The effect of ovarian steroid deficiency on regeneration of oviductal mucosa following reconstructive surgery

Andrzej Starczewski², Wojciech Głąbowski¹,³, Maria Laszczyńska³,
Sylwia Słuczanowska-Głąbowska⁴
²Clinic for Reproduction and Gynecology,
³Department of Histology and Embryology,
⁴Department of Physiology, Pomeranian Medical University, Szczecin, Poland

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SUMMARY

In patients with distal tubal occlusion a microsurgical oviductal reconstruction is, apart from the in vitro fertilization, the only treatment option. Unfortunately, the results of reconstructive surgery are often unsatisfactory. The effects of sex steroids on the regeneration process after reconstructive surgery have not been well investigated. This study was aimed to evaluate the effect of decreased concentrations of ovarian sex steroids (castration) on regeneration of the oviduct mucosa after the reconstructive surgery of distally occluded oviducts. The study was performed on 32 female rabbits that underwent unilateral oviduct ligature and resection of fimbriae. The occlusion lasted six (group I) or twelve weeks (group II). After this time the animals were re-operated, and allocated into 4 groups: castration with reconstructive surgery (IA, IIA), reconstructive surgery only (IB, IIB). After next six or
twelve weeks the fallopian tubes were examined under light, scanning and transmission electron microscopes. An immunohistochemical reaction for Ki-67 proliferative antigen was also performed. Ovarian steroid levels were evaluated by radioimmunoassays. The castrated animals had significantly lower levels of estradiol, progesterone and 17-hydroxyprogesterone than the control groups. Long lasting tubal occlusion caused pronounced histological changes of tubal mucous membrane (group II). In the rabbits with preserved ovaries and twelve-week long oviductal occlusion (group IIB), the regeneration of the distal end and restoration of fimbria were not complete twelve weeks after microsurgical reconstruction. In castrated animals with long-lasting occlusion (group IIA) the destructive changes, found in the mucosa of tubal ampullas of occluded oviducts before reconstruction, were still present and even intensified twelve weeks following reconstructive surgery. The castration hampered proliferation of the mucosa cells, thus no fimbriae were restored. Low levels of ovarian steroids were found to have adverse effect on fallopian tube regeneration following reconstructive surgery. The effect was noted even in cases with minor preoperative fallopian tube damage. Therefore, the treatment of concomitant endometriosis or uterine fibroids with GnRH analogues should not be recommended simultaneously with microsurgical tubal reconstruction. Reproductive Biology 2003 3 (3): 197-214.

Key words: castration, distal tubal occlusion, reconstructive tubal surgery

INTRODUCTION

Distal tubal occlusion is a very common cause of oviductal infertility [5, 27]. Most often it is related to pelvic inflammatory disease (PID; [12, 21, 27, 35]), presence of postoperative adhesions or endometriosis within the pelvis [22, 24, 27, 31, 35]. For patients infertile due to tuboperitoneal cause, microsurgical oviductal reconstruction is one of the treatment options. The operation usually consists of an incision in the occluded terminal end of the oviduct, resection of destroyed portion of the oviduct and reconstruction of the stroma with longitudinal incision creating flaps. The flaps are everted and secured to the ampullary seromuscularis with sutures or coagulation of the serosa [10, 14, 27].
The results of reconstructive surgery are often remain unsatisfactory. They depend on the nature and the extent of the tubal damage [4] as well as on the efficiency of regeneration processes of the oviduct mucosa after the surgery. In spite of achieving the tubal patency, there is frequently no restoration of mucosal folds and tubal fimbria following microsurgery. In such cases the patient remains infertile. The significance of sex steroids on the regeneration process after reconstructive surgery has not been well investigated.

As mentioned previously the distal tubal occlusion may be related to endometriosis. In such cases it often requires the subsequent treatment with GnRH analogues which produce the status of chronic ovarian-steroid deficiency. However it is not clear, how a long-term decrease of ovarian steroids and especially estrogen serum levels influence the regeneration process of the tubal mucosa after reconstructive surgery. In the current study an effort was made to evaluate the effects of 1/ decreased concentrations of ovarian sex steroids (castration) and 2/ morphological status prior to the surgical management on regeneration of the oviduct mucosa after the reconstructive surgery of distally occluded oviducts.

