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# Effects of fibrin matrix and Ishikawa cells on in vitro 3D uterine tissue cultures on a rat model: A controlled study

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**Ethics Committee Approval**
The ethical approval of this study was authorized by the Acibadem Mehmet Ali Aydinlar University Local Ethics Committee for Animal Experiments (ACU-HADYEK) with the decision number 2018/16 on the 9th of April 2018.
All procedures in this study were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

**Conflict of Interest**
No conflict of interest was declared by the authors.

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## Abstract

**Background/Aim:** In recent years, developing an embryo in in vitro conditions has been one of the most challenging and popular objectives in reproductive biology. In vitro models make observing the relationship between the two possible. Various cell culture and matrix models have been created to overcome embryonic disorganization during culturing. The primary aim of this study was to evaluate and compare the effects of fibrin, Ishikawa cell line, and a combination of both on the 3D multilayer uterine tissue cultures on a rat model, including a control group.

**Methods:** This study was designed as a prospective controlled cohort study. The standard uterine culture model [CNT] (N: 3) constituted the control group. In addition, fibrin matrix-supported [FIB] (N: 3), Ishikawa cells-supported [ISH] (N: 3), and a combination of both [FIB+ISH] (N: 3) culture models were designated as the exposures. All models were cultured for 14 days. Afterwards, the optimal model was determined regarding glucose consumption, lactate production, endometrial thickness and gland count (primary outcomes) with semi-quantitative and statistical methods. Finally, the optimal model was implanted with blastocytes, and the survival duration was observed (secondary outcome).

**Results:** There were significant differences between the groups in terms of glucose, lactate, endometrial thickness (millimeter), and the number of endometrial glands ($P$<0.05). FIB had the least glucose consumption, and the least lactate production was in CNT. The thickest endometrium and most endometrial glands were detected in FIB when all groups were compared, allowing for 14 days of embryo survival.

**Conclusion:** In embryogenesis research, the fibrin-matrix-supported culture model could be a satisfactory 3D uterine tissue culture model.

**Keywords:** Cell line, Tumor, Co-culture, Extracellular matrix, Fibrin, In vitro, Tissue culture techniques

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## Introduction

Complete embryogenesis has been an important goal in reproductive medicine for many years. The primary determinant in embryogenesis is the quality of the relationship between the embryo and endometrium. Therefore, both a healthy embryo and endometrium are crucial for healthy embryogenesis. In vitro models make observing the relationship between the two possible. The previous studies carried out on rat models indicate that the embryos could survive in the uterine tissue and matrix and cell lines without degeneration until 14 days, and even late organogenesis was achieved with the addition of a roller culture incubator platform with a gas and pressure modulator [1, 2]. However, complete embryogenesis is yet to be achieved. Severe conditions such as infertility, implantation failures, and recurrent pregnancy losses could be prevented when long-term in vitro culturing techniques advance that far.

Some in vivo culture models that enabled the development of the post-blastocyte phase have increased the implantation, therefore, success in embryological studies. In addition, the culture models made differentiation of the three germ layers, epiblast development, amnion, and yolk sac formation possible. However, the disorganization of the embryos in these models began on days 12-13 [3, 4].

Various kinds of cell culture and matrix models have been created to overcome embryonic disorganization. These models have been studied on mice, rats, pigs, and monkeys due to the ethical difficulties regarding humans. It could be observed that the tested feeder cell culture and matrix models have been focused on three types: Embryo + cell co-culture models [5-7], 3D multilayer/organoid models [8-11], and whole organ culture models [12, 13].

Implanting embryos in different cell cultures is hypothesized to provide better receptivity and longer viability in embryo + cell co-culture models. For example, Niu et al. [5] detected gastrulation, but their co-culture model collapsed before it reached day 20. Nowotschin and Hadjantonakis [6] also studied in vitro pre- and post-implantation with different cultures until the blastocyst stage with a mouse model. Additionally, there are co-culture models derived from the endometrial stromal cells from the regularly menstruating patients obtained via endometrial biopsy. However, in vitro embryos in these models had a maximum lifespan of six to eight days [7].

Secondly, the embryo co-culture model was developed into a complex 3D multilayer/organoid structure containing multiple cell types and biomaterials to investigate whether it could prolong embryo survival even more. Bentin-Ley et al. [8] detected trophoblast invasion due to embryo implantation in their multilayer human endometrium culture model. Wang et al. [9] attempted to improve this model by combining endometrial and trophoblastic cell lines with a fibrin-agarose matrix. Different from other models, Rozner et al. [10] created a 3D model consisting of peripheral blood immune cells and decidual macrophages in combination with Matrigel® with a rhesus monkey model. Ten days of embryonic development was achieved in both studies. Afterwards, Chang et al. [11] carried out blastocyte implantations in a microenvironment consisting of Buffalo Rat Liver feeder cells with Matrigel®. 21-45 days of embryonic development was achieved. It is essential to consider the anatomical, physiological, and developmental milestones between mammals such as rats, monkeys, and humans [14-16]. These differences must be observed in molecular and histologic processes in in vitro studies to allow for better translation to clinical medicine.

Lastly, there have been two studies on whole organ culture models with swine and pig uterus. Geisler et al. [12] aimed to sustain the swine uterus ex vivo long-term with open perfusion for 8-13 hours. Han et al. [13] achieved spontaneous in vitro fertilization using pig uterus and enabled the embryo's development until the blastocyst stage. Even though the information regarding the early stages of embryogenesis is improving, the knowledge about the post-implantation phase needs further research.

The primary aim of this study was to evaluate and compare the effects of fibrin, Ishikawa cell line, and a combination of both on the 3D multilayer uterine tissue culture model in a rat model with a control group. The secondary aim of the study was to determine the duration of blastocyte survival that the optimal model enables.

## Materials and methods

The ethical approval of this study was authorized by the Acibadem Mehmet Ali Aydinlar University Local Ethics Committee for Animal Experiments (ACU-HADYEK) with the decision number 2018/16 on the 9th of April 2018. The study was conducted in Acibadem Mehmet Ali Aydinlar University Laboratory Animal Application and Research Center (ACU-DEHAM).

**Selection and description of rats**

Twelve healthy 6-8 weeks old anestrous adult female Wistar Albino rats with an average weight of 350-400 g were purchased from ACU-DEHAM, which follows the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The inclusion criterion was being healthy, and the exclusion criterion was being pregnant. The rats were kept at a room temperature of 24 °C, with a 12:12 day/night cycle with adequate water and food supply. Uteri of the Wistar Albino rats have two horns per uterus (bicornuate uterus) due to their typical anatomic structure, which provided 24 uterine horns in total.

**Technical information**

Two hypotheses and their research questions were developed:

**Hypothesis 1:** Fibrin matrix, Ishikawa cell line, and a combination of these are associated with a better uterine metabolic activity and morphology.

1. Is there a significant difference between the models in terms of metabolic activity (glucose and lactate)?
2. Is there a significant difference between the models in terms of uterus morphology (endometrial thickness, number of endometrial glands; uterine histology; estrogen and progesterone affinity of the endometrial glands)?

**Hypothesis 2:** If an optimal model is determined in the testing of Hypothesis 1, how long will this model enable a blastocyte to survive?

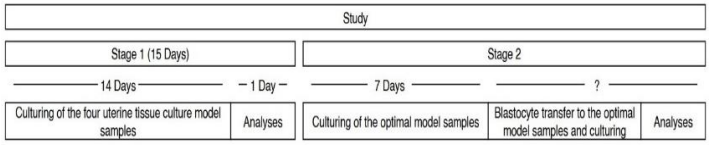
The primary outcomes were glucose and lactate measurements, endometrial thickness, number of endometrial glands, estrogen, and progesterone affinity of the endometrial glands. The secondary outcomes were embryo implantation and embryonic marker positivity.

**Study Design**

This study was designed as a controlled study and in two stages. Four groups, each consisting of three rats, were randomly formed. In Stage 1 (Primary Outcome), the standard uterine culture model [CNT] was the control. In addition, fibrin matrix-supported [FIB], Ishikawa cells-supported [ISH], and a combination of both [FIB+ISH] culture models were designated as the exposures. Each model was tested with three cultures. A total of 12 uterine tissues were assigned to all cultures randomly by the researcher. All models were cultured for 14 days. Lastly, metabolic and microscopic (primary outcome) analyses were carried out at the end of the 14th day (15th day).

In Stage 2 (Secondary Outcome), three uterine tissues were assigned randomly by the researcher to the model determined optimal in Stage 1. The three cultures of the optimal model were cultured for seven days. Afterwards, three blastocytes were implanted in each one of the cultures. The duration of survival of the implanted blastocytes was observed. Microscopic and molecular genetics (secondary outcome) analyses were carried out at the end. The researchers were not blinded to any data. The study flow is presented in Figure 1.

Figure 1: The flow of the study.




