

Changes in the proliferation activity of the ovarian surface epithelium during the ovulatory cycle, pregnancy and after GnRH-antagonist treatment in the primate

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Abstract: Objectives: To investigate the effect of Gonadotrophin releasing hormone (GnRH) antagonist treatment on the proliferative activity and morphology of the primate ovarian surface epithelium (OSE); the main origin of ovarian cancer. **Methods:** This study was conducted at the Biological Sciences Institute at The University of Edinburgh, Edinburgh, United Kingdom from 2009-2010. Evaluation of OSE cell proliferation was assessed using 5-bromo-2-deoxyuridine (BrdU) labeling in paraffin- embedded tissue sections of marmoset ovaries obtained from previously treated marmoset monkey (n=44) with Antarelix (GnRH antagonist). Treatment administration was at zero time and on 5th day of ovulation. The extent of proliferation was also determined using same marker in cycling and pregnant animals in the OSE layer in proximity to stroma, antrum and corpus luteum (CL). The staining was performed at early, mid and late follicular and luteal phases and at early pregnancy. **Results:** GnRH antagonist administration at any time during follicular phase suppresses OSE cells proliferation. The epithelia overlaying the large antral follicles at pre-ovulatory stage and CL at early luteal phase displayed the maximum staining, while hardly few cells atop the stroma reacted with the marker. The morphology of the epithelial cells in contact with stroma was typical resting cuboidal shaped with tight attachment to basement layer. The cells above CL were prominently squamous type with loosened attachment, and those over large antral follicle were flattened and disorganized. Apparently, the cell multiplication and intercellular adhesion was distorted in the layers above CL and antrum, which correlates with their proliferative activity. **Conclusion:** This study concludes that GnRH antagonist treatment can alleviate the OSE proliferation, therefore can reduce the risk of ovarian cancer development.

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1. Introduction

Gonadotrophin releasing hormones (GnRHs) is a short-acting decapeptide that is secreted by the hypothalamus, which could induce the secretion of Luteinizing Hormone (LH) and follicle stimulating hormone (FSH) in the pituitary gland.¹ FSH is a pleiotropic hormone produced by the pituitary and exerts its action on the ovary by inducing proliferation, differentiation and steroidogenesis in the granulosa cells of growing preovulatory follicles. The initial development of primordial follicles (PF) is believed to be FSH independent. Elevated FSH levels are found to be associated with altered ovarian function including diminished ovarian reserve, premature ovarian failure, infertility, ovarian hyperstimulation syndrome, poor quality of eggs, menopause and also a huge body of literature exists suggesting an association with ovarian cancers.² Over 20 different forms of GnRH in vertebrate species have highly conserved structure. Plasma gonadotrophin levels regulate the overall reproductive process, especially the gonadal steroidogenic and gametogenic functions.³ The GnRH-I and II receptors are also found in almost all types of normal and cancerous epithelial cells, including those present in gonads.

Prior studies suggest that GnRH-II has a role in suppression of cell proliferation and apoptosis by triggering a G-protein (G_i) mediated signal pathway in the target cells.⁴⁻⁵ During the last four decades, several thousand peptide and non-peptide GnRH agonists and antagonists have been synthesized and tested for clinical efficacy. These compounds competitively bind to pituitary GnRH receptors and down-regulate gonadotrophin synthesis and secretion. Clinically, the agonists/antagonists are used for the treatments of prostatic, breast and endometriotic cancers, uterine fibroids, precocious puberty, premenstrual and polycystic syndromes, and infertility.⁵⁻⁹ Ovarian epithelial proliferation is believed to be primarily regulated at the level of gonadotropin's, which stimulate follicular oestradiol production. As a mitogen, β -oestradiol and other localized factors such as insulin-like and hepatocyte-growth factors (IGF, HGF) trigger proliferative response in post-ovulatory OSE layer at the sites of rupture. Poly-ovulatory mammals like marmosets have repeated ovulations and therefore repeated rupture and healing of the OSE layer. These features accelerate OSE cell multiplication and transformation to neoplasia at the rupture sites.¹⁰

