Presentation of a New Endometriosis Mice Model Through Rat to Mice Xenograft Transplantation of Endometrium with some Modifications; A Pilot Study

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Research Article

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Abstract

Human endometriosis can lead to many complications, including permanent infertility. Rodent model of endometriosis is complicated due to the impractical isolation of myometrium. In this method, we defined a new model of endometriosis with some modifications. 40 female mice were grouped as; 1st sham, 2nd mice-mice allograft uterus transplantation of mice to anterior abdominal wall of mice, 3rd mice-mice allograft uterus transplantation of mice to mesentery of mice, 4th rat-mice xenograft endometrial transplantation of rat to anterior abdominal wall of mice, 5th rat-mice xenograft endometrial transplantation of rat to mesentery of mice. In recipient animals, estrous cycle was synchronized. Endometrium was dissected in rats and transplanted in abdominal wall or mesentery layer. Angiogenetic and inflammatory factors, serum levels of CA125, oxidative stress in peritoneal fluid, physical and histopathological features, and genes expression in uterus were assessed. Peritoneal concentrations of VEGF-A, TNF-α, NO, MDA, and serum levels of CA125, IL-37 were increased significantly ($P < 0.05$) in transplanted groups, especially in the 4th group than control. Total body weight of animals was decreased significantly ($P < 0.05$), while weight and size of endometrial lesions were increased significantly ($P > 0.05$). Genes expression of HOXA10 and HOXA11 were decreased in endometrium of uterus. Finally, histopathological features of endometrial lesions were confirmed by H&E and Perl staining. Xenograft transplantation of endometrium from rat to anterior abdominal wall of mice can potentially mimic the human endometriosis morphologically, histologically and genetically.

Introduction

The endometrium is the inner epithelial layer of the mammalian uterus divided into thin basal and thick functional layers. The presence of a functional layer strictly depends on the menstrual (in human) or estrous (in rodents) cycles.

Endometriosis is a pathologic condition in which the endometrial cells grow outside the uterus in abdominal or pelvic cavities. These cells reside on peritoneum layer located on organs (like ovaries, fallopian tubes, uterus, and intestine), attract adjacent vessels (angiogenesis), and grow by inflammation triggering. This pathologic condition leads to formation of new endometrial lesions. Thus, angiogenesis and inflammation are two crucial biological phenomena in endometriosis. Complications of this disease include presence of scar tissue, organs adhesions, pus-filled cysts, and adhesion-related obstruction (in lumen of the intestines or uterus). Also, following scar formation, anatomical disposition, and uterus obstruction, female infertility is probable, which is considered an end-stage outcome of endometriosis [1].

The most common theory of human endometriosis is a retrograde route of endometrial cells from intrauterine cavity to the peritoneal fossa. Although most studies in the field of infertility and reproduction are performed in mice models than other animals, the presence of intrinsic endometriosis in mice is impossible because there are no menstrual cycles and endometrium abscission in rodents and these integrated cells are absorbed by adjacent tissues. Thus, endometriosis induction by a surgical procedure in mice seems necessary to assess human endometriosis aspects [2].

There are different animal models for endometriosis, especially in rodents. One of the most common types is transplantation of endometrial layer of uterus to various abdominal organs or abdominal wall in mice or rats. According to many studies, endometriosis induction is seen commonly in mice, during which the endometrium of uterus is dissected and transplanted to the abdominal wall or various organs. Endometrium in mice is a thin microscopic layer which its dissection is often impossible; besides the endometrium is a fragile tissue with no suturable property. However, endometrial layer in sexual stimulation is thick enough to be dissected and transplanted. Besides, transplantation to vital organs such as mesentery, as a routine protocol of endometriosis induction, can potentially damage vessels, leading to internal haemorrhage and animal death. These complications caused endometriosis induction a complicated process in mice.

According to the facts mentioned above, presenting a new animal endometriosis model and introducing a proper anatomical side of endometrial implantation with fewer complications that completely mimic human endometriosis conditions seems necessary. Thus, in the present study, we compared three models of animal endometriosis (rat-rat and mice-mice allografts, and rat-mice xenograft) transplanted to the anterior abdominal wall and mesentery layer. Finally, we introduced a new model of animal endometriosis through xenograft transplantation of endometrium of rat to anterior abdominal wall in mice. In this experimental animal study, we assessed the recipient animals biochemically, genetically, and histologically to ensure the accurate imitation of a new endometriosis model similar to human type.

Materials And Methods

Laboratory animals

40 females adult NMRI mice (25-30 gr, 8-12 weeks) were purchased from the animal house of the university. To be acclimatized with the new living environment, the animals were kept in animal house for two weeks with no experimental treatment. Also, all standard living conditions (22 ± 2°C, 12-hour light/12-hour dark, and free access to water and food pletes) were provided.

Study animal groups

40 female mice were divided into 5 experimental groups (n=8 in each group); 1st sham (application of laparotomy followed by abdominal wall suture with no experimental treatments), 2nd mice-mice allograft uterus transplantation of mice to anterior abdominal wall of mice, 3rd mice-mice allograft uterus transplantation of mice to mesentery of mice, 4th rat-mice xenograft endometrial transplantation of rat to anterior abdominal wall of mice, 5th rat-mice xenograft endometrial transplantation of rat to mesentery of mice. In all treatments groups, laparotomy was applied in anterior midline region of animals. Due to the thin and inseparable layer of mice endometrium, uterus of mice with whole layers (endometrium, myometrium, and perimetrium) was dissected and

transplanted in allografts. Also in rats, the endometrium was dissected from total uterus tissue and transplanted to recipient animals in xenograft procedure. Two transplantation locations were used; anterior abdominal wall and mesentery of small intestine.

Hormonal and estrous cycle synchronization in recipient animals

According to the Whitten effect and based on estrogen-depended nature of endometriosis in rodents, the female recipient animals were exposed to males (in nested cages) before and during of endometrial induction. Also, to estrus cycle synchronization, daily vaginal cytology was checked for a week before the surgery. Cotton swab impregnated with normal saline was used to collect vaginal smear from vaginal orifice. The samples were stained by Papa Nicola staining method, and endometriosis induction was applied in estrus stage for all animals. The mice were sexually receptive in estrus cycle characterized by estrogen secretion. In this study, the estrus cycle was detected by the presence of whole types of superficial cells through microscopic examination of vaginal cytology [3].

Preparation of donor animals

To increase the endometriosis thickness of rats and facilitated endometrium dissection, as the main modification in this method, three main conditions were provided for donor rodents; all animals were in adult age, they had a previous pregnancy experience, and they were exposed to male animals for two weeks. Exposure of female animals with males (divided by metal grid for mating prevention) was conducted with one male / two female ratio for sexual stimulation and endometrial growth.