MATERIALS AND METHODS

Experimental design

The study was performed on 32 sexually mature female rabbits weight- ing 3500 g - 3959 g (mean 3720 g). At the beginning of the experiment the first surgical operation was performed under the general anesthesia and consisted of a unilateral ligature of the distal parts of oviducts and resection of fimbria (one oviduct in each animal). The animals were divided into two groups based on the duration of occlusion. In group I the occlusion was continued for six weeks and in group II for twelve weeks. Then, the second surgical operation was performed in animals of both groups. This operation included oviduct biopsy for morphological assessment (from the occluded as well as healthy oviduct on the other side) and reconstructive surgery of occluded oviducts (salpingostomy
with fimbrioplasty). In addition, excision of ovaries in eight individuals from each group was carried out producing four groups: IA, IIA without ovaries and IB, IIB with preserved ovaries (fig. 1). In each of the four groups (IA, IB, IIA, IIB) the microsurgery was followed by a period of six (4 animals) or twelve weeks (4 animals). Then, bilateral salpingectomy was performed and the fallopian tubes were taken for morphological studies. The oviducts were evaluated under light, scanning electron and transmission electron microscopy. The specimens for morphological evaluation were collected from the distal end of the oviductal ampulla. In case of light microscopy, paraffin sections were stained with hematoxillin-eosin.

**Radioimmunoassays**

Serum levels of estradiol, progesterone (Orion Diagnostica, Finland) and 17-hydroxyprogesterone (Immunotech, France) were evaluated by radioimmunoassay in each animal. Blood was collected at the time of microsurgical operation (and castration in groups IA and IIA) as well as three, six, nine and twelve weeks following this operation.
Statistical analysis

Serum ovarian steroids concentrations were analyzed by the Kruskal-Wallis’ test. Statistical significance was accepted at p<0.05.

Transmission electron microscopy (TEM)

Tissues for TEM were cut into 1 mm³ pieces, fixed in 0.25 mol/l glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.4) for 2 h at 4°C, postfixed in 1% OsO₄, dehydrated in ethyl alcohol (30-96%) and 100% acetone, and subsequently embedded in Epon [7]. The blocks were cut with Reichter OmU₂ ultramicrrotome. The ultra-thin sections were contrasted with uranyl acetate as well as lead citrate, and assessed under a JEM-1200 EX transmission electron microscope.

Scanning electron microscopy (SEM)

The specimens for SEM were washed with phosphate buffered solution (PBS), fixed in 0.25 mol/l glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.4) for 3 h at 4°C. The material was washed again with the buffer and postfixed in 1% OsO₄, dehydrated in ethyl alcohol (30-96%) and 100% acetone [7]. The slides were dried with liquid CO₂ and covered with gold and palladium. The material was examined under a JEOL JSM-6100 scanning electron microscope.

Immunohistochemistry

To evaluate the proliferation of mucous membrane cells, the immunohistochemical reaction for Ki-67 antigen was performed [6, 29]. The paraffin sections (5 μm) were deparaffinized, rehydrated and microwaved (2 x 4 min in citrate buffer, pH 6, 700W). Nonspecific staining was blocked with 3% H₂O₂ in methanol to inhibit the endogenous peroxidase. The sections were incubated with MIB-1 monoclonal antibody against Ki-67 at the dilution of 1:30 for 60 minutes, (Dianova, Hamburg) followed by streptavidin-biotin-
peroxidase complex (Histostain-SP kit, Zymed Laboratory, San Francisco, USA). The colour reaction was developed by chromogen AEC-kit (Zymed Laboratory, San Francisco, USA). After each step the sections were rinsed with phosphate buffered solution.

RESULTS

Morphology of the oviducts at the time of reconstructive surgery

The oviduct samples were obtained at the time of microsurgery after six (group I) or twelve weeks (group II) of occlusion. The first group’s occluded oviducts were evaluated six weeks after surgical ligature and
showed minimal morphological differences compared to non-occluded oviducts. Investigations showed only slightly reduced number of ciliated cells (fig. 2) as well as diminished number of secretory granules within the glandular cells. The thickness of the epithelium was normal. At this time the Ki-67 antigen expression has not been found in any mucous membrane cells.