**Chemicals, Reagents, Equipment, and Zygotes**

Ishikawa cell lines (Sigma-Aldrich, St. Louis, Missouri, United States of America, SKU number: 99040201, Ishikawa Cell Line human) were tested against fungal contamination with tryptic soy broth, mycoplasmal contamination with polymerase chain reaction (PCR) method, and bacterial contamination with MALDI Biotyper® (Bruker Corporation, Billerica, MA, United States of America). Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mixture (DMEM/F12) medium (Sigma-Aldrich, St. Louis, Missouri, USA) and DMEM - low glucose (DMEM-LG) (Sigma-Aldrich, St. Louis, Missouri, USA), and Dulbecco's phosphate-buffered saline (DPBS) without Ca and Mg were used to culture the tissues.

ADVIA 1800 Clinical Chemistry System (Siemens Health Care Diagnostics Inc, Tarrytown, New York, USA) was used for the metabolic activity evaluations. Anti-estrogen receptor-α antibody produced in rabbit (Sigma-Aldrich, St. Louis, Missouri, USA, product number: SAB4500810), anti-progesterone Receptor antibody produced in rabbit (Sigma-Aldrich, St. Louis, Missouri, USA, product number: SAB4502184), anti-human chorionic gonadotropin (hCG) beta antibody [5H4-E2] (Abcam, Cambridge, United Kingdom, catalogue number: ab185628, mouse monoclonal), anti-cytokeratin 1 antibody [EPR17744] (Abcam, Cambridge, United Kingdom, catalogue number: ab185628), anti-vimentin antibody (Abcam, Cambridge, United Kingdom, catalogue number: ab137321, rabbit polyclonal), anti-Ki67 antibody (Abcam, Cambridge, United Kingdom, catalogue number: ab833, rabbit polyclonal) were used for immunohistochemical staining.

Nine Sprague Dawley® Rat (Strain name: RjHan:SD) zygotes were purchased from the same laboratory (Janvier Labs, Le Genest-Saint-Isle, France) to achieve standardization throughout the study. In addition, the zygotes were tested against mycoplasmal contamination with the PCR method.

Anti-Nanog antibody [23D2-3C6] (Abcam, Cambridge, United Kingdom, catalogue number: ab173368, mouse monoclonal), anti-SOX2 antibody (Abcam, Cambridge, United Kingdom, catalogue number: ab239218, goat polyclonal), Alexa Fluor® 488 (Abcam, Cambridge, United Kingdom, catalogue number: ab150077, goat anti-rabbit IgG H&L), and

Alexa Fluor® 647 (Abcam, Cambridge, United Kingdom, catalogue number: ab150079, goat anti-rabbit IgG H&L) were used for molecular genetics evaluations.

**Stage 1 (Primary Outcome)**

**Obtainment of Uterine Tissue**

All steps were carried out using sterilized surgical equipment. The rats were fixed in a supine position following general anesthesia, and the abdominal wall was shaved and disinfected with povidone-iodine. The abdominal walls were incised with a transverse incision, and total hysterectomies were performed. The removed tubular uteri were placed in sterile Petri dishes. The uteri were dissected from the corpora to the uterine horns with a single transverse incision, leaving the endometrium facing up and the parametrium down. The following dissected uteri were further dissected in approximately 10 mm in length, 5 mm in width, and 3 mm in height. The removed full-layer uterine tissues were put in a sterile isotonic solution containing 0.9% NaCl and placed in the cultures.

**Standard Uterine Culture Model Preparation**

The further-dissected uterine tissue measuring 10 mm in length, 5 mm in width, and 3 mm in height (3D uterine tissue) was placed in a 3 cm deep flask containing DMEM/F12 medium consisting of 1% antibiotics, and 10% fetal bovine serum (FBS) was placed in an incubator at 37 °C, 5% $O_2$, 5% $CO_2$, and 90% $N_2$ for the culture process. The DMEM/F12 medium was changed entirely every three days until the 14th day to prevent contamination.

**Fibrin Matrix-supported Culture Model Preparation**

**Fibrin matrix formation:** 45 ml plasma component and 4,5 ml CaCl are combined in a 50 ml Falcon tube. This mixture is then transferred to a 10 $cm^2$ petri dish. Afterwards, 0,5 ml transamine is added, and the mixture is quickly stirred. Finally, the Petri dish containing the mixture was placed in an incubator at 37 °C, 5% $O_2$, 5% $CO_2$, and 90% $N_2$ for 20 minutes for the fibrin matrix to form.

**Combination of the fibrin matrix with the uterine tissue:** The 3D uterine tissue measuring 10 mm in length, 5 mm in width, and 3 mm in height is gently placed in the Petri dish containing the formed fibrin matrix. 15 ml plasma component, 1.5 ml CaCl, and 0.16 ml transamine is added to the Petri dish until the upper side of the endometrium tissue is covered with the fibrin matrix solution. Next, a 10 ml DMEM-LG medium containing 1% penicillin/streptomycin and 10% FBS is added. Lastly, the final fibrin matrix-endometrium mixture is placed in an incubator at 37 °C, 5% $O_2$, 5% $CO_2$, and 90% $N_2$ for the

culture process. The DMEM-LG medium was changed entirely every three days until the 14th day to prevent contamination.

**Ishikawa Cells-supported Culture Model Preparation**

**Ishikawa cell formation:** The Ishikawa cells frozen in a nitrogen tank are thawed at 37 ºC. Then, they are transferred to a falcon tube in a laminar airflow cabin. Afterwards, DPBS is applied, and the mixture is centrifuged at 400 G for 10 minutes. Following this, the formed supernatant at the top of the tube is removed. The medium application and centrifuge steps are repeated three times. Next, the cells are combined with DMEM-LG in T300 flasks. The cells are cultured in an incubator at 37 ºC, 5% $CO_2$, and 5% $O_2$ for 72 hours. When the confluency of the flasks reaches 70%, the cells' mitotic ability is blocked with ten micrograms/ml mitomycin C in an incubator for 3 hours. Subsequently, the cells were removed with trypsin-EDTA and placed in a flask so that every $cm^2$ of the flask is coated with 3 to 5 million cells, forming the Ishikawa cell layer.

**Combination of the Ishikawa cells with the uterine tissue:** The 3D uterine tissue measuring 10 mm in length, 5 mm in width, and 3 mm in height is gently placed in the Petri dish containing the Ishikawa cells. 10 ml DMEM-LG medium containing 1% penicillin/streptomycin and 10% FBS is added. Lastly, the final Ishikawa cells-endometrium mixture is placed in an incubator at 37 ºC, 5% $O_2$, 5% $CO_2$, and 90% $N_2$ for the culture process. The DMEM-LG medium was changed entirely every three days until the 14th day to prevent contamination.

**Fibrin matrix and Ishikawa Cells-supported Culture Model Preparation**

The 3D uterine tissue measuring 10 mm in length, 5 mm in width, and 3 mm in height is gently placed in a Petri dish containing the fibrin matrix and Ishikawa cells which the preparations were described above. Next, a 10 ml DMEM-LG medium containing 1% penicillin/streptomycin and 10% FBS is added. Lastly, the fibrin matrix-Ishikawa cells-endometrium mixture is placed in an incubator at 37 ºC, 5% $O_2$, 5% $CO_2$, and 90% $N_2$ for the culture process. The DMEM-LG medium was changed entirely every three days until the 14th day to prevent contamination.

**Stage 1 Analyses**

**Metabolic Activity (Glucose and Lactate Measurements)**

On the 7th and 14th days, the mediums of the uterine tissue culture models were sampled with pipettes, and the glucose levels were measured with quantitative methods such as Trinder's glucose oxidase method using a Glucose hexokinase-3 kit and the lactate levels were measured with quantitative methods using a lactate kit.

**Uterus Morphology (Histopathologic Analyses)**

Cultured uterine tissues were fixated in 10% neutral formalin. Following this, the tissues were embedded in paraffin blocks following dehydration and paraffin inclusion steps. Finally, 4-micron sections were taken and stained with hematoxylin and eosin [17].

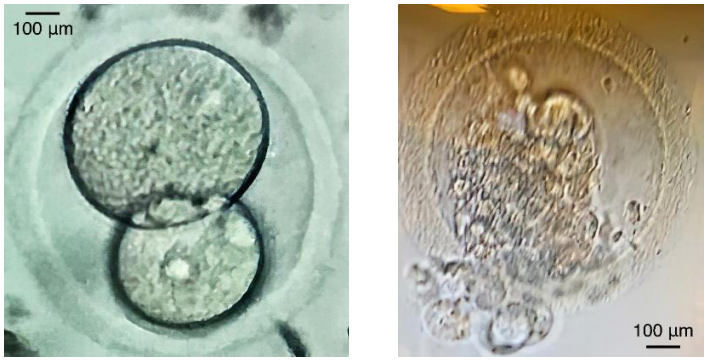
Markers selected for demonstrating the embryonic and extra-embryonic cell increase were visualized via immunohistochemical staining. The 4-micron sections were cleaned of paraffin and rehydrated following the immunohistochemical staining. Afterwards, they were stained using the streptavidin-biotin-peroxidase method [18]. The sections which were going to be applied with primary antibodies were placed in a citrate buffer solution and put in a microwave oven for 15 minutes. Subsequently, they were bathed with phosphate-buffered saline (PBS) three times. Afterwards, the endogenous peroxidase activity was blocked by applying a 3% hydrogen peroxide solution. Next, a single drop of designated primary antibodies was applied and waited for 60 minutes. The following steps were repeated with the same method for the five µL of the designated stains: ERα, PR, hCG, cytokeratin, vimentin, and Ki-67. Finally, the sections were bathed with tris buffer solution for the removal of the primary antibodies.