Implantation of endometrium to abdominal wall and mesentery

Donor animals (mice and rats) were euthanized through cervical dislocation. Uterus (of mice) and endometrium (of rats) were dissected, and all surrounding attached connective tissues were removed under a loop microscope (1A). Dissected tissues were placed in DMEMF12 / FBS 5% cell culture solution to preserve cell viability (1B). Round grafts were prepared using a 3-mm diameter punch and were sutured (nylon, 5-0 USP, SUPA medical devices, Iran) to anterior abdominal wall or mesentery layer of small intestine in recipient animals (1C and 1D). Recipient mice were weighed and anaesthetized through intraperitoneal injection of ketamine/xylazine (20 IU per 25 gr animal of a mixture 10 IU ketamine/90 IU xylazine). Small intestine was explored, and the inner epithelial surface (containing endometrium) of grafts was sutured in direct contact with the peritoneal surface of mesentery or abdominal wall. Also, for preparation of blood supply, the grafts were transplanted exactly close to mesentery vessels. Peritoneal and muscular layers of abdominal wall were sutured using absorbable strings (chromic 5-0 USP, SUPA medical devices, Iran), and the skin was closed by nylon (Nylon, 5-0 USP, SUPA medical devices, Iran). A day after the recovery from surgery, the recipient animals were exposed to males (for 4 weeks) surrounded by metal cages (to prevent mating) for endometriosis induction (Fig. 1).

Animals weighting, dissection, and tissue sampling

4 weeks after surgery, the recipient mice were euthanized using cervical dislocation procedure. Immediately, 1 ml of distilled water was injected into the peritoneal cavity. 2 min later, the peritoneal fluid was aspirated. After laparotomy procedure, the endometrial lesions and uterus were dissected. Thoracotomy was also conducted, and the blood was aspirated from right ventricle and followed by centrifugation (3000 g, 15 min) to separated blood serum. All biological samples were frozen in nitrogen liquid for future biochemical and genetic analysis or were fixed in formaldehyde 10% for histopathological assessments. Total body weight was also recorded.

Morphometric assessments of endometrial lesions

During tissue sampling procedure, diameter of endometrial lesions was calibrated (Calliper, Sana`t Co, Iran), and the weight of the grafts was calculated (Laboratory scale, Model GR 202, Tajhizat Co, Iran) after complete excision of surrounding connective tissues. The presence or absence of pus-filled cysts was also detected. These factors were considered growth markers of endometrial lesions, and they were compared with primary size (3mm) and weight (0.01 gr) of endometrial lesions exactly prior to implantation.

Assessment of angiogenesis in endometrial lesions and peritoneal fluid

To evaluate angiogenesis rate in endometrial lesions, number of new generated vessels around endometrial lesions were counted using histopathological sections (Nicon biology microscope, E200). Also, peritoneal concentration of VEGF-A, as a peritoneal angiogenic biomarker, was assessed biochemically using ELISA kit (Abcam, ab100662, USA) according to the routine procedure and based on the manufacturer`s instruction [4].

Assessment of inflammation status in peritoneal fluid and blood serum

Concentration of TNF-α (Abcam, ab193687, USA) in peritoneal fluid was considered an inflammation statue induced by endometrial lesions and produced by peritoneal macrophages. Also, IL-37 (Abcam, ab213798, USA) was measured as a serum biomarker for endometriosis diagnosis. These measurements were done by ELISA kit according to the protocol [4].

Status of oxidative stress in peritoneal fluid following endometriosis induction

Generated oxidative stress following hyper-activation of macrophages and high rate proliferation of endometrial cells was measured in peritoneal fluid using ELISA kit. In this process, NO concentration (Abcam, ab272517, USA) was evaluated using Griess techniques, and MDA (Abcam, ab238537, USA) levels (representing lipid peroxidation status) were also measured [4].

Serum levels of CA-125

Cancer antigen 125 is a member of the mucin family glycoproteins. CA-125 is a biomarker which is elevated in the blood of some types of cancers and endometriosis. This factor was measured using ELISA kit (Abcam, ab108653, USA) [4].

Histopathological assessments using H&E and Perl`s staining

Right horn of uterus and half of endometriosis lesions were fixed in formaldehyde 10% for H&E and Perl staining. H&E staining was used for glandular and stromal assessments of endometriosis, and Perl's staining was also applied for hemosiderin deposition in macrophages as a crucial factor of endometriosis. Tissue processing was applied, and paraffin blocks were prepared. Thin sections (5 µm) were cut (Microtome, Leica RM 2125, Germany) and stained using H&E and Perl staining. Finally, the slides were assessed by a research microscope (Olympus, BX-51T-32E01) based on the histopathological variations, including; epithelium and stroma of endometrium, endometrial glands, blood vessels, and macrophages loaded with hemosiderin [4].

RNA extraction and Real-Time Quantitative PCR of HOX genes expression in uterus

Variation of HOX genes (HOXA 10 and 11) on endometriosis and as an indicator of embryo implantation during fertility was approved by literature. In order to assess the probable pathologic effects of endometriosis on uterus, the genes expression of HOXA10 (F: GCCCTTCCGAGAGCAGCAAAG, R: AGGTGGACGCTGCGGCTAATCTCTA) and HOXA11 (F: GATTTCTCCAGCCTCCCTTC, R: AGAAATTGGACGAGACTGCG) were assessed. Left horn of uterus was dissected, and total RNA was extracted (QIAGEN RNA purification kit), and a spectrophotometer checked the quality of the extracted RNA (UV1240, Shimadzu, Kyoto, Japan) in 260/280 nm wavelength absorbance ratio. DNA was synthesized using a commercial BioFact kit (BioFact RT Series, Korea). The genes expression was evaluated using High ROX BioFact™ 2X Real-Time PCR Smart mix SYBR Green PCR master mix. Also, β-actin (F: GGCACCACACCTTCTACAATG, R: GGGGTGTTGAAGGTCTCAAAC) was used as a housekeeping gene. Gene expression levels were measured by using Ct ($2^{-\Delta\Delta t}$) method (fold changes).

Ethical Considerations

The present investigation was in compliance with ethical and human principles of research and approved by the Ethics Committee of the University of Medical Sciences.

Statistical analysis

After extracting the information, the Kolmogorov–Smirnov test was first conducted to confirm data compliance of the normal distribution. One-way analysis of variance (one-way ANOVA) was used for statistical analysis, and the Tukey post hoc test was used to determine the difference between the groups. Statistical Package for the Social Sciences 16 (SPSS Inc., Chicago, IL) was used for data analysis, and the results were expressed as mean ± standard error, and $p < 0.05$ was considered significant.

Results

Angiogenesis rate

This value was assessed according to peritoneal concentration of VEGF-A and vessels count in microscopic sections. Peritoneal fluid concentration of VEGF-A was increased significantly ($p < 0.05$) in mice-mice allograft and rat-mice xenograft transplantations in comparison with control group. Also, the levels of VEGF-A were accelerated significantly ($p < 0.05$) in rat-mice xenograft groups than mice-mice allograft animals representing high levels of VEGF-A in xenograft animals than allografts. Newly formed vessels also showed the incremental trend in whole groups of allograft and xenograft transplantations significantly ($p < 0.05$) compared to the control group. No significant ($p > 0.05$) differences were detected in vessels count in xenograft animals than allografts (Table 1).

