

Original article

Histological Evaluation of Implantation Deficit in Experimentally Induced Endometriosis Model of Rats

Özlem YILMAZ DİLSİZ¹, Murat ULUKUŞ², Eser SÖZMEN³, Utku ATEŞ¹, Mine ERTEM¹

¹ Ege University Faculty of Medicine, Department of Histology and Embryology, İZMİR

² Ege University Faculty of Medicine, Department of Obstetrics and Gynaecology, İZMİR

³ Ege University Faculty of Medicine, Department of Biochemistry, İZMİR

ÖZET

Deneysel olarak indüklenmiş sıçan endometriyozis modelinde implantasyon kusurunun histolojik değerlendirilmesi

Giriş: Deneysel hayvan modelleri insan endometriyozisinin anlaşılabilmesi için önemlidir.

Amaç: Deneysel endometriyoziste implantasyon kusuruna etkili faktörlerin tanımlanması amacıyla yapılmıştır.

Materyal ve Metot: Cerrahi olarak tetiklenmiş endometriyozis (n=20), sham operasyon (n=10) ve opere olmamış kontrol grubu (n=10) olmak üzere 3 gruba ayrılan deneklerde desidua oluşumu tespit edilerek, rutin ışık mikroskopik, elektron mikroskopik ve immünohistokimyasal tekniklerin yanı sıra histolojik, morfolometrik ve biyokimyasal teknikler ile değerlendirildi.

Bulgular: Cerrahi olarak tetiklenmiş endometriyozis grubunda desidualizasyon ve pinopod ekspresyonu göreceli olarak azalmış ve yeterli endometrial morfolojik bulguların izlendiği hallerde her zaman normal endometrial reseptivitenin oluşamayabileceğini göstermektedir. Her ne kadar mitotik figürler tanımlanmış olsa da, deney grupları arasında istatistiksel olarak anlamlı, morfolometrik kalınlık farklarının bulunduğu gösterilmiştir.

Sonuçlar: Alternatif yollar ile endometrial reseptivite düzenlenirken, uterin reseptivite problemleri varlığını sürdürebilmektedir. Buna ek olarak, immünohistokimyasal olarak gruplar arasında anlamlı fark göstermeyen belirteçlerin varlığı, bu belirteçlerin sadece uterin reseptivitenin tanımlanması için uygun olmadıklarını gösterebilir. Yine de morfolometri ile elde edilmiş olan anlamlı bulguların gelecekteki ultrasonografik çalışmalara kaynaklık edebileceği göz ardı edilmemelidir.

Anahtar Kelimeler: Cerrahi olarak tetiklenmiş deneysel endometriyozis; embriyo implantasyonunun baskılanması; pinopod; uterin reseptivite; morfolometri; sıçan

ABSTRACT

Background: Experimental animal models are important in understanding human endometriotic disease.

Aims: This study aims to describe factors influencing the implantation deficit in experimental endometriosis.

Material and Method: Decidua reaction were detected in surgical endometriosis induction group (n=20), sham operated (n=10) and non-operated control (n=10) groups and studied with routine light microscopic, electron microscopic and immunohistochemical techniques as well as histologic, morphometric and biochemical analysis.

Results: Decidualisation and pinopode expression of eutopic endometrium in surgically induced endometriosis relatively decreased and adequate endometrial morphology may not always display normal endometrial receptivity. Although mitotic figures were described, study groups revealed statistically significant thickness differences in morphometry.

Conclusions: Uterine receptivity defects are regulated differently while other alternative mechanisms effecting endometrial receptivity sustained. In addition, insignificant differences of immunohistochemical expressions are non-descriptive due to the antibodies used for uterine receptivity in experimental endometriosis. Yet, morphometry may pioneer novel ultrasonographic modules in future.

Key Words: Surgically induced experimental endometriosis; implantation defects; pinopodes; uterine receptivity; morphometry; rat

INTRODUCTION

Endometriosis is a disorder in which abnormal growths of tissue, histologically resembling the endometrium, are present in locations other than the uterine lining¹. It is present in 3-10% of women in the reproductive age and 25-35% of infertile women^{2,3}.

Experimental animal models play an important role in understanding the nature of the disease.

Invasive properties, decreased apoptosis, alterations in expression of specific gene and proteins, and increased production of steroid and cytokines were described in eutopic and ectopic endometrium⁴⁻¹³.

Furthermore, significant biochemical differences exist even between ectopic and autologous eutopic endometrium. Yet differences in pregnant eutopic endometrium and regular endometrium has not been demonstrated before¹⁴. Pregnancy results in a change in number and function of immune cells which potentially affects fetal survival and status of postpartum uteri. These

Correspondence:

Özlem Yılmaz DİLSİZ M.D.
Ege University Faculty of Medicine Department of Histology and Embryology, İzmir
e-mail: ozlem.yilmaz.dilsiz@gmail.com
Arrival date : 17.09.2012
Acceptance date : 04.10.2012

changes are driven both by the conceptus and the maternal-placental system¹⁵.