Afterwards, drops of the secondary antibody and streptavidin peroxidase solutions were applied and waited for 10 minutes. Next, the sections were bathed with PBS. Diaminobenzidine chromogen was applied for 20 minutes. After bathing the sections with deionized water, hematoxylin was applied for contrast staining for one to three minutes. After that, the sections were applied with a series of alcohol solutions with increasing density and xylene to achieve transparency. Subsequently, the microscopic slides were closed following the application of Entellan®. In the immunohistochemical semi-quantitative evaluations, the cells which showed positive staining in the minimum of three randomly chosen fields were evaluated under a light microscope.

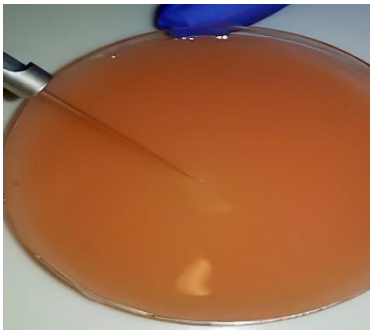
Figure 2: Embryo culturing and implantation.

A. Rat embryo on the second day of the culturing (light microscopy, x400 magnification)

B. Rat embryo on the fifth day of the culturing (light microscopy, x400 magnification)




C. Implantation of the embryo to the endometrial field in the uterine culture



**Stage 2**

In this stage, following the determination of an optimal uterine culture environment at stage 1, three culture models of the optimal model were prepared. First, seven days of culturing with DMEM-LG (with the medium changed every three days) in an incubator at 37 ºC, 5% $CO_2$, and 5% $O_2$ was performed. Next, the zygotes designated for implantation were cultured for five days in an incubator at 37 ºC, 5% $CO_2$, 5% $O_2$, and 90% $N_2$ (Figure 2.A and Figure 2.B) [19]. Afterwards, on the 7th day,

three 5-days-old blastocytes were transferred to the endometrial surface below each of the models using a Pasteur micropipette for achieving minimal trauma (Figure 2.C). Lastly, the medium was changed every 24 hours.

**Stage 2 Analyses**

At the end of Stage 2, evaluations were carried out macroscopically and microscopically using hematoxylin and eosin, immunohistochemical staining (ERα, PR, hCG, cytokeratin, and vimentin stains), and molecular genetics markers (NANOG and SOX2). In addition, immunohistochemical staining was carried out with the same protocol mentioned above [17].

**NANOG and SOX2 Staining**

The microscopic slides were fixated with paraformaldehyde solution at room temperature for thirty minutes and washed twice with DPBS ($Ca^-$ & $Mg^-$ free). Next, 1,5 ml phosphate-buffered saline with Tween™ was applied to the slides for 10 minutes at room temperature. Afterwards, the slides are washed with DPBS ($Ca^-$ & $Mg^-$-free) twice. Afterwards, 1,5 ml bovine serum albumin (BSA) solution is applied to the slides, and they are incubated at 37℃ for 30 minutes. Subsequently, the BSA solution is removed without any contact with the drop in the middle. Finally, the periphery of the drop is dried without any contact with the middle drop itself.

Anti-NANOG and anti-SOX2 stains are diluted in a 1:50 fashion for creating a 400 µl antibody solution. Afterwards, the solution is applied to the slides, and they are incubated at 37℃ in the dark. Following primary antibody incubation, the slides are washed with DPBS ($Ca^-$ & $Mg^-$-free) twice. For the secondary antibody incubation, Alexa Fluor 488 and Alexa Fluor 647 are diluted in a 1:200 fashion. Afterwards, the solution is added to the slides, and they are incubated at 37℃ in the dark. Following primary antibody incubation, the slides are washed with DPBS ($Ca^-$ & $Mg^-$-free) three times. Subsequently, the slides are incubated in a DPBS solution containing DAPI for 10 minutes at room temperature and stained. The slides are washed with DPBS ($Ca^-$ & $Mg^-$-free) two times to remove the residue. Lastly, the slides are left in DPBS ($Ca^-$ & $Mg^-$-free) and examined under a confocal microscope.

**Statistical analysis**

The normality test was done with the Shapiro-Wilk test. Non-parametric statistical methods were used for values with skewed (non-normally distributed, Shapiro-Wilk $P>0.05$) distribution. Descriptive statistics were presented using median and percentile 25 and 75 (Q1 and Q3) for non-normally distributed variables. Non-parametric statistical methods were used for values with skewed distribution. For comparison of more than two non-normally distributed independent groups, the Kruskal Wallis was used. For comparison of two non-normally distributed independent groups, the Mann Whitney U test was used.

Statistical analysis was performed using the MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013).

## Results

**Stage 1 (Primary Outcome)**

The groups are described in Table 1. All analyses were carried on the 15th day. There was a statistically significant difference in terms of glucose, lactate, endometrial thickness (millimeter), and the number of endometrial glands between the groups (Kruskal Wallis test, $P<0.05$) (Table 2).

Table 1: The distribution of the experiment groups

| Groups | Number | % |
|---|---|---|
| CNT | 3 | 25 |
| FIB | 3 | 25 |
| ISH | 3 | 25 |
| FIB+ISH | 3 | 25 |

CNT: Standard Uterine Culture Model, FIB: Fibrin Matrix-supported Culture Model, ISH: Ishikawa Cells-supported Culture Model, FIB+ISH: Fibrin matrix and Ishikawa Cells-supported Culture Model

Table 2: Comparison of parameters according to the groups

| | CNT N=3 Median (Q1-Q3) | FIB N=3 Median (Q1-Q3) | ISH N=3 Median (Q1-Q3) | FIB+ISH N=3 Median (Q1-Q3) | *P*-value |
|---|---|---|---|---|---|
| Glucose | 76 (62-84) | 87 (74-100) | 33 (30-45) | 4 (4-5) | 0.021 |
| Lactate | 54.4 (35.5-75.6) | 107.0 (89.5-112.0) | 87 (65.6-95.2) | 132.0 (120.0-135.0) | 0.023 |
| Endometrial Thickness (millimeter) | 0.2 (0.1-0.25) | 0.65 (0.55-0.7) | 0.35 (0.3-0.4) | 0.35 (0.1-0.45) | 0.046 |
| Number of Endometrial Glands | 0 (0-0) | 5 (4-6) | 0 (0-0) | 3 (2-4) | 0.015 |

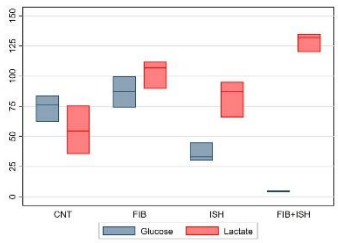
Kruskal Wallis test, CNT: Standard Uterine Culture Model, FIB: Fibrin Matrix-supported Culture Model, ISH: Ishikawa Cells-supported Culture Model, FIB+ISH: Fibrin matrix and Ishikawa Cells-supported Culture Model, N: Number

**Metabolic Activity**

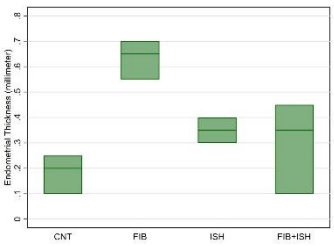
Glucose and lactate measurements were calculated as the average of the 7th and 14th-day measurements and are presented in Figure 3.A. The most glucose consumption and lactate production were detected in FIB+ISH. Conversely, FIB had the least glucose consumption, and the least lactate production was in CNT.

Figure 3: Boxplots. (CNT: Standard Uterine Culture Model, FIB: Fibrin Matrix-supported Culture Model, ISH: Ishikawa Cells-supported Culture Model, FIB+ISH: Fibrin matrix and Ishikawa Cells-supported Culture Model)

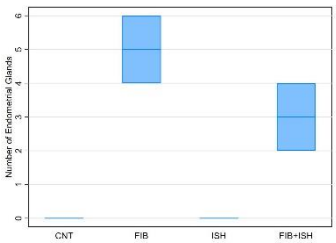
A. Glucose and lactate measurements




B. Endometrial thickness




C. The number of endometrial glands



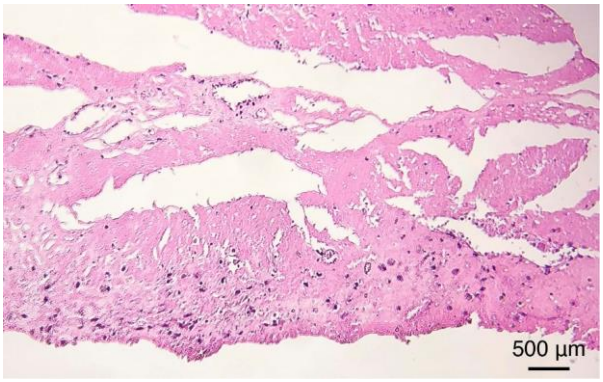

**Uterus Morphology**

**Endometrial Thickness and the Number of Endometrial Glands**

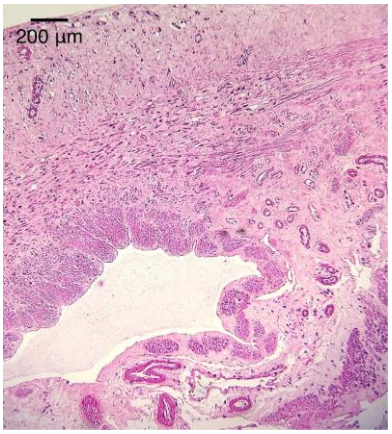
Endometrial thickness and the number of endometrial glands are presented in Figure 3.B and Figure 3.C. The thickest endometrium and most endometrial glands were detected in FIB, whereas the thinnest endometrium was in CNT. The least number of glands was detected in CNT and ISH, the gland count being zero.