Table 1
Rate of angiogenesis, inflammation, CA125, oxidative stress, and alteration of physical features in control and various transplanted groups

NO	Angiogenesis		CA-125	Inflammation		Oxidative stress		Physical features		
	VEGF-A (pg/ml)	Vessels count	(pg/ml)	TNF-α (pg/ml)	IL-37 (pg/ml)	NO (OD%)	MDA (nm/mg)	Total body weight (gr)	En lesions weight (gr)	En diameter (mm)
1	190.1 ± 7.02	0	8 ± 0.2	1.1 ± 0.2	37 ± 4.1	4.8 ± 0.7	2.1 ± 0.3	25.8 ± 2.4	0	0
2	432 ± 43.5*	8 ± 2*	34.01 ± 1.2*	3.8 ± 0.9*	54.02 ± 3.4*	7.3 ± 1.7*	8.3 ± 1.1*	21.2 ± 2.2*	0.8 ± 0.01	8 ± 0.1
3	398 ± 21.1*	5 ± 1*	29.4 ± 2.3*	3.3 ± 1.01*	48.2 ± 2.9*	6.2 ± 0.9*	6.2 ± 2.01*	24 ± 1.3	0.19 ± 0.1	5 ± 0.2
4	512 ± 25.8*	9 ± 2*	35.8 ± 1.6*	3.5 ± 1.2*	68.3 ± 4.1*	7.4 ± 2.2*	8.9 ± 2.4*	19.8 ± 2.3*	1.8 ± 0.04	12 ± 0.03
5	490 ± 50.4*	4 ± 1*	28.2 ± 0.9*	2.8 ± 0.4*	59.1 ± 3.3*	6.8 ± 109*	5.9 ± 1.4*	23.6 ± 2.6	0.70 ± 0.01	4 ± 0.1

Number of exprimental groups; 1st sham, 2nd mice-mice allograft uterus transplantation to anterior abdominal wall, 3rd mice-mice allograft uterus transplantation to mesentery, 4th rat-mice xenograft endometrial transplantation of rat to anterior abdominal wall of mice, 5th rat-mice xenograft endometrial transplantation of rat to mesentery of mice. Data were presented as mean ± SD. * indicated $P < 0.05$ compared to sham group, indicated $P < 0.05$ compared to allograft mice-mice groups, indicated $P < 0.05$ compred to the primary size of endometrial lesions (3mm) prior to implantation, indicated $P < 0.05$ compared to the primary weight of endometriosis lesions (0.01 gr) prior to implantation. VEGF-A; vascular endothelial growth factor A, CA-125; cancer antigen 125, TNF-α; tumor necrosis factor α, IL-37; interleukin 37, NO; nitric oxide, MDA; Malondialdehyde, En; endometriosis.

Serum concentration of CA-125

CA-125 factor, which represented the cancer antigen in blood serum, was elevated in allograft and xenograft transplantations in comparison with control animals significantly ($p<0.05$). Also, no significant ($p>0.05$) alteration was found among rat-mice xenograft transplantation than allograft group (Table 1).

Inflammation status

Inflammation was assessed using peritoneal concentration of TNF-α and serum levels of IL-37 as crucial inflammatory markers in endometriosis. In whole groups of treatments (allografts and xenografts), serum levels of TNF-α were increased significantly ($p<0.05$) in peritoneal fluid than control group. Also, non-significant ($p>0.05$) differences were detected in xenograft animals than allograft mice regarding TNF-α concentration. Serum levels of IL-37 were increased in all animals of allograft and xenograft transplantations significantly ($p<0.05$) in comparison with control group. This serum value showed significant ($p<0.05$) incremental changes in rat-mice xenograft group in comparison with mice-mice allograft animals than allograft cases (Table 1).

Oxidative stress status

Concentration of all oxidative stress markers (NO and MDA) was increased significantly ($p<0.05$) in whole transplanted animals (both treatment groups of allografts and xenograft) than control group. No significant ($p>0.05$) alteration was found among the animals in xenograft group than allografts (Table 1).

Physical features

Following endometriosis induction, total body weight of recipient animals was decreased significantly ($p<0.05$) in 2nd (mice-mice allograft uterus transplantation to anterior abdominal wall) and 4th (rat-mice xenograft endometrial transplantation of rat to anterior abdominal wall of mice) groups of treatment in comparison with control group. In other transplanted groups (transplanted to serous mesentery layer), non-significant ($p>0.05$) changes in the field of animal`s weight were observed than control group. Also, the weight of endometrial lesions was increased significantly ($p<0.05$) in allograft and xenograft mice at the end of surgery than the primary weight (0.01 gr) at the start of induction. In this value, the animals in 4th group of treatment (rat-mice xenograft endometrial transplantation of rat to anterior abdominal wall of mice) showed a significant ($p<0.05$) increase in weight of endometrial lesions than animals with allograft transplantation. Significant ($p<0.05$) increase in diameter of lesions was found in whole animals with allograft and xenograft transplanted tissues than the primary size of lesions prior to transplantation. More obviously, the size of the transplanted endometrial lesion was increased significantly ($p<0.05$) in rat-mice xenograft transplantation to anterior abdominal wall than allografts (Table 1).

Histopathological variations

As it was depicted in Fig. 2, the endometrial grafts of rat (with dissected layers of myometrium and perimetrium) were stained with H&E staining prior to implantation. Obvious epithelium (purple arrow) with compact stromal tissue (green rectangular) were seen located on lamina properia layer (2A). Also, stroma had coronal sections of endometrial glands (2A, black circle). After implantation of mice uterus to abdominal wall of recipient mice, pathologic [illegible] arrow) had no villi, stroma (2B, green rectangular) was detected thin layer with multiple cavities, and

less number of coronal sections of blood vessels were found. Also, in Fig. 2B, the thick layer of myometrium and perimetrium were degenerated following uterus transplantation. These histopathological changes led to less secretion of pus in luminal space of endometrium, decreased size of grafts, and reduced weight of lesions after 4-week implantation (2B). Fig. 2C showed a complete section of rat-mice endometrial xenograft transplantation. Although the endometrial layer showed an irregular pattern than normal histology, thick and glandular epithelial formation caused the generation of a lumen full of secretion and haemorrhages (2C, yellow star). Outer part of this type of endometrial transplantation (xenograft rat-mice implantation) had a thick attachment layer as a common feature of endometriosis (2C) with many coronal sections of blood vessels (2C, red circle). There were many glandular sections (black circle) in thickness of stromal layer (2C, green rectangle), there were many glandular sections (black circle). Fig. 2D represented Perl`s staining in a part of connective tissue around endometrial lesions containing blood vessels. Black arrow (2D) showed macrophages loaded with hemosiderin following haemorrhages in endometrial lesions of xenograft transplantation to anterior abdominal wall.

HOX gene expression in uterus of recipient animals

Following induction of endometriosis in two recipient groups of 2 (mice-mice allograft uterus transplantation to anterior abdominal wall) and 4 (rat-mice xenograft endometrial transplantation of rat to anterior abdominal wall of mice), low decreased levels of HOXA10 and HOXA11 genes expression was found significantly ($P<0.05$). Also, in other treatment groups, no significant ($P>0.05$) alterations in genes expression of HOXA10 and HOXA11 were detected than β-actin baseline (Fig. 3).

Discussion

As human endometriosis causes irreversible complications such as permanent female infertility, thus a comprehensive study in the field of endometriosis and discovery of modern treatments seem necessary. For this purpose, application of animal endometriosis model for assessment of molecular pathways involved in this disease and innovation of modern treatments are crucial. The most common model of endometriosis is transplantation of endometrium to mesentery of recipient mice [5]. These mice to mice endometrial transplantations are practically difficult in two ways; it is impossible to separate the microscopic monolayer of endometrium, and thin mesentery layer as a background for endometrial implantation. Thus, in this pilot study, we particularly proposed a xenograft (rat to mice) model of endometriosis through implantation of endometrium to anterior abdominal wall. In this experiment, through exposure of adult female rats (with previous pregnancy experience) to males, estrogen production was stimulated, leading to increased thickness of endometrium. The recipient mice were also stimulated (using exposure to males) for estrogen production to achieve the transplantation of endometrial grafts. Finally, we examined all histological, biochemical, and genetic factors to confirm the correct and proper induction of endometriosis. Finally, we concluded that due to the estrous cycle and induction of estrogen production in donor rat body, it is possible to increase the thickness of the rat endometrium, which can be dissected easily. Implantation of these dissected lesions to the anterior abdominal wall of a recipient mouse can successfully mimic histological and serological changes similar to human endometriosis.

