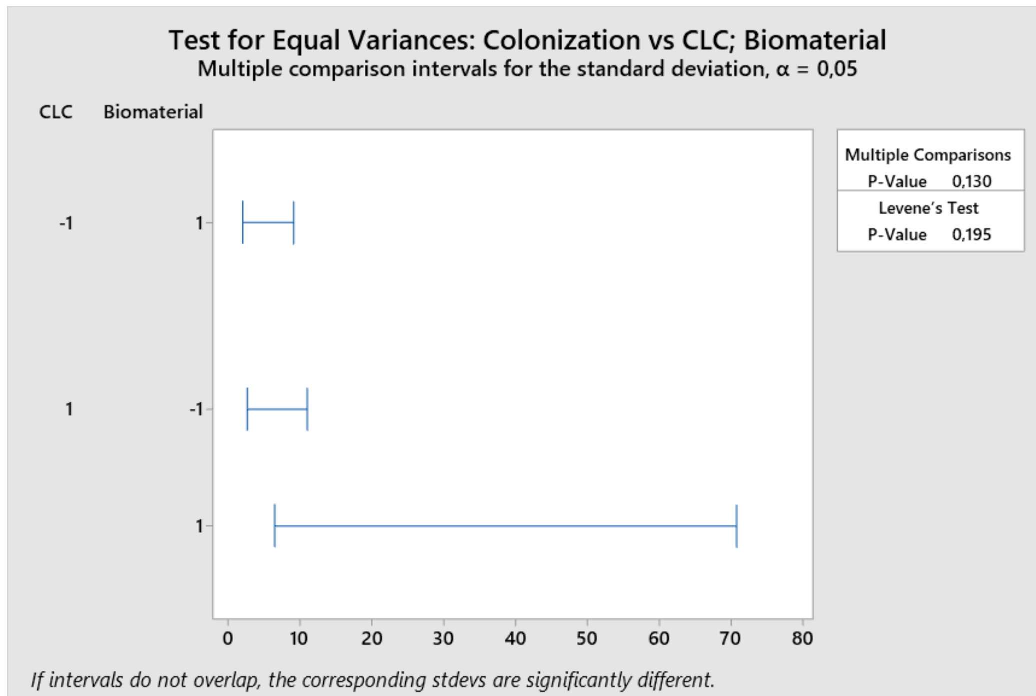

Figure 2. Test of equal variances between the factors analysed

APPENDIX E. Main effects and interaction plots

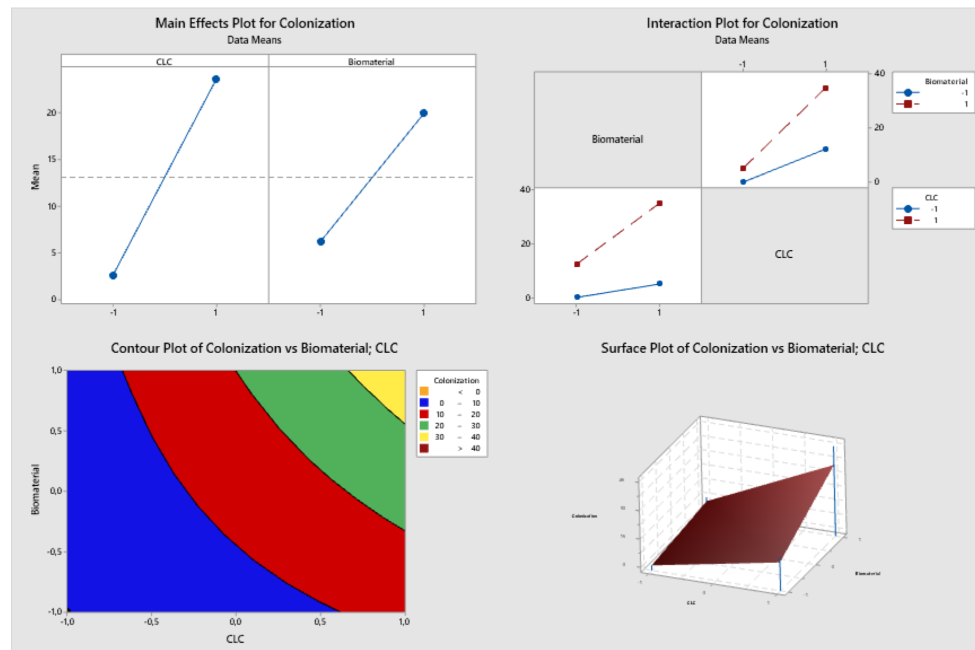

Figure 3. 3A. Graphic of principal effects, 3B. Graphic of interaction between factor over the % of cellular