Figure 4: Histologic evaluations (1 high-power field, hematoxylin & eosin).

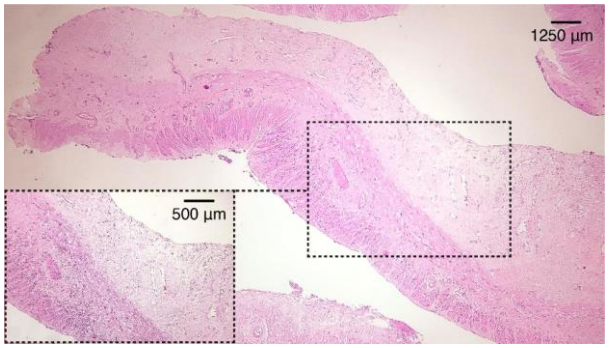
A. The control group is demonstrating a lack of development of the endometrium, myometrium, perimetrium, uterine glands, and uterine vasculature (x100 magnification)



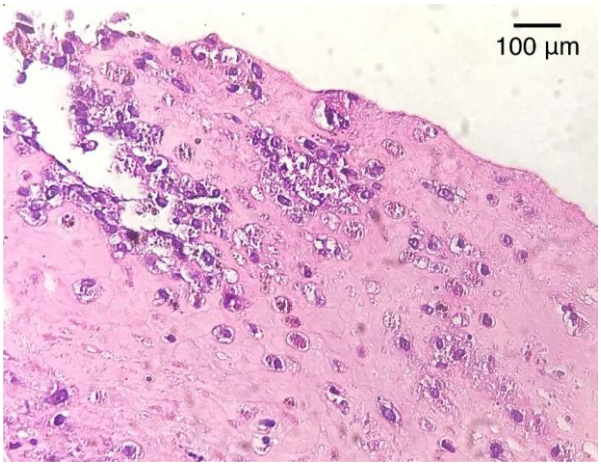

B. Fibrin-matrix supported culture model (FIB) exhibiting uterine walls composed of three layers: the endometrium, myometrium, parametrium, and containing blood vessels and uterine glands. FIB exhibiting formation of uterine vasculature (7-8%) and endometrial glands (<5%) (x200 magnification)




C. Ishikawa cells-supported culture model (ISH) displaying telangiectatic vasculature and (main image, x40 magnification; lower-left corner image, x100 magnification)




D. ISH is showing the presence of pyknotic cells (77%) (x400 magnification)




E. FIB+ISH is exhibiting uterine wall composed of three layers and connective tissue lamina propria containing large microvessels, endometrial glands, and large endometrial glandular formation. (x100 magnification)

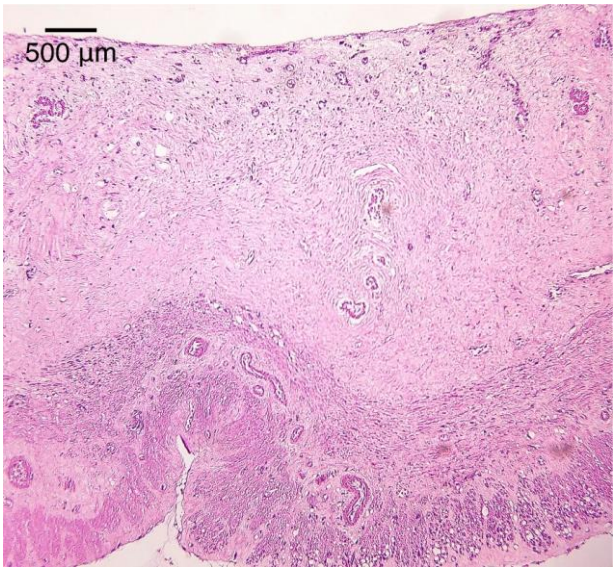
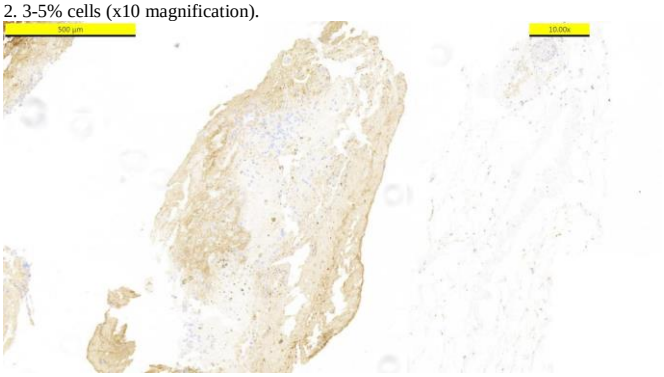
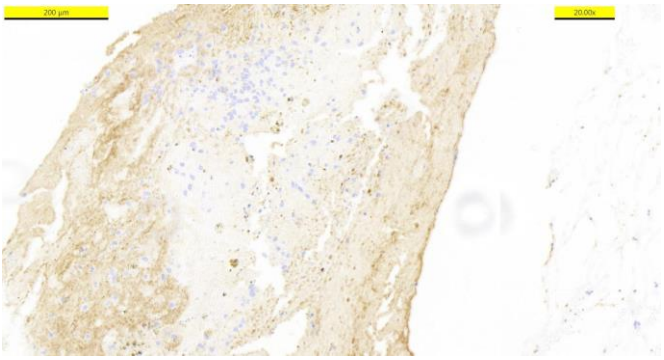


Figure 5: Estrogen affinity findings [1 high-power field, anti-estrogen receptor α (ERα) antibody]. A. Control group showing low nuclear and non-specific background staining. 1. 10-15% nuclear staining (x4 magnification)

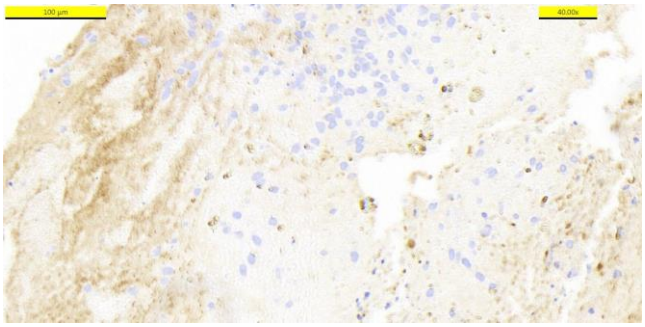
2. 3-5% cells (x10 magnification).



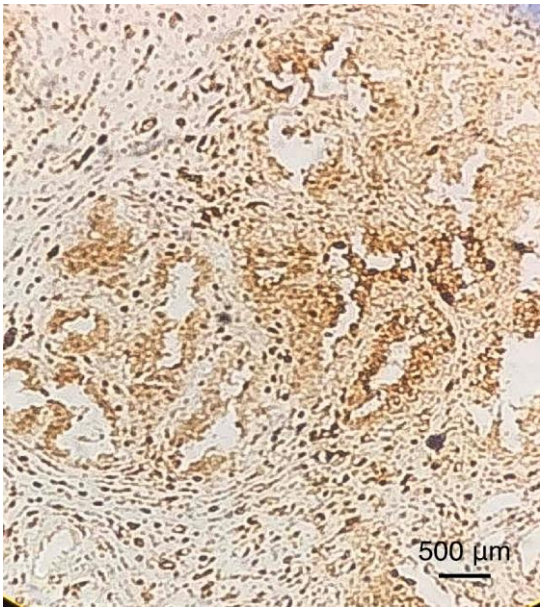
1. 3-5% cells (x20 magnification)



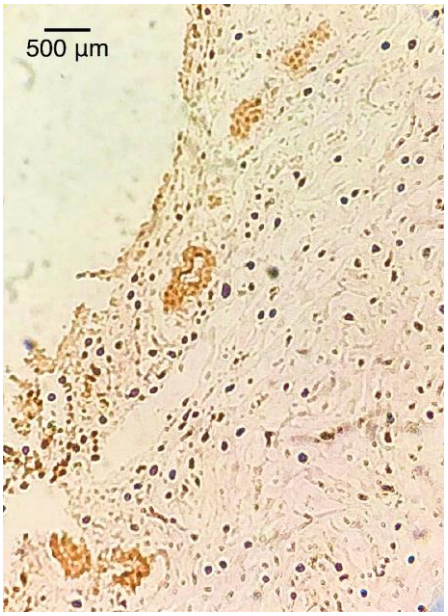
2. 3-5% cells (x40 magnification)