Two main factors, as the basic features of endometriosis, are; angiogenesis and inflammation. Through the inflammation process, the successful implantation of endometrial lesions occurs, and the angiogenesis process approves the viability and the growth of endometrial lesions. A successful inflammatory process can potentially guarantee the precise implantation of endometriosis lesions. Inflammation increases the capacity of lesion to attach to the peritoneal layer (in serous layer or abdominal wall) and increases the ability of endometrial cells to survive through induction of vessels formation. Macrophages located in the peritoneal layer are essential elements involved in onset of inflammation. With the permanent attachment of endometrium to peritoneal cells, macrophages begin to invade this area, leading to the release of several inflammatory factors. These inflammatory factors also enter into the peritoneal fluid; thus in addition to triggering the inflammation in other parts of abdomen or pelvis, these substances can be measured within the peritoneal fluid [6]. TNF-α is an important initiator of inflammation which is secreted by peritoneal macrophages into endometrial lesions and peritoneal fluid. It has been shown that the higher level of TNF-α in peritoneal fluid causes the severity of inflammation, which indicates a high level of endometriosis lesion attachment. As it was seen in this study, the level of TNF-α in peritoneal fluid was increased significantly in all transplanted groups. This incremental change indicated the successful existence of an inflammation model of endometriosis. Thus, allograft or xenograft endometrial transplantations can provide acceptable inflammation to initiate a successful endometriosis induction. In a meta-analysis study, Cao and coworkers investigated the association of TNF-α gene T-1031C polymorphism with endometriosis. As we found the high levels of TNF-α in this study, they also concluded that TNF-α is considered as an important factor in the advancement of endometriosis in females [7]. Although their study was conducted on human cases, the final result was parallel to our findings in which the peritoneal levels of TNF-α is increased in animals with endometriosis. TNF-α is an inflammatory cytokine secreted by peritoneal macrophages that induces proliferation, differentiation and migration of endometrial cells in endometrial pathophysiology. According to the in vitro studies, this cytokine stimulates the expression of matrix metalloproteinase. Functional studies in the field of endometriosis modelling also suggest the vital role of TNF-α in endometriosis progression. Thus, it was proposed that the blockage of TNF-α activity can potentially reduce development of endometriosis [8]. Chronic inflammation following endometriosis induction, in addition to affecting the abdominal and pelvic organs, can also alter inflammatory factors in the blood serum. IL-37 is one of the important blood factors that indicate the presence of inflammation in a part of the body. Although this cytokine is considered as an anti-inflammatory agent, but various studies reported high serum levels of IL-37 in endometriosis conditions. Through examination of the blood serum of transplant recipients, the serum level of IL-37 showed a significant increase in allograft and xenograft animals. Also, the level of this factor (IL-37) in the blood of xenograft transplant recipients was considerably higher than the allograft group. This difference approved the higher inflammation rate in xenograft implantation than allografts implantation. The levels of IL-37 in blood serum correlates with endometriosis severity [9]. Thus, as we found in this experimental study, high levels of IL-37 are more prominent in xenograft transplanted animals than other allografts. Jiang and coworkers introduced IL-37 as a biomarker for endometriosis diagnosis because they found that this factor is increased in endometriosis cases [10]. The study of inflammatory cytokines following endometrial induction confirmed the important role of TNF-α and IL-37 in inflammatory phase of endometriosis. Totally it can be concluded that inflammation as a crucial factor can be induced through this xenograft transplantation.

Angiogenesis is another critical factor in successful induction of endometriosis. In this study, the rate of angiogenesis was determined by two factors; VEGF-A and the number of blood vessel sections per unit area. This glycosylated mitogen has proliferative effects on endothelial cells leading to angiogenesis. Thus, also the VEGF-A is essential during organ remodelling, but this type of growth factor is widely involved in pathologic condition with the high rate of blood vessels formation (such as endometriosis). The presence of macrophages at the site of vascular lesion transplantation has been suggested as a source of VEGF-A secretion. Thus, it seems that the higher presence of macrophages in the tissue can lead to the higher probability of growth factor secretion and angiogenesis rate. Thus, through initiation of inflammation following transplantation of endometrium, the angiogenesis subsequently occurs following VEGF-A secretion by macrophages. In all lesions induced by allograft and xenograft transplantation methods, the levels of VEGF-A were increased in peritoneal fluid. This signalling factor was observed more prominent in both xenograft groups than allograft animals. In addition, the highest presence of VEGF-A in peritoneal fluid belonging to the xenograft group was found in the anterior abdominal wall transplantation. With the presence of increased levels of VEGF-A in peritoneal fluid, the rate of angiogenesis was also increased significantly around the lesions. This means that this growth factor by the effects on the vessels in the abdominal wall or mesenteric vessels induces their growth and accelerates the rate of angiogenesis. As it was obtained by microscopic examination, vessels sections in endometriosis lesions were significantly increased compared to the control group. Due to the tissue structure in the anterior wall of the abdomen, more blood flow and blood vessels were seen in this layer than the mesenteric vessels. This condition increased the rate of angiogenesis in endometrial lesions grafted to the anterior abdominal wall than implantation to mesentery. This study also observed that in the allograft and xenograft groups, the number of the blood vessel in tissue sections was much higher in grafts transplanted to the anterior abdominal wall than mesenteric sections. Based on the findings of biochemical and microscopic studies, it can be mentioned that in xenograft transplant model, a high rate of angiogenesis and factors involved in this process were observed. As Takehara and coworkers found, high levels of VEGF-A were also detected in peritoneal fluid. The results of Takehara`s study suggested that endometriosis may arise from eutopic endometrium with higher levels of angiogenic activity possibly induced by VEGF-A.

CA125 is one of the surface components of epithelia in female reproductive tract. This protein is highly glycosylated creating a hydrophilic barrier [11]. Cytoplasmic domain of CA125 interacts with cytoskeleton. In this case, it is affected from intracellular mechanisms and transmits extracellular signals to the intracellular matrix. CA125 has been shown to play a role in advanced tumorigenesis and tumor proliferation. This factor is considered a serum biomarker of endometriosis [12]. The expression of CA125 is altered in several types of cancers and endometriosis [13]. Since this protein is located on the cell surface of endometrial lesions, thus in high grades of endometriosis, serum levels of this protein had elevated concentration [14]. In the present study, significant accelerated serum levels of CA125 was also found in all cases of endometriosis (including allografts and xenografts). This finding showed that all implantations of endometrial lesions with a one-month duration of adaption could completely increase the serum levels of CA125 as a marker of endometriosis. CA125 also acts in cell-to-cell interaction for metastasis of cancerous tumors or proliferation of transplanted masses. This factor selectively binds to mesothelin (a glycoprotein expressed by mesothelial cells of peritoneum) to be implanted to peritoneal layer [15]. Rokhgireh et al. evaluated the probable role of CA125 as a non-invasive protocol for endometriosis diagnosis. They concluded that following endometriosis induction, high levels of CA125 in blood serum is also probable, which is parallel to our results [16].