Women with idiopathic recurrent pregnancy loss have been categorized on the basis of their endometrial response in the peri-implantation period¹⁶. A possible association between endometriosis and spontaneous abortion has been suggested in mostly uncontrolled and/or retrospective studies, most of which lack evidence in this field¹⁷⁻¹⁹. Therefore, other studies to determine pregnancy loss in endometriosis via immune response mechanisms or optimal immune functions^{20,21} are needed.

A rat model of surgically-induced endometriosis was developed about 20 years ago, where uterine fragments are auto-transplanted on the mesenteric arteries supplying the bowels or the abdominal viscero-peritoneum^{22,23}. This study was originally designed to assess histological and biochemical alterations in experimental endometriosis on non-implanted areas of eutopic endometrium for fertility. Histological evaluation has been accomplished via histochemical determination of stromal elements as well as ultrastructural analysis and immunohistochemistry of cellular intermediate filament elements like *Desmin*; stromal connective tissue marker like *Fibronectin*; oxidative stress determinants like *iNOS* and *eNOS*; and immunoreactivity of anti-apoptotic elements like *Anti-Bcl-2*. Accordingly, this is the first 'morphometric histological study' of experimental endometriosis model in rats as far as we know. The aim of this study is to investigate the role of an autograft of the uterine horn; assumed as endometriotic model, has an impact on eutopic endometrium considering the decidualisation and implantation processes.

MATERIALS AND METHODS

Animals and experimental design

All the animals were kept in the same laboratory conditions of temperature (25±2°C) and light (14:10 h light:dark cycle), and were fed *ad libitum* with chow and water. The experimental protocol was approved by the Local Animal Care Committee (2006/46). Experimental study groups consists of surgical endometriosis induction group (n:20), sham operated (n:10) and non-operated control (n:10) groups of 4-6 months old female albino (Wistar) rats (180-200g), housed as four rats per cage.

Induction of experimental endometriosis

Endometriosis was surgically induced by transplanting autologous endometrium as previously proposed by Vernon and Wilson²².

Briefly, each rat was anesthetized with intraperitoneal injection of a mixture of Ketamine

(0.15 mg/100 g body weight, Alfamine®, Ege Vet, Izmir, Turkey) and Xylazine (0.02 ml/100 g body weight, Alfazyne®, Ege Vet, Izmir, Turkey). A 5cm vertical midline incision was made and both uterine horns were exposed. A distal segment (~1cm) was resected and placed in phosphate-buffered saline (PBS). Longitudinally, a 5 mm piece was sectioned and transplanted onto the inner surface of the right abdominal wall with the serosal surface apposed and secured with single Polyglactin 4/0 suture (Vicryl®, Ethicon Inc, USA).

The abdomen was closed with 4/0 Polyglactin suture for peritoneum-fascia and skin. All surgeries were performed by the second author. The rats were individually caged afterwards for 6 weeks to recover^{22,24}. No antibiotherapy was required.

Daily vaginal smears were performed to ensure the permanence of the estrous cycle. The female rats without normal periods were excluded²⁵. All groups were induced for ovulation²⁶. Six weeks after the first surgery, couples were mated. The day of mating was termed day 0.5. In result, non-pregnant females were accepted as foster-surrogate mothers. On day 11.5, the animals underwent on exploratory laparotomy, during which implants were evaluated for acceptance/rejection. Ectopic uterine tissue were photographed and eutopic uteri were excised, then all subjects were sacrificed.

No surgery was applied in controls while in sham, abdomen was opened and then sutured accordingly in laparotomy. Sham and controls were also mated and induced for ovulation likewise²⁶.

Histology

Tissue processing and histocytochemical technique

Uterine samples were fixed in cold Cacodylate-buffered Paraformaldehyde+Glutaraldehyde solution. After fixation, frozen tissue were stained with Gomori's Trichrome stainings for stromal evaluation. All histological procedures applied in this study are conventional and well-established methods²⁷.

Tissue processing and Application of immunohistochemistry

Paraffin wax blocks were cut in 3µm thickness and immunoreactivity of **Desmin** (Desmin Ab-1 Neomarkers, Fremont, CA; prediluted); **Fibronectin** (polyclonal rabbit anti-human Fibronectin, Dako, USA; 1:100); **iNOS** (Zymed, South San Francisco, CA; 1:100); **eNOS** (Zymed, South San Francisco, CA; 1:1000); and **Anti-Bcl-2** (Neomarkers, Fremont, CA; 1:100) were analyzed under light microscope (Olympus BX-51) particularly for description of the eutopic endometrial status whilst implantation and inflammation.