B. Fibrin-matrix supported culture model (FIB) exhibiting strong staining for ERα in the uterine glands (80% nuclear staining, x100 magnification)




C. Ishikawa cells-supported culture model (ISH) demonstrating ERα in the endothelial cells of the blood vessels (95% nuclear staining, x100 magnification)




D. ERα presence in the endometrial stroma and uterine glands of FIB+ISH (70% nuclear staining, x100 magnification)

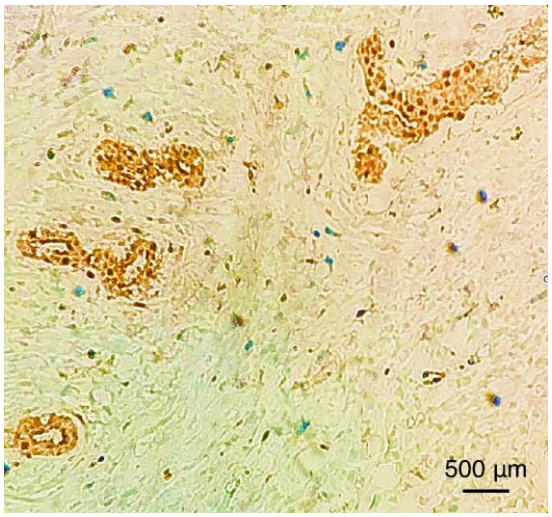
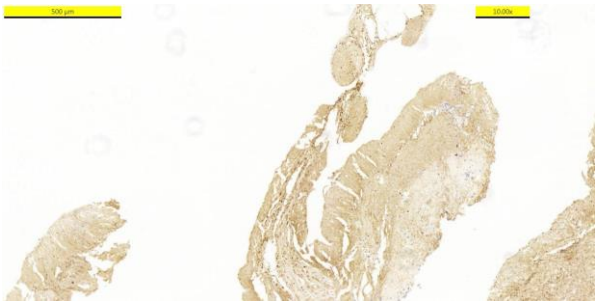
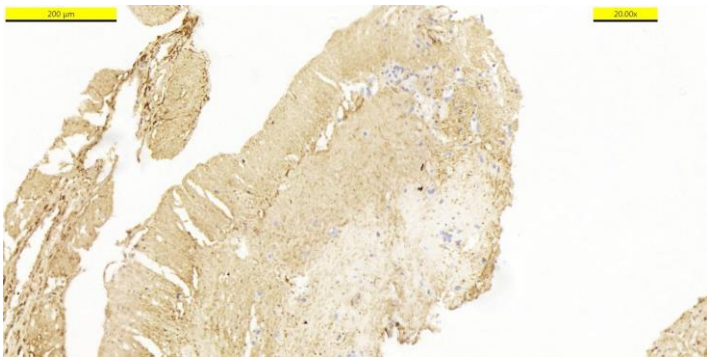


Figure 6: Progesterone affinity findings [1 high-power field, anti-progesterone receptor (PR) antibody]. A. CNT is showing no staining. 1. Diffuse background staining (x4 magnification)

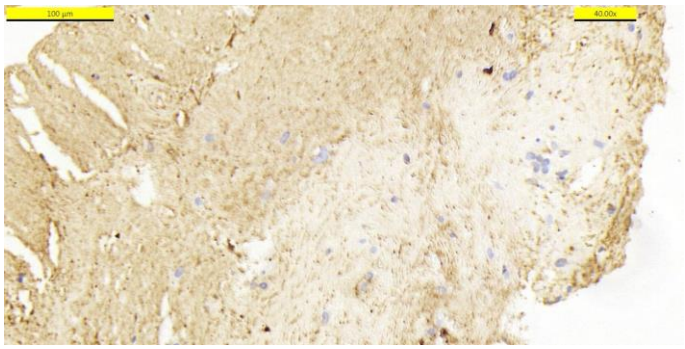
2. x10 magnification



3. x20 magnification

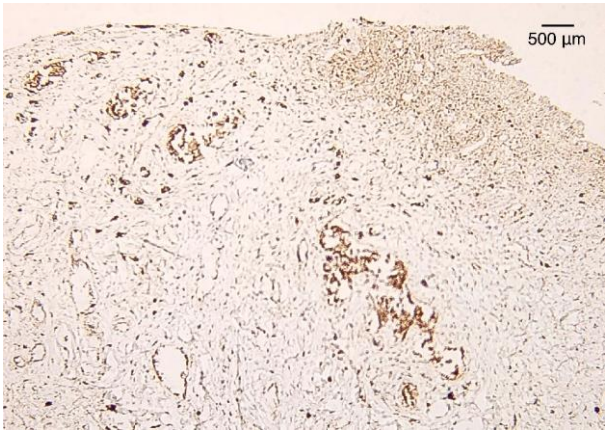


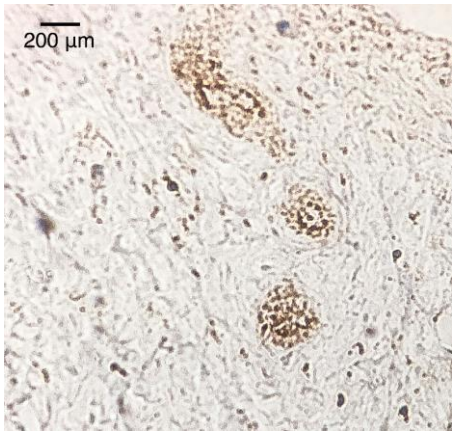
4. x40 magnification



B. Fibrin-matrix supported culture model (FIB) exhibiting PR presence in the endometrial epithelial cells and the uterine glands (55-60% nuclear staining, x100 magnification)

C. Ishikawa cells-supported culture model (ISH) demonstrating PR presence in the stromal cells and the uterine glands (70% nuclear staining, x200 magnification)




D. FIB+ISH displaying smooth muscle fibers of the myometrium and the uterine glands demonstrating weak positivity for PR antibody (55-60% nuclear staining, x200 magnification)

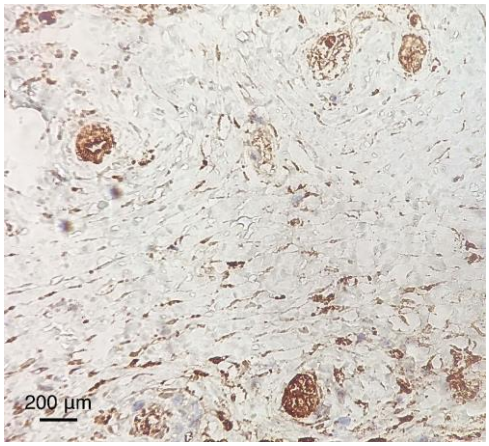
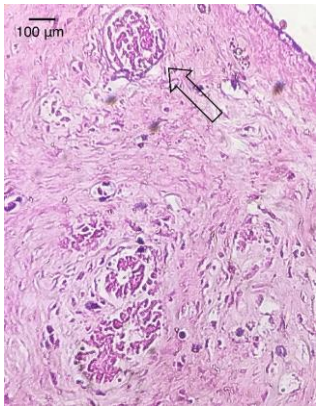


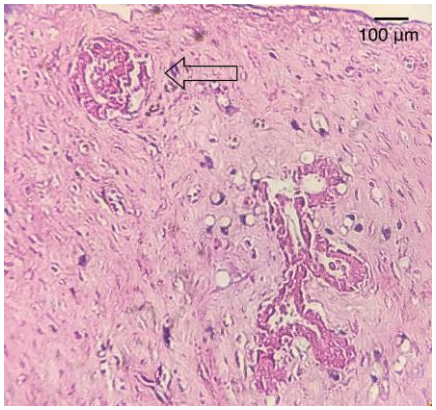

Figure 7: Implantation reaction in the Petri dish.

Figure 8: Embryos in the fibrin matrix-supported culture model (1 high-power field).