Recent studies have focused on the role of oxidative stress on endometriosis pathophysiology. In oxidative stress condition, an imbalance among reactive oxygen species and antioxidants occurs causing a general inflammatory response in endometrial implanted region. Reactive oxygen species are intermediaries produced by normal oxygen metabolism and are physiological inflammatory mediators to modulate cell proliferation; besides, they have deleterious effects in uncontrolled activity or high concentration. The progression of endometriosis is clearly related to oxidative stress. The connection between endometriosis and ROS production is widely accepted and deeply studied [17]. In endometriotic cells, just similar to cancerous cells, increased ROS production is associated with an increase in cellular proliferation rate [18]. Peritoneal oxidative stress is a major constituent of endometriosis-associated inflammation regulating expression of numerous genes of immune-regulators, cytokines, and cell adhesion molecules which are necessary for ectopic endometrial lesions progression. Peritoneal aggregation of macrophages is at a higher rate in women with endometriosis. Macrophages release prostaglandins, cytokines, and growth factors. In the present study, we found high levels of NO in peritoneal fluid. Osborn et al. assessed the expression of nitric oxide synthase by peritoneal macrophages in endometriosis-associated infertility. The higher levels of NOS2 and subsequently higher NOS enzyme activity in these cases. Osborn concluded that in addition to the effects of NO on reproduction, peritoneal NO levels could potentially serve inflammation in women with endometriosis [19]. Also, oxidative mechanisms involving LDL oxidation are higher in women with endometriosis. In this process, oxidation of polyunsaturated fatty acid-containing lipids of the lipoprotein occur. MDA, as a marker for oxidative stress, results from lipid peroxidation of polyunsaturated fatty acids. Reactive oxygen species degrade polyunsaturated lipids, forming MDA. This compound is a reactive aldehyde and is one of the many reactive electrophile species causing detrimental cellular stress. Thus, the level of MDA production is considered as a biomarker to measure the level of oxidative stress in peritoneal cavity following endometriosis induction. In the present study, we also detected high levels of MDA in peritoneal fluids of cases of allograft and xenograft endometriosis. We found that animals with endometriosis have enhanced oxidative stress indices including NO and MDA.

According to the published articles, the physical changes following endometriosis induction are probable in humans, such as decreased total body weight, increase in size of endometriosis lesions, and weight gain of endometrial lesions. In this study, total body weight of mice showed a significant decrease in animals with transplanted endometrial lesions to anterior abdominal wall than non-endometriosis animals or even in comparison with the animal groups with transplanted endometrial lesions attached to mesentery. Amaral et al. developed an experimental model of endometriosis in rats. They implanted total sections of uterus to abdominal wall of rats. In their allograft transplantation, they found non-significant differences in the total weight of animals [20]. In contrast, we found weight loss in allograft and xenograft transplantations to the abdominal wall of mice than transplantation to mesentery layer. It seems that by attachment of endometrial lesions to the anterior wall of abdomen, the processes of inflammation and angiogenesis initiate entirely leading to the total growth of lesions. Finally, these molecular mechanisms caused animal weight loss in unknown mechanism. In addition, by microscopic and macroscopic examinations of endometriosis lesions, it was found that angiogenesis occurs in lesions attached to the anterior abdominal had more blood supply than the lesions attached to the mesentery. This finding probably led to complete growth of lesions attached to anterior abdominal wall through proper blood supply leading to increased size and weight of lesions than other animals with endometrium attached to the mesentery. The weight of endometrial modelled lesions in all groups showed weight gain compared to the initial size prior to transplantation. Meanwhile, in the xenograft endometrium attached to the anterior

abdominal wall, the increase in size of endometrial lesions was at a maximal level compared to other treated groups. According to these findings, it is concluded that by implantation of endometrial xenograft lesions to the anterior wall of the abdomen, the larger endometrial masses with more weight and pus secretions can be modelled, all of which physical features are crucial factors similar to endometriosis in humans.

A successful endometriosis transplantation should ultimately lead to the production of an endometriosis lesion with specific histopathological features. All-important histopathological factors following endometriosis induction are related to the changes in epithelium, stroma, endometrial glands, surrounding connective tissues, blood vessels, serous or pus discharge into the lumen of endometriosis cavity, haemorrhage, and presence of hemosiderin disposition. Epithelium in normal rat endometrial grafts prior to transplantation was regular with elongated columnar cells. Thick stroma also contained numerous coronal sections of endometrial glands (2A) which is responsible for secretion of pus or serous liquid by endometrium. After transplantation of this xenograft tissue to the anterior abdominal wall of recipient mice (2C), the normal and physiological arrangement of epithelium was disrupted, and the pathological form was obtained, including irregular epithelium formation and non-columnar epithelial layer. But in this way, high pus secretion still remained in xenograft transplantation to anterior abdominal wall animals. It showed that, although there were various pathological changes in endometrial glands in stroma of lesions, but the pus secretion continued. Besides, probably this deformed epithelium caused high levels of CA125, as a cell surface marker, in blood serum. High proliferation rate in stromal cells also covered most of the thickness of grafts with different irregular sections of endometrial glands. In allograft endometriosis lesions (2B), a thin layer of primitive and immature epithelium and a thin and degenerated stroma were detected. As it was depicted in Fig. 2B, shrinkage stroma following uterine allograft transplantation contained numerous gaps instead of typical endometrial glands. This pathologic degenerated formation of allografts (2B) caused generation of a lumen of endometrial lesions with no pus or serous secretions in almost all cases. Growth of endometrial glands (2C) resulted in production of a large space containing multiple purulent secretions (2C, yellow star). While the analysis of epithelium and stroma in allograft lesions (2B) showed absence of purulent discharge into the cavity of endometrial lesions. In some cases, serous discharge was seen in a low inseparable amount. Examination of the outer layer of grafted specimens (2C) showed that transplantation of xenograft tissues to anterior wall of abdomen could induce proliferation of connective tissue in the form of scars or extensive dissectible adhesions. This connective tissue (2C) had many blood vessels supplying the nutrition and oxygen for lesion growth. While in the allograft groups, although the uterus was transplanted with all layers (endometrium, myometrium, and perimetrium), but the myometrium and perimetrium layers were degenerated. Besides, although myometrium and perimetrium layers were dissected in xenograft transplantation, but extensive outer connective tissue was generated. Presence of blood vessels in connective tissue (2C) led to vast haemorrhage in endometriosis lesions which were detected by Perl staining. As shown in Fig. 2D, following haemorrhage in endometriosis lesions, the macrophages were activated to regulate RBC phagocytosis. During this stage, hemosiderin granules were deposited in macrophages which were detected by pearl staining against Fe atoms. This feature, which is seen only in xenograft endometriosis lesions attached to the anterior abdominal wall, was a prominent feature of successful induced endometriosis lesions. Finally, it was concluded that based on the histopathological findings, the xenograft endometrial lesions transplanted to anterior abdominal wall seems more resemble the morphology of endometriosis in human.