For this, slides were pretreated with 2% Trypsin and endogenous peroxidase activity were inhibited in 3% H₂O₂. The sections were exposed to the primary antibodies overnight and then the secondary antibody (Zymed Laboratories, USA) were added. Then sections were incubated with the enzyme complex (Zymed Laboratories, USA) and DAB-substrate with HRP-conjugated antibodies (Zymed Laboratories, USA) were applied. Next, sections were counterstained and mounted.

Table 1. Distribution of Bcl-2, iNOS, eNOS, Desmin and Fibronectin Immunoreactivity

Antibodies/Groups	Control	Sham	Experimental Endometriosis
bcl-2	Endometrium + Decidua +	Endometrium + Decidua +	Endometrium +++ Decidua ++
iNOS	Endometrium ++ Decidua +	Endometrium +++ Decidua ++	Endometrium +++ Decidua +++
eNOS	Endometrium + Decidua +/-	Endometrium ++ Decidua +	Endometrium +++ Decidua +++
desmin	Endometrium + Decidua ++	Endometrium ++ Decidua +	Endometrium +++ Decidua +++
fibronectin	Endometrium ++ Decidua +	Endometrium + Decidua +	Endometrium +++ Decidua ++
The intensity of immunostaining is grouped in the following categories: - no staining; + weak staining; ++ moderate staining; +++ strong staining			

Histologic and morphometric analysis

In histological assessments, specimens were analysed for cellular integrity and mitosis as well as pinopode development via light and electron microscopy. For morphometry, random areas were captured via light microscope integrated colour digital camera (Olympus C-5050). Images were then saved onto Image-Pro Express (Media-Cybernetics, 2002, USA) image analysis software loaded computer. Two investigators, blinded to the groups, performed 3-5 measurements from 10 non-consecutive sections under 40x objectives. Mucosal depth was measured as the thickness of endometrial lining. The endometrial surface and glandular epithelium depths were measured from the luminal side to the basal lamina. Numeric variables were analysed statistically for normal distribution in each group with Shapiro-Wilk test.

Normally distributed endometrial surface depths were compared with One-way ANOVA while Dunnett T3 was used as a Post Hoc test for non-homologous variants. Abnormally distributed glandular epithelium depths and myometrial thickness were compared in control, sham and experimental groups with Kruskal Wallis and dual comparisons were analysed with Mann-Whitney U test. All hypothesis were analysed using SPSS (Statistical Package for the Social Sciences) for Windows version 17.0 and significance was defined as a *P* value of <0.05.

Immunohistochemical evaluation and analysis

Two sections per each animal and ten random fields per slide were analysed in all study groups in order to identify the areas bearing the densest immunoreactivity. Only the cytoplasmic immunostaining was considered as positive for Desmin, bcl2, iNOS and eNOS (endothelial cells) while membranous immunostaining was considered as positive for bcl-2 and immunostaining of extracellular matrix and connective tissue was considered as positive for iNOS and Fibronectin.

Transmission electron microscopic (TEM) procedures

Ultrastructurally, tissues were fixed, washed in Cacodylate buffer (0.1M) and then postfixed in Osmium Tetraoxide+Cacodylate-buffer solution (1:1). Afterwards, routine protocols were used for epon embedding and then semithine sections were stained in toluidine blue and ultra-thin sections were taken onto 3,05 mm; G2300C Square mesh Copper grids (Agar)²⁷.

Image analysis and photography were made with a Zeiss LIBRA 120 TEM and attached digital camera.

Biochemical analysis

Development of oxidative injury was estimated by TBARS, SOD and CAT levels in uterine homogenates. The statistical significance was determined by Kruskal Wallis test and significance was defined as a *P* value of <0.05.

Preparation of tissue homogenates and analytical methods

Samples were homogenized in cold phosphate-buffer (1/10; 0.5M, pH=7.0). The homogenates were centrifuged at 700xg.

Quantification of TBARS

A colorimetric assay according to the protocol by incubation with TBARS solution (0.12M TBA in 15%TCA and 1%HCl) previously used by Sozmen et al.²⁸ were applied and results were calculated as nmol/mg protein using standard curve.