In group II the morphological examination of occluded oviducts carried out twelve weeks after ligature revealed substantial distension of ampullas, shortening and smoothening of folds of the ampullary mucosa (light microscopy), numerous cells deprived of cilia (fig. 3) and a reduction of secretory granules within the glandular cells. As with group I, no mucous membrane cells expressed the Ki-67 antigen in occluded oviducts of the group II at the time of surgical reconstruction.

Fig. 3. The oviductal epithelium 12 weeks following distal ligature in rabbits from group II; numerous cells deprived of cilia can be observed. SEM, magnification x 5000.
Fig. 4. Immunostaining for Ki-67 in the mucous membrane of the rabbit oviducts 6 weeks after reconstructive surgery; group IB (duration of occlusion: 6 weeks, preserved ovaries); the Ki-67 proliferative antigens are visible in numerous cells. Light microscope, magnification x 670.

Fig. 5. Immunostaining for Ki-67 in the mucous membrane of the rabbit oviducts 6 weeks after reconstructive surgery; group IA (duration of occlusion: 6 weeks, removed ovaries); very few cells express positive immunostaining. Light microscope, magnification x 670.
Morphology of the oviducts 6 or 12 weeks following reconstructive surgery

The effect of oviduct microsurgical reconstruction

In group IB (occlusion lasting six weeks; preserved ovaries), six weeks after reconstructive surgery, numerous epithelial cells expressed the Ki-67 antigen indicating the increased mitotic activity within the mucus membrane (fig. 4). The small number of secretory granules within epithelial glandular cells...
was still present (fig. 6A). After six weeks the fimbria had been partially restored. Twelve weeks following microsurgery the restoration of fimbria was almost complete. At the same time we found a larger quantity of secretory granules in comparison to the number observed in non-occluded oviducts (fig. 6B). Thus, 12 weeks following reconstructive surgery, the morphology and anatomy of occluded and non-occluded oviducts (on the other side of the same animals) did not differ in this group.

In group IIB (duration of occlusion 12 weeks; preserved ovaries) the immunoractivity for Ki-67 observed six and twelve weeks after microsurgical management revealed only a sporadic presence of positive cells. This indicated poor proliferative activity within tubal mucous membrane. Twelve weeks after this operation small fimbria at the terminal ends of previously occluded oviducts were restored but the morphological evalu-
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The folds were smooth, poorly branched and slightly protruding. The areas with epithelial cells deprived of cilia were still present (fig. 7). The secretory granules in secreting cells of the epithelium were scarce. Thus, neither the regeneration of mucous membrane of the oviducts nor restoration of fimbria (to the status present before the ligature) was completed twelve weeks after microsurgery.

The effect of oviduct microsurgical reconstruction and castration

In healthy, non-occluded oviducts of castrated animals, a reduction in both the number of cilia in the epithelial cells (fig. 8) and secretory granules within glandular cells was found.

Fig. 8. The epithelium of non-occluded oviduct 12 weeks after castration; reduced number of ciliated cells is observed. SEM, magnification x 5000.
In group IA (duration of occlusion 6 weeks; removed ovaries) the morphology of distal ends of previously ligated oviducts six and twelve weeks after reconstruction was similar to that observed in healthy oviducts of castrated rabbits. Very short, atrophic fimbria as well as short folds of mucosa covered with thin epithelium with decreased secretory activity and highly reduced number of ciliated cells were observed (fig. 9). The histology of the ampullas of these oviducts generally did not differ from non-occluded ones. Thus, the morphological abnormalities were related not only to previous occlusion but also to ovarian steroids deficiency. In the ampullas and atrophic fimbria of these oviducts only few cells expressed Ki-67 antigen six and twelve weeks after reconstruction (fig. 5).

Fig. 9. The oviductal epithelium 12 weeks following reconstructive surgery and castration in group IA (duration of occlusion: 6 weeks); large areas of deformed cells deprived of cilia and ciliated cells with small number of cilia are visible. SEM, magnification x 2000.
In group IIA (duration of occlusion 12 weeks; removed ovaries) there was no restoration of fimbria six as well as twelve weeks after reconstruction. The immunostaining did not reveal any expression of Ki-67 proliferative antigens in the ampullar mucosa of these oviducts at both times. The destructive changes, found in the mucosa of tubal ampullas before reconstruction, were still present and even intensified twelve weeks following microsurgery. Only single ciliated cells with very few cilia (fig.10) were observed. Moreover, a lack of secretory granules within the secreting cells was noted.