In high grades of endometriosis, the gene expression of HOX in uterus could be altered. HOXA10 and HOXA11 are homeobox genes that act as transcription factors essential to embryonic development. The role of each of these two genes in regulation of endometrial development was approved. Both HOXA10 and HOXA11 are essential for embryo implantation and appear to play a similar role in women [21]. Aberrant HOX gene expression following endometriosis contribute to the aetiology of female infertility in these patients. Thus it was approved that high grade and chronic presence of endometrial lesions can finally lead to changes in morphology and molecular characteristics of endometrium in uterus. In the present study, gene expression of HOXA10 and HOXA11 in uterus tissue were decreased in all allograft and xenograft transplanted to the anterior abdominal wall. This difference was more prominent in xenograft transplantation than allografts. Altered gene expression in uterus is considered as the main factor in the end stage of endometriosis pathology. As we found in the present study, genes expression of HOXA10 and 11 were altered, which represented successful implantation of endometrial lesions and endometriosis induction in xenograft transplantations.

Conclusions

This study aimed to determine the most similar model of animal to human endometriosis regarding molecular, histological, and genetic characteristics. Main modifications to accelerate the success rate of xenigraft implantation were; previous pregnancy experience and exposure to male animals for two weeks. These important conditions led to increased levels of estrogen and high thickness of endometrium for proper dissection. In this study, blood and peritoneal factors affecting inflammation and angiogenesis, physical characteristics of lesions, oxidative stress levels, histopathological changes, and alteration in genes expression of uterus were analyzed and compared. Endometrial lesions isolated from stimulated uterus of a rat and implanted in the anterior abdominal wall of mice in a xenograft surgery could potentially be an appropriate option for endometriosis modelling in rodents. In this transplantation, the inflammatory process occurred, which led to a deep change in all inflammatory factors and oxidative stress in the peritoneal cavity. Subsequently, angiogenesis occurred with emergence of different layers of blood vessels to supply nutrition and oxygen to the implanted lesions. Thus, the lesions showed a higher growth rate. All these changes led to the histopathological alteration of lesions in which the initial and physiological form of lesions prior to implantation was shifted to the pathological form. At this stage, the lesion grew, scar tissue and adhesions appeared, and its lumen was filled with purulent or serous secretions. Eventually, this xenograft modelling also affected the uterine endometrium regarding the HOXA11 and HOXA12 genes expression. The whole above findings confirmed that xenograft transplantation from rat endometrium to the anterior abdominal wall of mice can be considered a proper model for endometriosis in rodents. In this study, we evaluated the morphometrical, histological and genetic features of implanted endometrial lesions, followed by some laboratory examinations to confirm this type of modelling accuracy. Application of more laboratory protocols and comparison with actual human samples were important limitations we faced to. For future studies, conduction of high levels of laboratory assays like immunoassay and protein immunoblotting is strictly recommended. Also, the use of a human endometriosis sample to compare with rodent`s endometriosis lesions seems necessary to upgrade the level of study.

Declarations

AUTHORS' CONTRIBUTIONS

M.B conceived and designed the study, supervised the data collection, interpreted the results, and revised the manuscript. AA conducted the experimental procedures, data collection analysis, and manuscript preparation. C.J was a scientific advisor for conduction of laboratory analysis. K.M was a scientific advisor in the filed of angiogenesis pathways and animal disease modelling. All authors read and approved the final manuscript prior to submission.

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AVAILABILITY OF DATA AND MATERIALS

The datasets used and analyzed for this are available from the corresponding author upon reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All assessments were conducted on in accordance with ethical principles and under the supervision of the University's Ethics Committee (Ethic NO. IR.KUMS.REC.1399.994). The study was reviewed by the appropriate ethics committee and had been performed in accordance with the ethical standards described in an appropriate version of the 1975 Declaration of Helsinki, as revised in 2000.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

References

1. Taylor HS, Kotlyar AM, Flores VA (2021) Endometriosis is a chronic systemic disease: clinical challenges and novel innovations. The Lancet 27(10276):397. 839 – 52. https://doi.org/10.1016/S0140-6736(21)00389-5
2. Arafah M, Rashid S, Akhtar M (2021) Endometriosis: A Comprehensive Review. Ad Ana Pathol 6(1):30–43. https://doi.org/10.1097/PAP.0000000000000288 28) .
3. Whitten WK (1956) Modification of the oestrous cycle of the mouse by external stimuli associated with the male. J Endocrinol 1:13(4):399–404. https://doi.org/10.1677/joe.0.0130399
4. May KE, Conduit-Hulbert SA, Villar J, Kirtley S, Kennedy SH, Becker CM (2010) Peripheral biomarkers of endometriosis: a systematic review. Human Reprod update 1(6):651–674. https://doi.org/10.1093/humupd/dmq009 16) .
5. Laganà AS, Garzon S, Franchi M, Casarin J, Gullo G, Ghezzi F (2018) Translational animal models for endometriosis research: a long and windy road. An Trans Med 6(22). https://doi.org/10.21037/atm.2018.08.24
6. Samimi M, Pourhanifeh MH, Mehdizadehkashi A, Eftekhar T, Asemi Z (2019) The role of inflammation, oxidative stress, angiogenesis, and apoptosis in the pathophysiology of endometriosis: Basic science and new insights based on gene expression. J Cell Physiol 234(11):19384–19392. https://doi.org/10.1002/jcp.28666
7. Cao XL, Chai J, Yu YY, Tian X, Zhao JY, Yu LY, Sun ZG (2020) Association of TNF-α gene T-1031C polymorphism with endometriosis: A meta-analysis. Am J Reprod Immunol 84(6):e13305. https://doi.org/10.1111/aji.13305
8. Zhou HE, Nothnick WB (2005) The relevancy of the matrix metalloproteinase system to the pathophysiology of endometriosis. Front Biosci 1:10: 569–575. https://doi.org/10.2741/1552
9. Kaabachi W, Kacem O, Belhaj R, Hamzaoui A, Hamzaoui K (2017) Interleukin-37 in endometriosis. Immunol Lett 1: 185: 52 – 5. https://doi.org/10.1016/j.imlet.2017.03.012
10. Jiang J, Jiang Z, Xue M (2019) Serum and peritoneal fluid levels of interleukin-6 and interleukin-37 as biomarkers for endometriosis. Gynecol Endocrinol 3(7):571–575. https://doi.org/10.1080/09513590.2018.1554034 35) .
11. Perez BH, Gipson IK (2008) Focus on molecules: human mucin MUC16. Exp Eye Res 87(5):400. https://doi.org/10.1016/j.exer.2007.12.008