Measurement of SOD activity

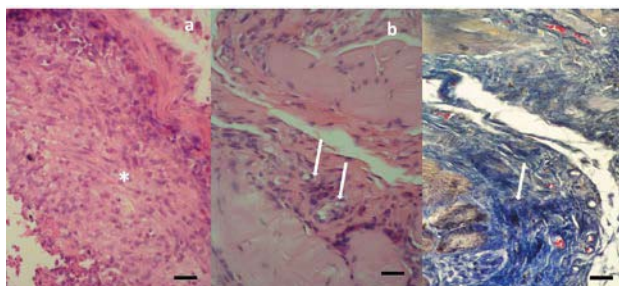
SOD activities were measured based on the inhibition of epinephrine autoxidation by SOD at 480 nm with a LKB Ultraspec-2-spectrophotometer (LKB Biochrom Ltd, Cambridge, England)²⁸. The assay was calibrated using purified SOD, and one unit of enzyme was defined as the amount required for inhibiting 50% of the autoxidation.

Measurement of CAT activity

CAT activities were determined as described previously²⁸. The degradation of H₂O₂ is recorded at 240 nm. One unit of CAT was defined as the amount of enzyme which decomposed 1 µmol H₂O₂/min under specific conditions.

RESULTS

In the exploration laparotomy, viable endometrotic implants were recognised as hemorrhagic liquid filled, cystic structures. Histologic assesment of endometriotic implants were not considered necessary (Suppl Figure 1).



Suppl Figure 1. Peritoneal endometrial implants exposed at laparotomy after first induction surgery

Light microscopic evaluation

Relatively decreased decidual reaction which revealed itself like regressed stromal proliferation by means of increased fiber synthesis and transmission of large epitheloid cells in endometrial stroma were detected in eutopic endometrium with light and electron microscopy compared to other groups. Due to changes in synthesis of fibers and stromal cells, Trichrome staining revealed cellular decrease in tissue thickness and stromal angiogenetic groups were demonstrated with both light and electron microscopy. In especially experimental endometriosis group (Figure 1), mitotic figures and significant thickness differences (for endometrium surface epithelium (Figure 2); endometrial surface stromal gland depths (Figure 3) and myometrium thickness (Figure 4) in morphometry were demonstrated.

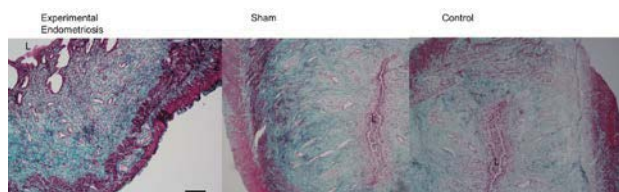


Figure 1. Trichrome Gomori stainings. (125µm, 10x)

Morphometric evaluation

Quantitative assesments revealed significant differences between groups (Figure 2, Figure 3 and Figure 4).

Electron microscopic evaluation

Ultrastructurally, heterochromatic nuclei, disorder of nuclear configuration and pycnosis and short microvilli of endometrial epithelium were demonstrated in experimental group. Eutopic

endometrial epithelium also revealed regressive pinopods. Stromal ultrastructural analysis showed increase in fibroblastic activity and angiogenetic pre-endothelial clumping of cells as well as other migrating cells in experimental endometriosis group. Tissue skeleton was also increased for collagen fibers in this group (Figure 5).

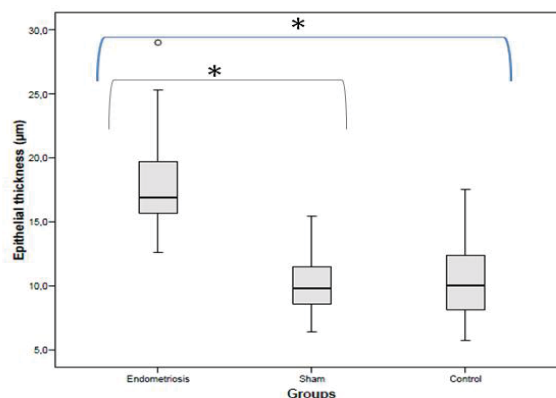


Figure 2. Mean endometrium surface epithelium thickness were compared by ANOVA test ($p=.023$). Dual analysis of 'endometriosis and sham' and 'endometriosis and control' groups with Dunnett T3 revealed statistically significant difference ($p=.00$ and $p=.00$ respectively), while control and sham were not different ($p>0.05$).

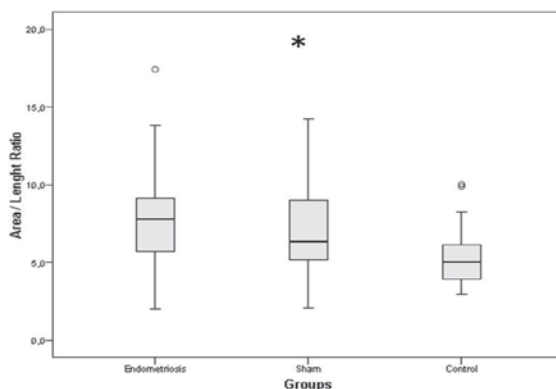


Figure 3. Depths for glandula uterina compared by Anova Test ($p=.011$)

Immunohistochemical evaluation

Increased expressions were determined for the experimental group for eNOS expression of vascular smooth muscles, stromal iNOS activity and bcl-2 expression of eutopic endometrium. Fibronectin and desmin were applied for demonstration of decidual reaction.