In this paper we have described only morphological features present in all animals of particular groups. Sporadically observed morphological abnormalities have not been mentioned.

Fig. 10. The oviductal epithelium 12 weeks after reconstructive surgery and castration in group IIA (duration of occlusion: 12 weeks); single ciliated cells with extremely few cilia are visible. SEM, magnification x 2000.
Castration and oviductal regeneration

The serum levels of estradiol, progesterone and 17α-OH progesterone were significantly lower in castrated female rabbits compared to animals with preserved ovaries (tab. 1). The decrease in steroid serum concentration was observed three weeks after castration and was present at the end of the experimental period.

**DISCUSSION**

The morphological destruction of oviduct mucosa caused by distal tubal occlusion as well as the outcome of tubal reconstructive surgery have been the subject of great interest recently. Vasquez at al. [36, 37] and Kleinstein at al. [19] reported more severe oviductal damage with longer period of tubal occlusion. This is consistent with the results of the current study. On the other hand, Gauwerky at al. [11] observed similar oviduct mucus membrane histological abnormalities four and sixteen weeks following distal occlusion.

In our previous studies (Starczewski, unpublished), by means of immunohistochemical reaction for the presence of Ki-67 proliferative antigen, we
confirmed that the regenerative process of fimbria and mucous membrane folds within the ampulla is most intensive during first six weeks after reconstructive surgery. Clinical observations as well as a few experimental studies showed that the effects of microsurgical reconstruction of distally occluded oviducts are related to the time of the occlusion and morphological status of the oviducts prior the surgical management [14, 15, 18, 20, 25, 33, 36]. However, Gauwerky at al. [11] did not find the correlation between the duration of occlusion and outcome of the reconstructive surgery. Nevertheless, they reported the persistent infertility in individuals with long lasting distal occlusion.

In our experiment we found less severe morphological changes of occluded oviducts at week 6 as compared to week 12 following distal tubal closure. Moreover, in latter group the microsurgical reconstruction did not satisfactorily improved the histological structure of the oviducts even twelve weeks after operation. The very poor immunoreactivity of Ki-67 was detected in the mucosa of longer occluded oviducts showing low proliferative activity [8, 13, 34]. It is likely that this inhibition of proliferation was caused by improper functioning of irreversibly destroyed secretory cells, which are responsible for synthesis and release of epidermal growth factor (EGF; [1, 3]). It corresponds to previously reported observations of the expression of the epidermal growth factor receptor as well as pro-mitotic effect of its activation in the epithelial cells of the oviduct [1, 2]. Thus, it is possible that poor success rates of microsurgical treatment of the infertility caused by long lasting distal tubal occlusion are related to the lack of improvement of oviductal mucous membrane morphology after reconstructive operations.

In the current experiment we found no immunoreactivity for Ki-67 and very poor restoration of fimbria and folds of mucosa in animals that underwent castration. These results confirm that ovarian steroids deficiency (castration) disturbs the regeneration of the tubal mucosa after microsurgical reconstruction of distal tubal occlusion, not only in cases of severe but also in those of moderate and mild morphological damage prior to the surgical treatment. It is well known that infertile women with tubal distal occlusion often suffer from concomitant diseases such as endometriosis or numerous uterine fibroids. In these patients treatment with GnRH analogues is frequently
applied following microsurgical reconstruction [9, 16, 17, 23, 26, 28, 30]. Our results suggest that GnRH analogues therapy, which actually causes the reversible, pharmacological castration, should not be recommended immediately after surgical treatment because it may frustrate the proper post-surgical regeneration process. Thus, in such cases the treatment with the GnRH analogues needs to be introduced and completed prior to the microsurgery. In fact such a management extends the duration of occlusion but it seems to be more adequate because it avoids the post-microsurgical ovarian steroids deficiency, and subsequently improves the regeneration process after microsurgery. Additionally, as it was reported previously [32], the use of GnRH analogues before surgery may prevent the progress of mucous membrane destruction related to tubal occlusion.

It may be concluded that chronic ovarian steroids deficiency (for instance after pharmacological castration with GnRH analogues) may annihilate the effects of microsurgical treatment of oviductal infertility.

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