12. Blalock TD, Spurr-Michaud SJ, Tisdale AS, Heimer SR, Gilmore MS, Ramesh V, Gipson IK (2007) Functions of MUC16 in corneal epithelial cells. Invest Ophthal Vis Sci 1(10):4509–4518. https://doi.org/10.1167/iovs.07-0430 48) .
13. Bafna S, Kaur S, Batra SK (2010) Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells. Oncogene 29(20):2893–2904. https://doi.org/10.1038/onc.2010.87
14. Barbieri RL, Niloff JM, Bast RC Jr, Schaetzl E, Kistner RW, Knapp RC (1986) Elevated serum concentrations of CA-125 in patients with advanced endometriosis. Fertil Steril 1(5):630–634. https://doi.org/10.1016/S0015-0282(16)49333-7 45) .
15. Kaneko O, Gong L, Zhang J, Hansen JK, Hassan R, Lee B, Ho M (2009) A binding domain on mesothelin for CA125/MUC16. J Biol Chem 6(6):3739–3749. https://doi.org/10.1074/jbc.M806776200 284) .
16. Rokhgireh S, Mehdizadeh Kashi A, Chaichian S, Delbandi AA, Allahqoli L, Ahmadi-Pishkuhi M, Khodaverdi S, Alkatout I (2020) The Diagnostic Accuracy of Combined Enolase/Cr, CA125, and CA19-9 in the Detection of Endometriosis. BioMed Res Int 2: 2020. https://doi.org/10.1155/2020/5208279
17. Vitale SG, Capriglione S, Peterlunger I, La Rosa VL, Vitagliano A, Noventa M, Valenti G, Sapia F, Angioli R, Lopez S, Sarpietro G (2018) The role of oxidative stress and membrane transport systems during endometriosis: a fresh look at a busy corner. Oxid Med Cell long 21: 2018. https://doi.org/10.1155/2018/7924021
18. Ngô C, Chéreau C, Nicco C, Weill B, Chapron C, Batteux F (2009) Reactive oxygen species controls endometriosis progression. Am J Pathol 1(1):225–234. https://doi.org/10.2353/ajpath.2009.080804 175) .
19. Osborn BH, Haney AF, Misukonis MA, Weinberg JB (2002) Inducible nitric oxide synthase expression by peritoneal macrophages in endometriosis-associated infertility. Fertil Steril 1(1):46–51. https://doi.org/10.1016/S0015-0282(01)02940-5 77) .
20. Amaral VF, Dal Lago EA, Kondo W, Guarita-Souza LC, Francisco JC (2009) Development of an experimental model of endometriosis in rats. Revista do Colegio Brasileiro de Cirurgioes 36(3):250–255. https://doi.org/10.1590/S0100-69912009000300012
21. Nazarenko TA, Kalinina EA, Knyazeva EA, Kiselev VI, Smolnikova VY, Sukhikh GT (2019) The role of abnormal hypermethylation of the HOXA10 and HOXA11 promoters in implantation failures in IVF programs. Gynecol Endocrinol 31(sup1):31–34. https://doi.org/10.1080/09513590.2019.1632087 35) .

Figures

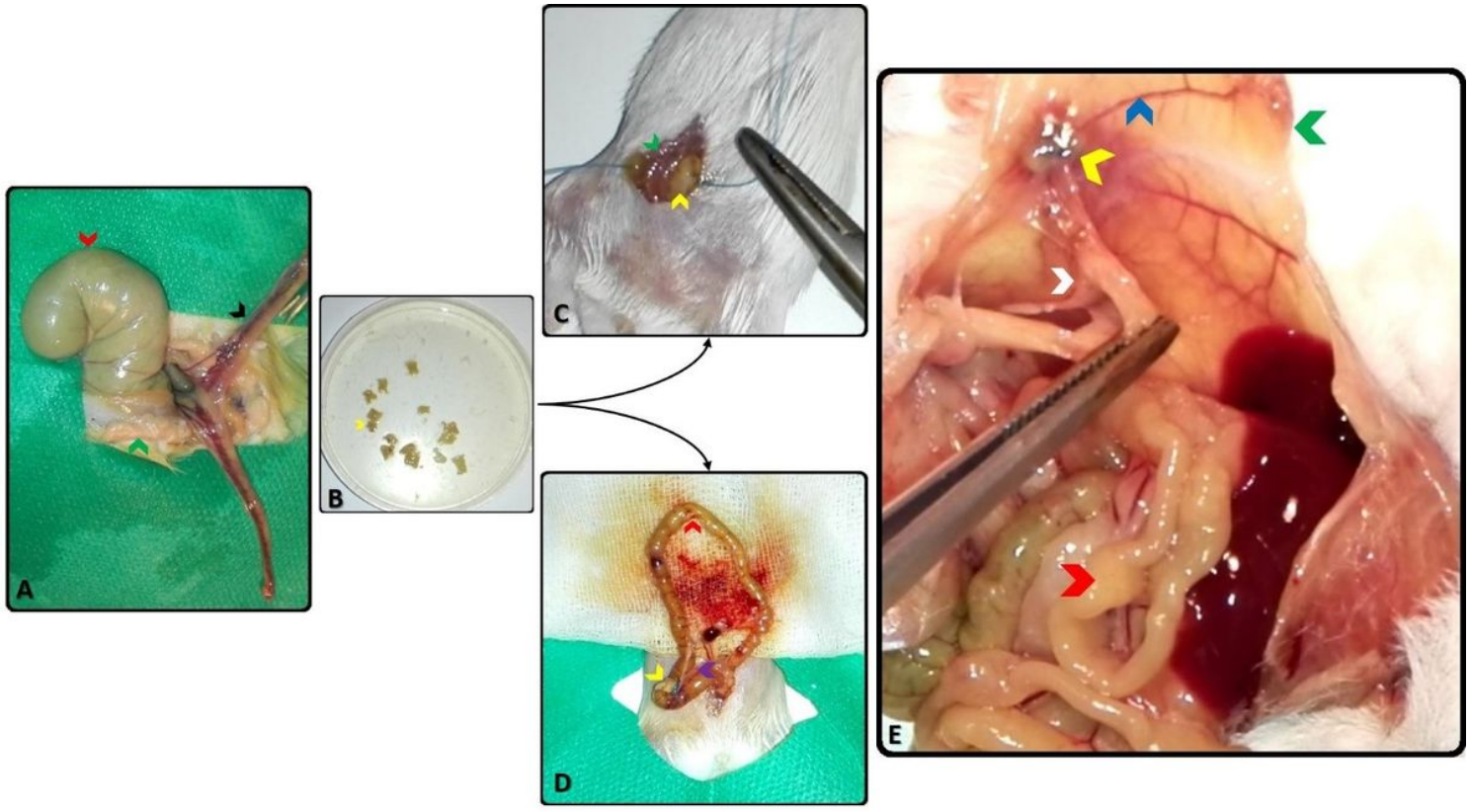

Figure 1

Rat-mice xenograft transplantation of endometrial segments (A and B) to abdomonal wall (C) and small intestine mesentery (D) transplantation for endoemtriosis induction (E). Arrows represent; intestine (red), uterus horn (black), abdominal wall (green), angiogenesis (blue), mesentery artey (purpale), endometrial lesions (yellow), and endometrial tissue attachemnts (white)