Immunohistochemical stainings did not reveal any difference between groups for fibronectin staining; but due to the pseudo-decidual reaction, decreased expression of both desmin and fibronectin were determined in presupposed embryonal regions (Figure 6).

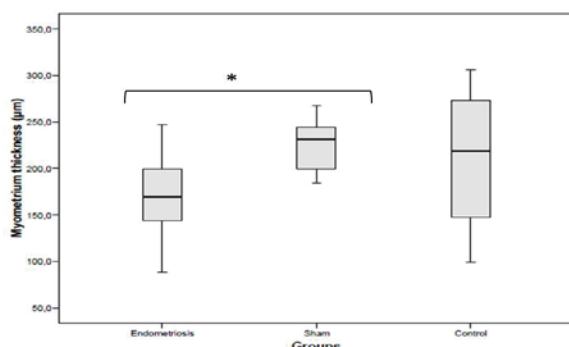


Figure 4. Mean myometrium thickness were compared by Kruskal Wallis test ($p=0.054$). Significant difference were determined between endometriosis and sham groups by Mann Whitney test ($p=.010$), while no significant difference were determined between sham and controls ($p>0.05$)

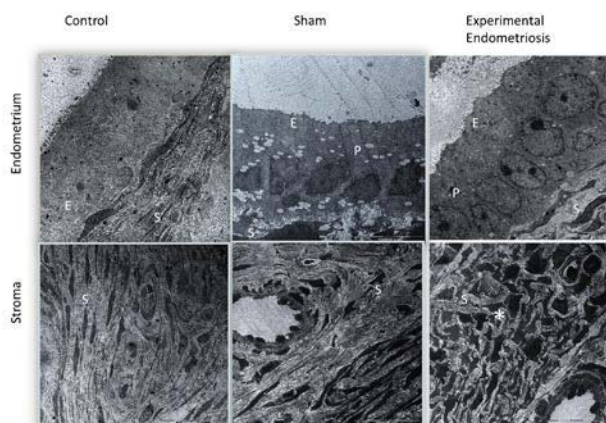


Figure 5. Ultrastructure of eutopic endometrium and stroma

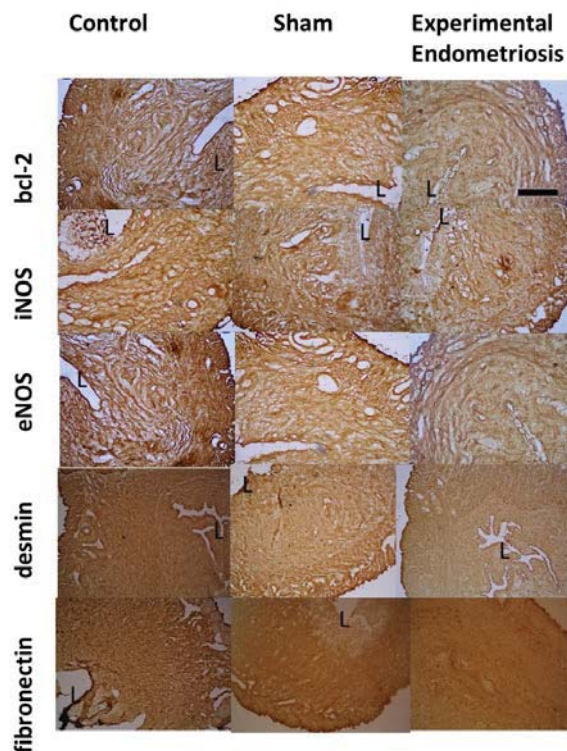


Figure 6. Immunohistochemical expressions (125µm; 10x)

Biochemical evaluation

There were not any statistically significant difference between TBARS levels; although there were apparently different values between groups. SOD and CAT enzyme levels were not any statistically different.

DISCUSSION

Endometriosis is thought to occur as a consequence of retrograde menstruation^{1,2,29}.

However, the fact that the retrograde menstruation is a common physiological phenomenon in reproductive women, other factors like genetic, hormonal, immune and environmental factors seem to be also involved in the pathogenesis³⁰.

The diagnosis is made visually at laparoscopy^{3,31}. Our model has been likewise confirmed with macroscopy. Animal models provide an invaluable tool to study endometriosis³¹. High costs of animal handling limit the use of monkeys as an experimental model. For this reason, the establishment of small laboratory animals¹¹, especially rodents, as models for endometriosis by the transplantation of pieces of endometrial tissue to ectopic sites has greatly extended in recent years^{10,13,15,22-24,32}.