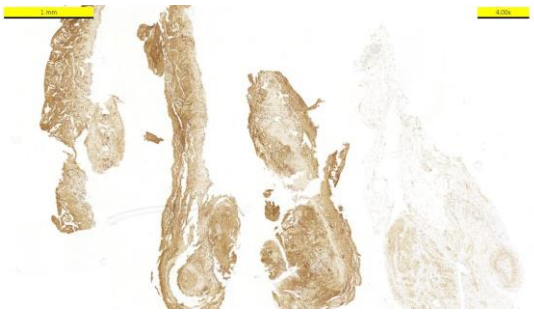
A. Embryonic body (arrow) (x400 magnification, hematoxylin & eosin)



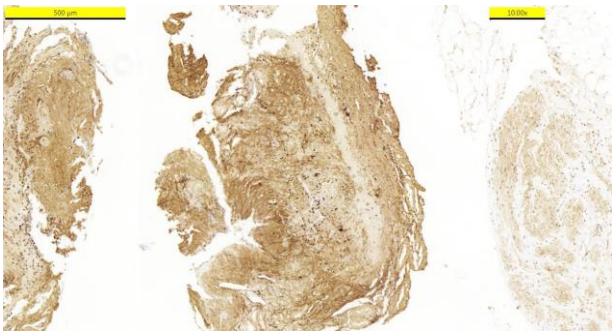

B. Embryonic body (arrow) (x400 magnification, hematoxylin & eosin)



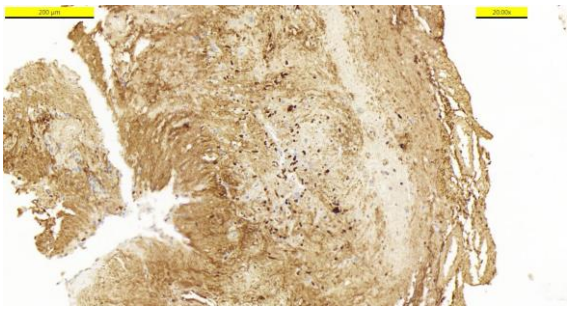

C. Estrogen receptor alpha positive cells around the uterine glands (60-70%) (x400 magnification, anti-estrogen receptor alpha). 1. x4 magnification



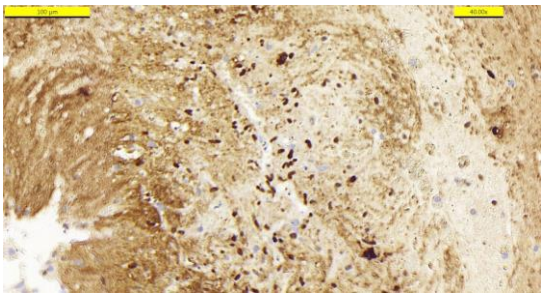
2. x10 magnification



3. x20 magnification

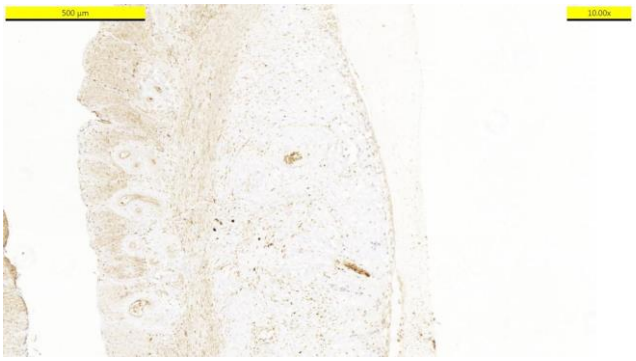


4. x40 magnification

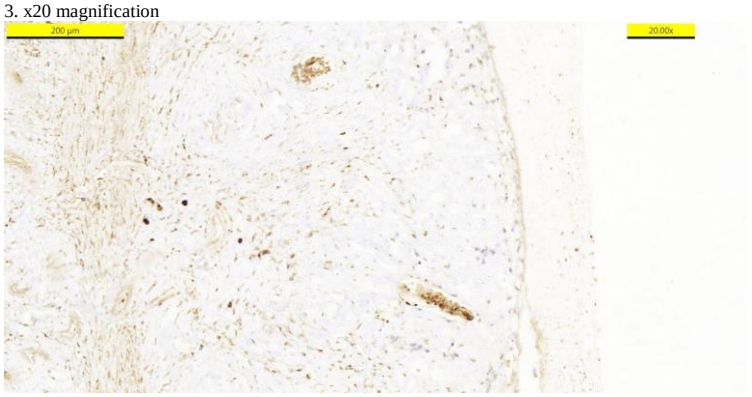


D. Progesterone receptor-positive cells in the nuclei of stromal cells (60-70%) (x400 magnification, anti-progesterone receptor). 1. x4 magnification

2. x10 magnification



3. x20 magnification



4. x40 magnification

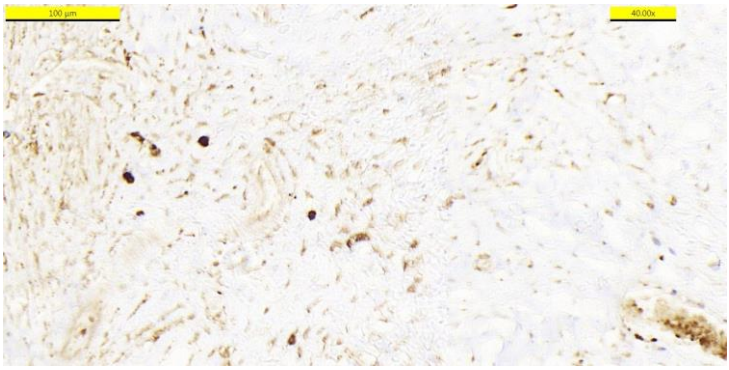
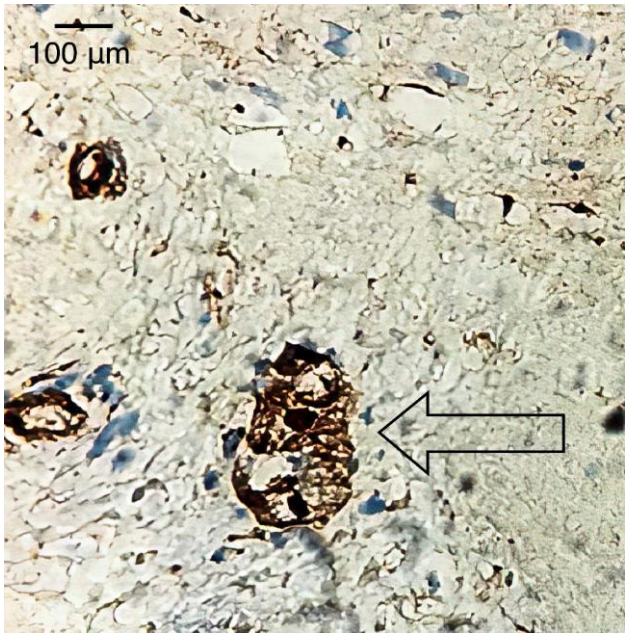
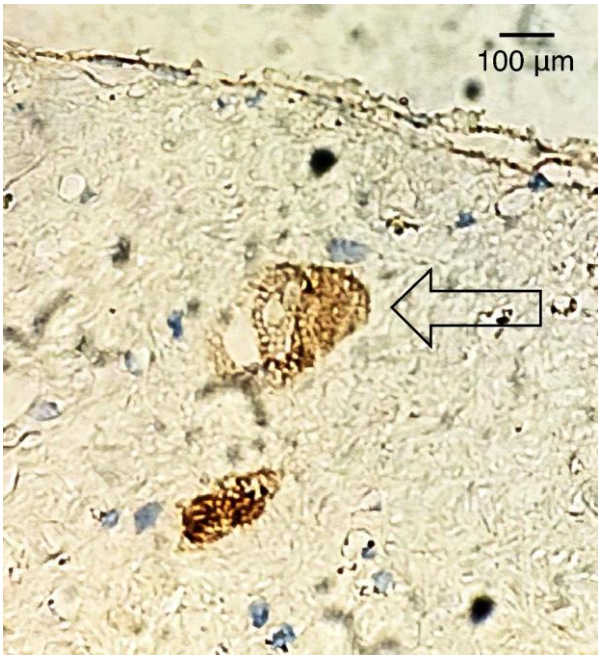


Figure 8: Embryos in the fibrin matrix-supported culture model (1 high-power field).

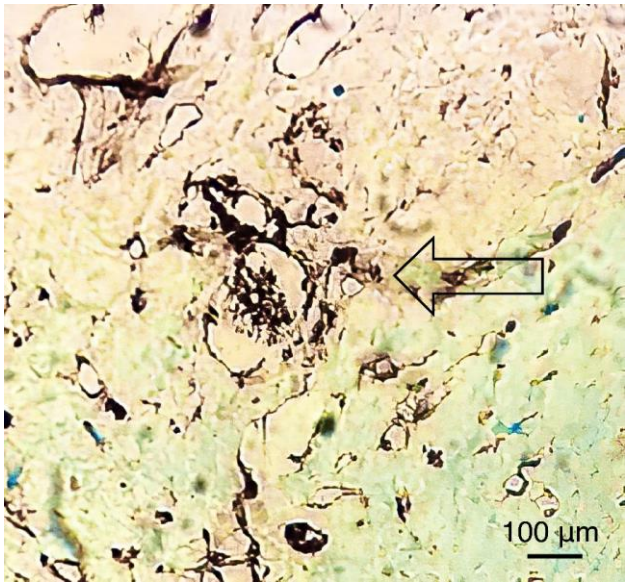
E. Embryonic body (arrow) and differentiated trophoblasts (anti-human chorionic gonadotropin antibody)