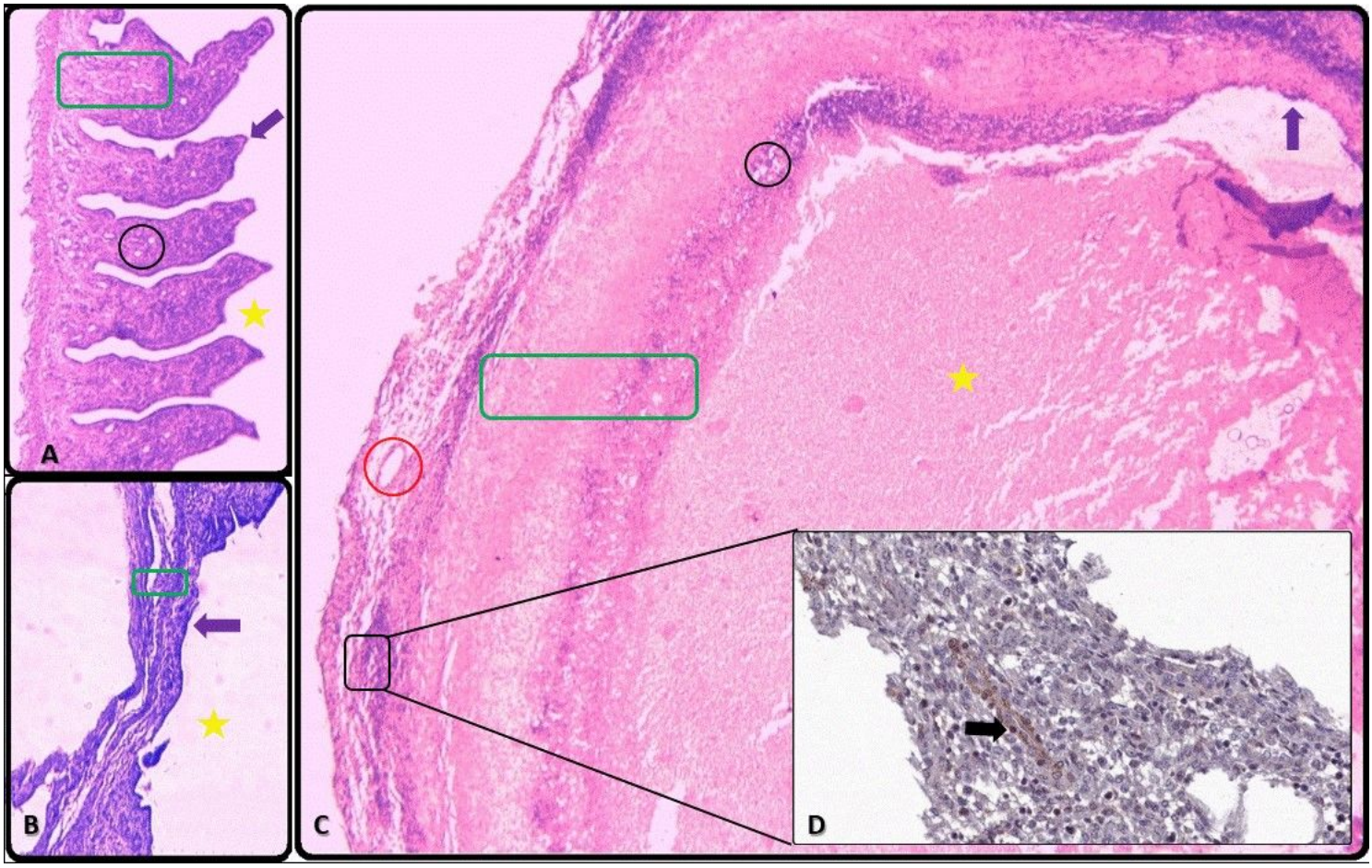

Figure 2

Histological H&E sections of thikened endometrium prior to implantation (A), allograft mice-mice tuerus transplantation (B), xenograft rat-mice endometrium transplantation (C), and Perl

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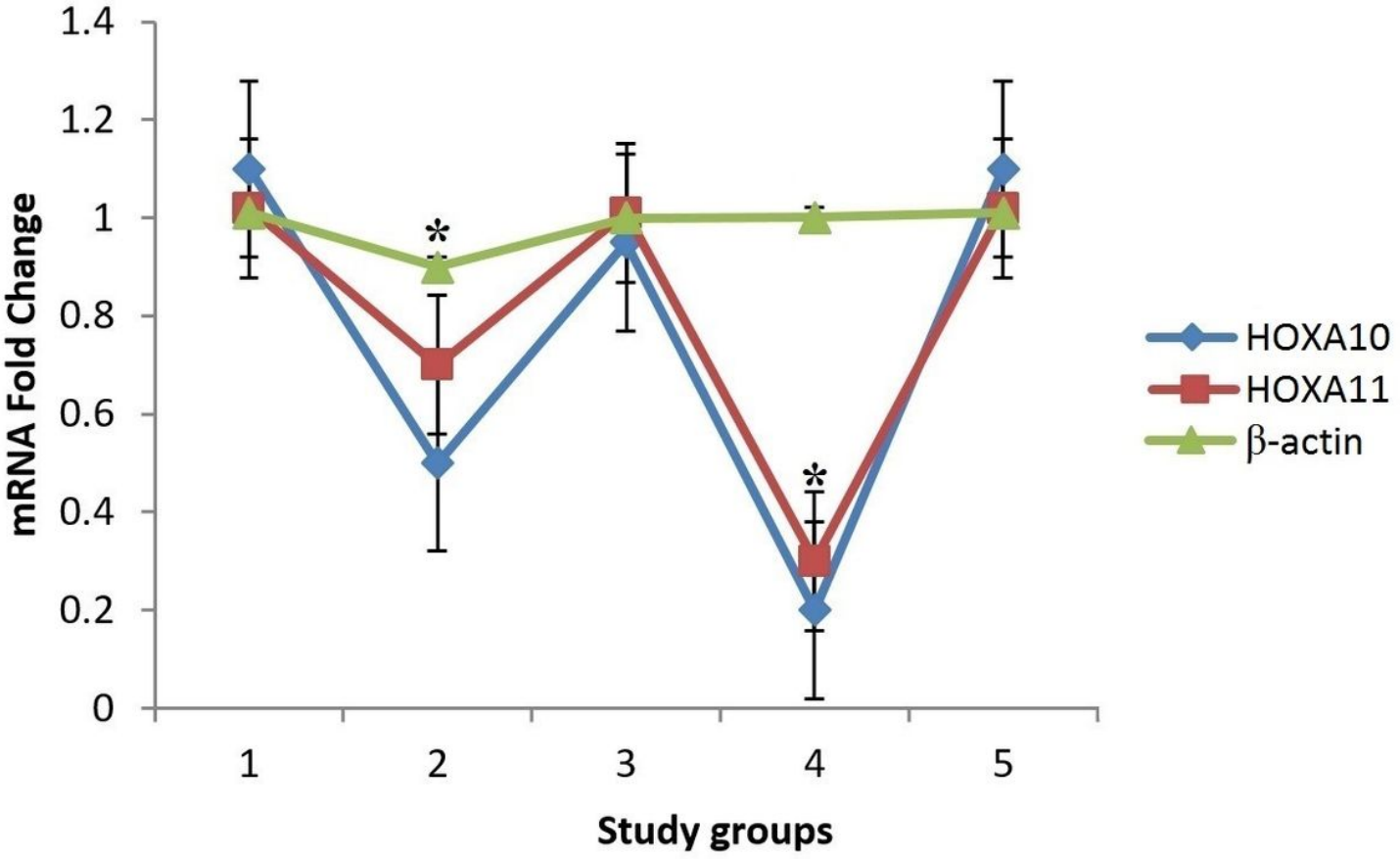

Figure 3

Rate of genes expression in uterus of graft-recipient animals. Number of exprimental groups; 1st sham, 2nd mice-mice allograft uterus transplantation to anterior abdominal wall, 3rd mice-mice allograft uterus transplantation to mesentery, 4th rat-mice xenograft endometrial transplantation of rat to anterior abdominal wall of mice, 5th rat-mice xenograft endometrial transplantation of rat to mesentery of mice. Data were presented as mean ± SD. * $P < 0.05$ compared to β-actine baseline