colonization, 3C. Contour plot and 3D. 3D Surface plot.

APPENDIX F. Histochemical Analysis

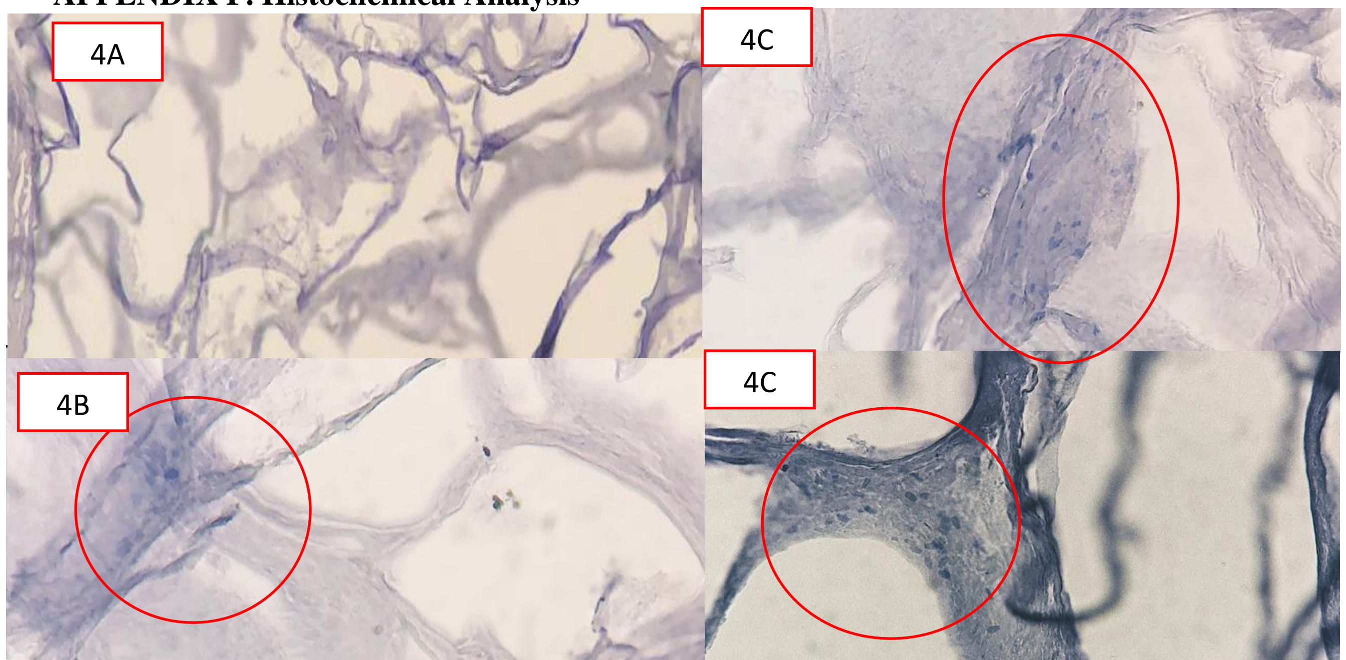

Figure 4. 4A. Cell culture sample at 3 days of incubation. 4B. Cell culture sample at 7 days of incubation, 4C. Cell culture sample at 11 and 14 days of incubation.