F. Embryonic body (arrow) and differentiated trophoblasts (x400 magnification, cytokeratin)




G. Embryonic body (arrow) (x400 magnification, vimentin)




H. Embryonic body (arrow) (x200 magnification, Ki-67)

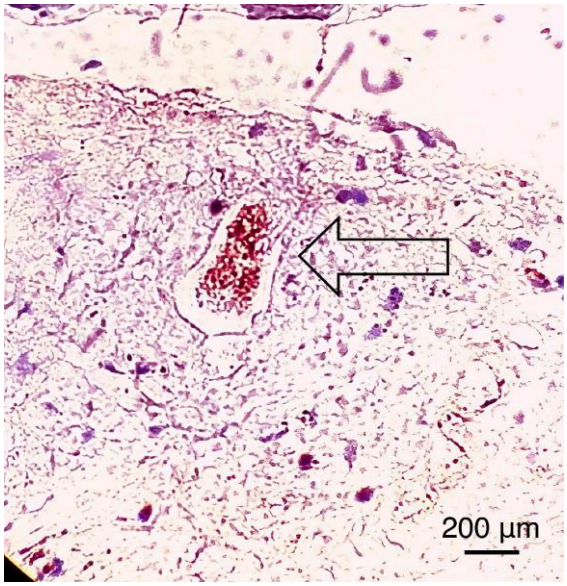
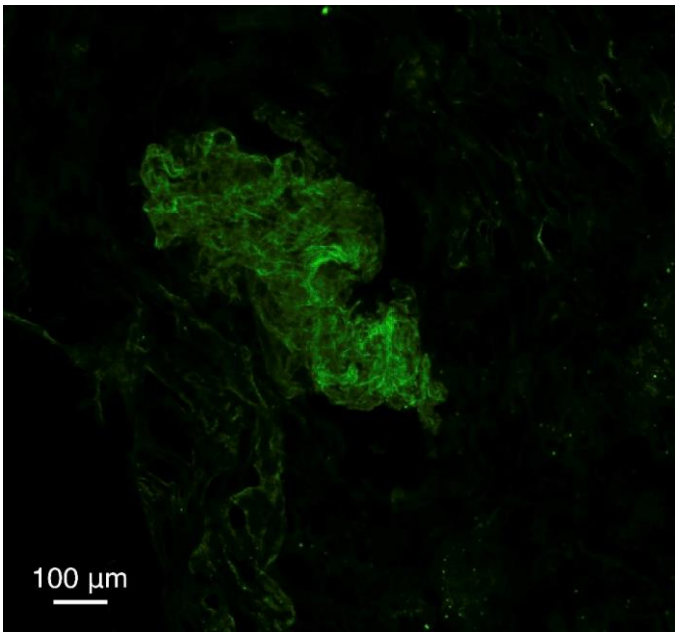
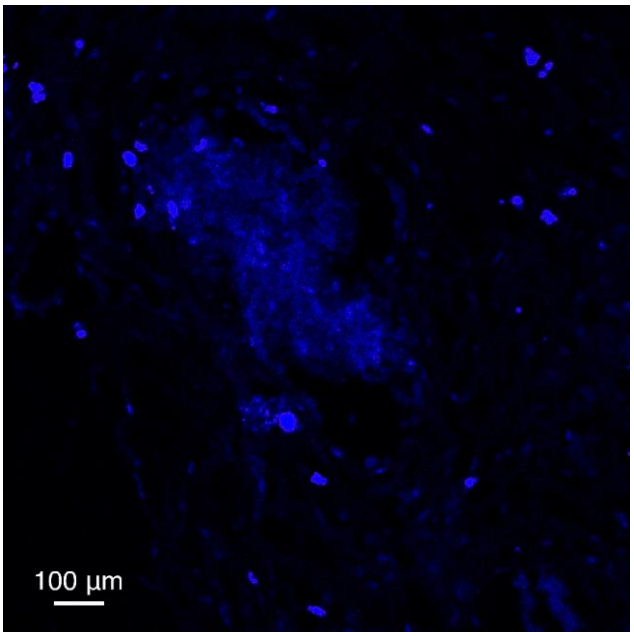



Figure 9: Embryo viability markers in the fibrin matrix-supported culture model (x100 magnification). A. Exhibiting gastrulation (NANOG)




B. Exhibiting gastrulation (SOX2)



**Uterine Histology**

In histopathologic semi-quantitative evaluations, in CNT, the three layers of the uterus were separated from each other, and the layers were not internally intact, and the tissue integrity was not preserved. The thickness of the endometrium was thin. These were attributed to the extensive necrosis in the endometrium and the myometrium. Additionally, the tissue itself was reduced in volume due to liquefaction. The tissue morphology was not preserved, and uterine glands were absent (Figure 4.A).

In FIB, the morphology of the endometrium, myometrium, perimetrium was intact. Additionally, uterine glands and vascularity were observed to be preserved (Figure 4.B).

In ISH, the absence of stromal cells, the sparsity of the vessels, and the presence of pyknotic cells were remarkable (Figure 4.C and Figure 4.D). Therefore, it was concluded that only the Ishikawa cells were not sufficient for uterine glands to form.

In FIB+ISH, the morphology of the endometrium, myometrium, perimetrium was intact. It was demonstrated that vascularity was preserved (Figure 4.E). Connective tissue lamina propria contained large microvessels and endometrial glands.

When all groups were compared, FIB had the thickest endometrium and the most endometrial glands. FIB+ISH was second in the number of endometrial glands, whereas CNT and ISH did not demonstrate any glands. CNT had the thinnest endometrium.

**Hormonal (Estrogen and Progesterone) Affinity of the Endometrial Glands**

ER-positive cells were detected in patches in the muscularis mucosa of CNT (Figure 5.A.1-4). Strong staining ER-positive cells were detected in the periphery of the submucosa and endothelium of FIB (Figure 5.B). ER positivity in the uterine glands of ISH were more prominent when compared with FIB+ISH (Figure 5.C and Figure 5.D).

PR expression was negative in CNT (Figure 6.A.1-4). PR positive cells were detected scarcely in the periphery of submucosa and endothelium in FIB (Figure 6.B). PR positive cells in ISH were more strongly stained when compared to FIB+ISH (Figure 6.C and Figure 6.D).

**Stage 2 (Secondary Outcome)**

All attempts had to be stopped due to macroscopic uterine tissue necrosis secondary to perfusion defect. The total duration of embryo viability was 14 days (21$^{st}$ day in the total duration of stage 2) in all cultures. Every culture was observed to have a single implanted embryo (Figure 7).

**Embryo Staining**

The embryos are shown in Figure 8.A and Figure 8.B. ERα and PR positive cells were detected around the uterine glands was present and regarded as embryonic implantation. (Figure 8.C.1-4 and Figure 8.D.1-4). hCG and cytokeratin staining detected the embryos and differentiated trophoblasts. Therefore, it was concluded as positive implantation (Figure 8.E and Figure 8.F). Additionally, vimentin and Ki-67 positive cells were detected in the laminar epithelia and trophoblastic differentiation zone (Figure 8.G, Figure 8.H).

**Embryo Viability**

The embryos were analyzed under a confocal microscope by staining with NANOG and SOX2 markers. The embryo tissue expressing positive signal was considered the continuing expression of three germ layers, and the integrity was preserved on the 14$^{th}$ day (21$^{st}$ day in the total duration of Stage 2). Additionally, the strong positive signals indicated that gastrulation was present (Figure 9). These correspond to embryonic day (E) 9 (E9) in embryo development. Thus, all three embryos were in stage E9.

## Discussion

The main parameters contributing to the success of in vitro embryogenesis studies are the integrity of the endometrium, the positivity of embryonic markers, the presence of gastrulation, and the duration of viability [6, 8, 9, 20]. This controlled study's primary outcome was the metabolic, morphologic, and histopathologic effects of fibrin matrix, Ishikawa cells, and both on in vitro 3D uterine tissue cultures. The secondary outcome was the duration of embryo survival. Ishikawa cells were used instead of the uterine cells of the same rats due to their high metabolic energy output and the presence of estrogen and progesterone receptors.

A statistically significant difference was present between the groups in glucose, lactate, endometrial thickness, and the number of endometrial glands. Detrimentally high glucose consumption and lactate production were detected in FIB+ISH. Additionally, it had adequately preserved morphology and histology. However, it was not the best group in terms of metabolic activity and uterus morphology. These could have been due to a couple of reasons. Firstly, the rat endometrium and Ishikawa cells (human adenocarcinoma) could have damaged each other by secreting cross-species soluble factors such as cytokines such as interleukin-1β [21]. Moreno et al. [22] studied the effect of different growth factors and cytokines in addition to surfactants, embryotropic recombinant albumin, and hyaluronan without fetal calf serum and BSA on in vitro embryogenesis. It was demonstrated that the addition of cytokines and other factors positively affects embryo development.

Block et al. [23] also studied the effect of insulin-like growth factor, colony-stimulating factor 2, and hyaluronan on in vitro embryogenesis. In vivo maternal environment constitutes various growth factors, cytokines, hormones, and other regulatory molecules that affect embryo development. Transferring these molecules to a culture environment aids the development of the embryo. However, cytokine measurements and interventions were not performed in the current study. Secondly, the Ishikawa cells, which are adenocarcinoma cells that consume more glucose, could be disarranging the histologic integrity of the uterine cells by creating metabolic deprivation and harmful metabolites for the endometrial tissue. It could be prevented by changing the mediums more frequently and allowing more glucose in the uterine tissue.