Throughout the reproductive years, the endometrium undergoes dramatic cycles of growth, differentiation, and breakdown under the influence of steroids. In response to changes in steroid exposure, the endometrium produces an array of bioactive growth factors and other cytokines that are critical for paracrine interactions^{20,33}. The endometrium does not only communicate within itself but also with the embryo even before implantation and this interaction is also reflected on the morphology. Pinopods are characteristic for mid to late secretory phase endometrium, which exhibit cycle-dependent changes and are most prominent during the putative implantation interval³³⁻³⁸.

Assessments regarding the implantation defects in this study, revealed regressive pinopod like structures ultrastructurally in the experimental endometriosis group while none was observed for sham and controls, which proves the cyclic delay in experimental endometriosis. Our findings and previous studies both pointed out that demonstration of pinopode structures in endometrial specimens is profitable in infertility studies. Yet due to other unknown reasons, endometrial receptivity may not be always pursued even with enough morphology³⁸⁻⁴¹. Implantation involves a complex sequence of signalling events, consisting in the acquisition of adhesion ligands together with the loss of inhibitory components, which are crucial to the establishment of pregnancy⁴². Lately, the development of microarray technology has allowed this process to be studied from a global molecular perspective. Previously published works have revealed a long list of genes, either upregulated or downregulated at the time of implantation including integrins, IL11 and LIF, etc⁴³.

Experimental endometriosis group also revealed significant endometrial depth differences probably due to mitotic proliferation. Uterine stromal and endometrial elements might be regulated with different mechanisms for uterin receptivity deficits. Zorn et al.⁴⁴ demonstrated mouse endometrial tissue to exhibit "thick" collagen fibrils between decidual cells from the 6th day of pregnancy onwards. Our evaluation also demonstrated tissue skeleton to increase on behalf of mostly collagen fibrils which is concordant with the literature.

Animal models of endometriosis are extremely valueable and indispensable for the evaluation of pathophysiology underlying the development of this prevalent disease. Story L and Kennedy S³¹

described that potential limitations of experimental studies have to be weighted up with regard to their species-specific characteristics of metabolism or reproductive physiology. Our findings are relevant with the literature about symptomatology of endometriosis and implantation deficit. Yet, both ultrastructure and immunohistochemistry differ from humans. Endometrial receptivity, in terms of pinopode expression, disagree the authors in human model. For this reason, it can be assumed that findings in rat pregnancy is not always adaptable to human pregnancy especially when complicated with such diseases. Abortus is an anticipatory result here. Studies have associated recurrent miscarriage with the abnormal expression of various endometrial molecules⁴⁷. Our morphometric perception scopes an adaptable vision of the subject. However new insights and more detailed analyses with novel approaches might be profitable.

Biochemical and immunohistochemical findings were not directly related for the oxidative status. Endometrium was morphologically normal in appearance and biochemically boundary results were recorded. These complied with the regressing endometrium due to the induction of the model artificially to an *in vivo* system with other unexpected interactions⁴⁸⁻⁵⁰.

Finally; relatively decreased decidualisation in eutopic endometrium defines once again that adequate endometrial morphology would not always display normal endometrial receptivity. However, these results are correlated to the reduction of embryo implantation in endometriotic human disease. Furthermore the weak evidence of differences in decidualisation, as demonstrated, is certainly associated with the lack of implantation but the relationship between implantation and decidualisation is not as evident as that between decidualisation and endometriosis or implantation and endometriosis.

Finally, this model is elegant but the intimate mechanism governing the embryo implantation in rat are different from that of the humans. So it is reported here that uterin receptivity defects are regulated differently. Additionally, primary antibodies used in this study were found to be non-descriptive for uterin receptivity in experimental endometriosis.

Morphometric evaluation of the subjects is the salient feature of this study, which can be related to future ultrasonographic devices with novel modules for related measurements.

REFERENCES

1. Vinatier D, Orazi G, Cosson M, Dufour P. Theories of endometriosis. *European Journal of Obstetrics, Gynecology, and Reproductive Biology* 2001;96(1):21-34.
2. DeCherney AH, Lauren, N. Endometriosis. in DeCherney AH, editors. 10th ed. *Current Obstetric & Gynecologic Diagnosis & Treatment*. Ohio, McGraw-Hill Companies; 2003.p.801-7.
3. Yao Z, Shen X, Capodanno I, Donnelly M, Fenyk-Melody J, Hausamann J, et al. Validation of rat endometriosis model by using raloxifene as a positive control for the evaluation of novel SERM compounds. *J Invest Surg* 2005;18(4):177-83.
4. Xue Q, Lin Z, Yin P, Milad MP, Cheng YH, Confino E, et al. Transcriptional activation of steroidogenic factor-1 by hypomethylation of the 5' CpG island in endometriosis. *J Clin Endocrinol Metab* 2007;92:3261-7.
5. Maruyama T, Yoshimura Y. Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium. *Endocrine Journal* 2008;55(5):795-810.
6. Nezhat F, Datta MS, Hanson V, Pejovic T, Nezhat C. The relationship of endometriosis and ovarian malignancy: a review. *Fertil Steril* 2008;90(5):1559-70.
7. Ohlsson Teague EMC, Van der Hoek KH, Van der Hoek MB, Perry N, Wagaarachchi P, Robertson SA, et al. MicroRNA-regulated pathways associated with endometriosis. *Mol Endocrinol* 23(2):265-75.
8. Zhou HE, Nothnick WB. The relevancy of the matrix metalloproteinase system to the pathophysiology of endometriosis. *Front Biosci* 2005;10:569-75.
9. Braundmeier AG, Fazleabas AT. The Non-Human Primate Model of Endometriosis: Research and Implications for Fecundity. *Mol Hum Reprod* 2009;15(10):577-86.
10. Bruner-Tran KL, Carvalho-Macedo AC, Duleba AJ, Crispens MA, Osteen KG. Experimental endometriosis in immunocompromised mice after adoptive transfer of human leukocytes. *Fertil Steril* 2010; 93(8):2519-24.
11. Rosa-e-Silva JC, Garcia SB, de Sá Rosa-e-Silva AC, Candido-dos-Reis FJ, Poli-Neto OB, Ferriani RA, et al. Increased cell proliferation in experimentally induced endometriosis in rabbits. *Fertil Steril* 2010;93(5):1637-42.
12. Chrobak A, Sieradzka U, Sozański R, Chelmońska-Soyta A, Gabryś M, Jerzak M. Ectopic and eutopic stromal endometriotic cells have a damaged ceramide signaling pathway to apoptosis. *Fertil Steril* 2009; 92(6):1834-1843.
13. Bruner-Tran KL, Eisenberg E, Yeaman GR, Anderson TA, McBean J, Osteen KG. Steroid and cytokine regulation of matrix metalloproteinase expression in endometriosis and the establishment of experimental endometriosis in nude mice. *J Clin Endocrinol Metab* 2002;87(10): 4782.
14. Ulukus M, Cakmak H, Arici A. The role of endometrium in endometriosis. *J Soc Gynecol Investig* 2006;13(7):467-476.
15. Hansen PJ. Regulation of immune cells in the uterus during pregnancy in ruminants. *J Anim Sci* 2007;85(13 suppl):E30-1.
16. Serle E, Aplin JD, Li TC, Warren MA, Graham RA, Seif MW, et al. Endometrial differentiation in the peri-implantation phase of women with recurrent miscarriage: a morphological and immunohistochemical study. *Fertil Steril* 1994;62(5):989-96.
17. Senturk LM, Arici A. Immunology of endometriosis. *J Reprod Immunol* 1999;43(1):67-83.
18. Selam B, Arici A. Implantation defect in Endometriosis: endometrium or peritoneal fluid. *J Reprod Fertil Suppl* 2000;55:121-8.
19. Vercammen EE, D'Hooghe TM. Endometriosis and recurrent pregnancy loss. *Seminars in Reproductive Medicine* 2000;18(4):363-8.
20. Sidell N, Han SW, Parthasarathy S. Regulation and modulation of abnormal immune responses in endometriosis. *Annals of the New York Academy of Sciences* 2002;955:159-73.
21. Vinatier D, Dufour P, Oosterlynck D. Immunological aspects of endometriosis. *Hum Reprod Update* 1996;2(5):371-84.
22. Vernon MW, Wilson EA. Studies on the surgical induction of endometriosis in the rat. *Fertil Steril* 1985;44(5):684-94.
23. Rajkumar K, Schott PW, Simpson CW. The rat as an animal model for endometriosis to examine recurrence of ectopic endometrial tissue after regression. *Fertil Steril* 1990;53(5):921-5.
24. Yang JZ, Foster WG. Continuous exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin inhibits the growth of surgically-induced endometriosis in the ovariectomized mouse treated with high dose estradiol. *Toxicol Ind Health* 1997;13(1):15-25.
25. Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. *Braz J Biol* 2002;62(4A):609-14.