The least glucose consumption was detected in FIB. It was considered a sign of adequate perfusion because the cells did not spend much energy to survive. It could also be attributed to the absence of glucose-depriving Ishikawa cells allowing the uterine tissue to survive without any deprivation and toxic

metabolites. In histopathologic evaluations, morphology and histology in FIB appeared to be preserved, and it had the thickest endometrium and the most endometrial glands. Additionally, ISH had the strongest staining for ER and PR. However, when every parameter was combined, FIB was better than ISH. Concurrent with the current results, Pelletier [24] has demonstrated positive staining for ER and PR in the pregnant rat uterine epithelial and stromal cell nuclei. Furthermore, Carvalho et al. [25] recorded that immunostaining for ERα was positive for glandular, epithelial, and stromal cell nuclei and cytoplasm of the uterus. As a result, FIB was determined as the optimal model (Stage 1).

When normal endometrium cells obtained via biopsy are cultured, it was defended that the endometrial, epithelial, and stromal cells were superior in terms of receptivity. Furthermore, it was determined that the natural killer and endometrial epithelial cells form a viable environment, especially for decidualization [26]. However, endometrial biopsies do not always provide a standard ratio of endometrial/epithelial cells. Therefore, artificial cell lines were created to overcome this issue and create a standard ratio of cells. Uchida et al. [27] created a new co-culture model using the Ishikawa cell line (human adenocarcinoma). They took advantage of the richness of estrogen and progesterone due to their adenocarcinoma nature and the adhesive properties of this cell line. However, as the current study demonstrates, this cell line was also restrictive due to the contamination caused by malignant cell formation and metabolically harmful.

Uterine tissue culture environments are attempting to be enhanced with co-cultures and biomaterials such as gels [5, 8-11], macrophages [10], stem cells [28 - 33], cell lines [34], and decellularized scaffolds [35-37]. Numerous researchers have combined uterine tissue with Matrigel®, a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, in different species' embryos [5, 8-11]. In the model by Rozner et al. [10], Matrigel® was combined with a matrix of maternal macrophages and pregnant monkey decidual cells. Additionally, stem cells are used and combined with Matrigel® for endometrial tissue regeneration studies [29].

In Stage 2, the study was terminated due to macroscopic necrosis on day 14. After evaluation, it was detected that each of the three models of FIB had successful implantation of a single embryo out of three. Additionally, estrogen and progesterone are essential markers of blastocyst development in addition to glandular epithelial proliferation. The endometrium is observed to have estrogen and progesterone receptors profusely when an embryo is held onto the uterus at the blastocyst stage. Blastocyte-implanted FIB was demonstrating stronger staining for estrogen and progesterone receptors than the non-blastocyte-implanted FIB. Thus, it points to successful implantation concurrent with the literature [24].

Each of the three models of FIB had successful implantation of a single embryo out of three implanted. The duration of embryo survival was 14 days. Moreover, immunohistochemical markers are essential in evaluating the multiplication and differentiation of the embryonic and extra-embryonic cells. Therefore, they were applied in the current study as well. Cytokeratin staining, a well-recognized marker to detect differentiated trophoblasts in placental cell isolates, showed areas positive for trophoblastic invasion [38]. Positive hCG staining points to the extra-embryonic cellular changes. It was also previously defined in the literature as a feature of differentiation of the trophoblasts into syncytiotrophoblasts [39]. Vimentin is a mesenchymal marker expressed from non-trophoblastic fibroblasts and endothelium when embryonic structures demonstrate growth [40]. In the current evaluations, the presence of vimentin-positive cells in the embryonic structure indicates mesenchymal growth. Ki-67 trophoblast proliferation marker was also positive [10]. In molecular genetics evaluations, NANOG and SOX2 staining demonstrated gastrulation and the embryo growth was deemed viable and classified as stage E9 (in 14-day old embryos). The literature also supported this as only onwards from stage E9 the NANOG and SOX2 markers were reported to be expressed [41]. A 14-day-old embryo matching the developmental stage, corresponding to day 9 (E9), could be contributed to the embryo's alteration of growth speed for adapting to environmental conditions [42]. It was also contributed to inadequate perfusion by the medium-only type of perfusion of the model. It is shown in the literature that when advanced perfusion tools with gas and pressure regulators are utilized, the in vitro embryo follows the same daily developmental course as a normal embryo [1]. The current study used a 3D uterine tissue culture system, and a continuous perfusion system was not used. Continuous perfusion systems are the next generation of 3D culture systems. Fibrin matrix-supported culture provides adequate perfusion for embryo development. Adding continuous gas perfusion to the current 3D model (FIB) could enable an in vitro embryogenesis model in which organogenesis could be observed. Combining the principles of the current study and Aguilera-Castrejon et al.'s [1] study could light the way for this.

In the organ perfusion models, the model by Geisler et al. [12] was designed for uterine transplant but is also considered valuable in embryonic development. In the model by Han et al. [13], monospermic insemination was achieved in ex vivo porcine uterus by in vitro fertilization. This model's embryonic development was slow; however, it is the first organ perfusion model to demonstrate monospermic blastocyte development. When the two models are evaluated, they need complex mechanisms to achieve long-term embryonic development since the organ in the model needs continuous perfusion to stay viable. Thus, the continuation of perfusion is the fundamental problem in whole organ models.

Enders et al. [43] have reported that rhesus monkey blastocyst in 2-D Matrigel® did not fully develop and had limited trophectoderm growth. Nevertheless, it was demonstrated that the trophoblastic activity was developing towards Matrigel®. Two of these embryos were followed until the 18$^{th}$ day, and the other two were followed until the 45$^{th}$ day. The embryo was sustained for a long time, but data regarding the embryonic markers in the study were not reported [11]. Rhesus monkey embryos' trophoblast development was detected until the 6$^{th}$ day. Afterwards, embryos were co-cultured in Buffalo rat liver cell-conditioned medium cultured using macrophages, and embryonic development was observed until the 9$^{th}$ day. hCG secretion was recorded until the 10$^{th}$ day, and the Ki-67 trophoblast

proliferation marker was detected due to staining. Thus, macrophage culture looks promising for placentation and gestation; however, long-term studies are required to validate this finding [10]. A review by Morris et al. [44] presents the culture models that support the human embryo until the gastrulation stage.

In recent years, a model by Niu et al. [5] containing Matrigel® allowed monkey embryogenesis and gastrulation findings were present after the 14th day. However, degeneration on the 20th day shows that the model enabled limited development. The current study demonstrated gastrulation similar to Niu et al.'s [5] study. However, considering the trimester of both species, 55 days per trimester for rhesus monkeys and seven days per trimester for rats, shows that when compared with the primate models, the current rat model corresponds to a more extended period of pregnancy when trimester durations between species considered. Additionally, one of the main differences of the current model with Niu et al.'s [5] study is that a uterine culture was not used for embryogenesis in the current study. Therefore, the current study's model is different due to the usage of the combined uterine culture.

There are strict ethical limits for human embryo studies, mainly focusing on the 14th day of development [45-54]. The embryo is accepted as an individual after the primitive streak is formed on day 14, and further research is prohibited [55]. Embryoids, which are animal cells taken from the intracellular matrix embryos and then cultured, behave like an early embryo (blastocyte and gastrulation stages) under culture conditions. The most significant advantage of the embryoids is that they do not possess an ethical issue, and in vitro embryogenesis research also focused on these [16, 33, 56-64]. Embryoids could also mimic specific developmental stages such as gastrulation [65, 66].

To summarize, the current results show that the fibrin matrix-supported uterine culture model supports embryogenesis and could be proposed as an adequate extracellular matrix for in vitro uterine tissue culture and embryogenesis studies. Furthermore, the embryonic cell markers being positive until the 14th day demonstrates that the model is satisfactory when the 21st-day uterus activity data of the current study and the pregnancy period of the rats are considered.

**Limitations**

The limitations of this study are, first, the limited number of uterine cultures and embryos. Secondly, only the optimally determined group was implanted with embryos instead of all the groups. Thirdly, cytokine and cross-species soluble factor measurements were not performed.

Further studies with larger sample sizes, including control groups, are needed for achieving a stronger generalizability. Future investigators could utilize continuous perfusion systems for maximum uterine culture survival and embryo viability. More studies on the effects of biomaterials such as cell lines and matrixes on uterine tissue cultures and implantation should be conducted on different mammalian species to better translate to clinical medicine.

**Conclusion**

FIB was demonstrated to be the best model for glucose consumption, endometrial gland count, and endometrial thickness. Furthermore, FIB enabled 14 days for a single embryo to survive when implanted with three embryos. In conclusion, the fibrin-matrix supported culture model could be a satisfactory 3D uterine tissue culture model for in vitro embryogenesis research.

Further studies utilizing advanced systems with continuous bioreactor systems supported by hormonal stimulation and perfusion are required. Additionally, cross-species soluble factors evaluation and adjusting are needed to achieve an even more efficient model.

**Acknowledgments**

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