26. Dursun A, Sendag F, Terek MC, Yilmaz H, Oztekin K, Baka M, et al. Morphometric changes in the endometrium and serum leptin levels during the implantation period of the embryo in the rat in response to exogenous ovarian stimulation. *Fertil Steril*. 2004 Oct;82 Suppl 3:1121-6.
27. Sheehan DC, Hrapchak BB. Theory and Practice of Histotechnology. Ohio, Battelle Memorial Institute;1987.
28. Sözmen EY, Tanyalçın T, Onat T, Kutay F, Erilaçın S. Ethanol induced oxidative stress and membrane injury in rats. *Eur J Clin Chem Clin Biochem* 1994;32(10):741-4.
29. Sampson JA. Perforating hemorrhagic (chocolate) cysts of the ovary. Their importance and especially their relation to pelvic adenomas of the endometrial type ("adenomyoma" of the uterus, rectovaginal septum, sigmoid, etc.). *Arch Surg* 1921;3: 245-323.
30. Ulukus M, Ulukus EC, Tavmergen Goker EN, Tavmergen E, Zheng W, Arici A, et al. Expression of interleukin-8 and monocyte chemoattractant protein 1 in women with endometriosis. *Fertil Steril* 2009;91(3):687-93.
31. Story L, Kennedy S. Animal studies in endometriosis: a review. *ILAR J* 2004;45:132-8.
32. Grummer R. Animal models in endometriosis research. *Hum Reprod Update* 2006;12(5):641-9.
33. O'Shea JD, Kleinfeld RG, Morrow HA. Ultrastructure of decidualization in the pseudopregnant rat. *Am J Anat* 1983;166(3): 271-98.
34. Usadi RS, Murray MJ, Bagnell RC, Fritz MA, Kowalik AI, Meyer WR, et al. Temporal and morphologic characteristics of pinopod expression across the secretory phase of the endometrial cycle in normally cycling women with proven fertility. *Fertil Steril* 2003;79(4):970-4.
35. Psychoyos A. Hormonal control of uterine receptivity for nidation. *J Reprod Fertil-Suppl* 1976; 25:17-28.
36. Psychoyos A. Uterine receptivity for nidation. *Ann NY Acad Sci* 1986;476:36-42.
37. Psychoyos A, Nikas G. Uterine pinopodes in peri-implantation human endometrium. Clinical relevance. *Ann N Y Acad Sci* 1997;816: 129-42.
38. Murphy CR. Understanding the apical surface markers of uterine receptivity: pinopods-or uterodomes? *Hum Reprod* 2000;15 (12):2451-4.
39. Murphy R. Junctional barrier complexes undergo major alterations during the plasma membrane transformation of uterine epithelial cells. *Hum Reprod* 2000;15(Suppl 3):182-8.
40. Martel D, Monier MN, Roche D, Psychoyos A. Hormonal dependence of pinopode formation at the uterine luminal surface. *Human Reprod* 1991;6(4):597-603.
41. Warren RH, Enders AC. An Electron Microscope Study Of The Rat Endometrium During Delayed Implantation. *Anat Rec* 1964;148: 177-95.
42. Achache H, Revel A. Endometrial receptivity markers, the journey to successful embryo implantation. *Hum Reprod Update* 2006;12(6):731-46.
43. Horcajadas JA, Riesewijk A, Domínguez F, Cervero A, Pellicer A, Simón C. Determinants of endometrial receptivity. *Ann NY Acad Sci* 2004; 1034:166-75.
44. Zorn TM, Bevilacqua EM, Abrahamssohn PA. Collagen remodeling during decidualization in the mouse. *Cell Tissue Res* 1986;244(2):443-8.
45. Ordí J, Creus M, Casamitjana R, Cardesa A, Vanrell JA, Balasch J. Endometrial pinopode and alphavbeta3 integrin expression is not impaired in infertile patients with endometriosis. *J Assist Reprod Genet* 2003;20(11): 465-73.
46. Garcia-Velasco JA, Nikas G, Remohí J, Pellicer A, Simón C. Endometrial receptivity in terms of pinopode expression is not impaired in women with endometriosis in artificially prepared cycles. *Fertil Steril* 2001;75(6):1231-3.
47. Tuckerman E, Laird SM, Stewart R, Wells M, Li TC. Markers of endometrial function in women with unexplained recurrent pregnancy loss: a comparison between morphologically normal and retarded endometrium. *Hum Reprod* 2004;19(1):196-205.
48. Welsh AO, Enders AC. Occlusion and reformation of the rat uterine lumen during pregnancy. *Am J Anat* 1983;167(4):463-77.
49. Welsh AO, Enders AC. Light and electron microscopic examination of the mature decidua cells of the rat with emphasis on the antimesometrial decidual and its degeneration. *Am J Anat* 1985; 172:1-29.
50. Welsh AO, Enders AC. Chorioallantoic placenta formation in the rat: I. Luminal epithelial cell death and extracellular modification in the mesometrial region of implantation chambers. *Am J Anat* 1991;192:215-33.