Antarelix has been used in marmosets to suppress LH/CG secretion from the pituitary gland.¹¹ It was found that upon such treatment the level of serum β -oestradiol rapidly falls and as a result proliferation of granulosa cells in antral follicle and CL diminishes. The study hypothesizes that Antarelix may also suppress the mammalian OSE proliferation and tumorigenesis in a dualistic manner. Either by inhibiting LH/CG secretion so that follicular development is inhibited and ovulation is prevented. Hence the number of re-epithelization events is reduced and OSE stays non-proliferative. Or, the antagonists may bind directly to OSE cell's GnRH receptors and impart anti-proliferative response through some unknown downstream signaling pathway. According to the gonadotrophin theory of ovarian cancer, a high level of gonadotropins at menopause has a role in the development of epithelial ovarian cancer. In this study, we test the hypothesis that GnRH antagonist administration would suppress endogenous gonadotrophin, resulting in inhibition of OSE proliferation in marmoset. We also hypothesize that changes in the ovarian environment during the ovarian cycle (follicular and luteal phase) and at early pregnancy might have an influence on the activity and morphology of the OSE cells. To our knowledge this is the first report of a GnRH antagonist treatment on OSE proliferation in a primate.

2. Materials and methods

Animals and tissue samples. Adult female common marmoset monkeys (*Callithrix jacchus*) (age 2-3 yrs) with body weight ~350 g, and having a regular 28 d ovulatory cycles or gestation periods between 141 d and 145 d, were housed in cages under standard conditions. The ovaries used for this study had been collected for analysis on day 0 ($n = 4$), day 5 ($n = 5$) and day 10 ($n = 6$) of the follicular phase,⁹ and at early ($n = 5$), mid ($n = 5$) and late ($n = 5$) luteal phase (14-22 d) of the ovarian cycle. Additionally, ovaries from the pregnant animals were collected from second ($n = 5$), third ($n = 3$) and fourth ($n = 6$) week of pregnancy.

Treatments. All experimental procedures of this work were carried out in accordance with the Animals (Scientific Procedures) Act 1986, and were approved by the Local Ethical Review Process Committee. In order to synchronize the timing of ovulation, marmosets were treated intramuscularly with a single *i.m.* injection of 1 μ g of a prostaglandin (PG) $F_{2\alpha}$ analogue (cloprostenol, Planate; Cheshire, UK), between days 12-15 of 20 d luteal phase to induce luteolysis. The day of the PG injection was designated as follicular day zero. A GnRH antagonist Antarelix (Europeptides, Argenteuil, France) was dissolved in water to reach a concentration of 10 mg/ml. To provide a slow-release depot, Antarelix was

administered at a dose of 12 mg/kg body weight by subcutaneously injecting on follicular day 0 (day of PG injection) ($n = 5$) or day 5 ($n = 5$).⁹ Ovaries were collected on day 10 following PG administration, corresponding to the periovulatory period in the control animals. Control marmosets were studied in parallel on day 0 ($n = 5$) and day 10 ($n = 5$) corresponding to PG administration. All animals were injected *i.v.* with 20 mg BrdU (Roche Molecular Biochemicals, Essex, UK) prepared in saline. After 1 h they were killed with an *i.v.* injection of 400 μ l of Euthetal (sodium pentobarbitone; Rhone Merieux, Harlow, Essex, UK). Both ovaries from each animal were removed, weighed and immediately fixed in 4% neutral buffered formalin. After 24 h, the ovaries were transferred to 70% ethanol, dehydrated and embedded in paraffin. Whole ovaries were serially sectioned at 5 μ m, prepared and then processed for immunohistochemical analysis as described.¹²

Immunohistochemistry. The changes in the proliferative activity of the OSE throughout the follicular phase, luteal phase and early pregnancy and after GnRH antagonist treatment were studied by quantifying the number of proliferating cells within the OSE area whose nuclei were stained with BrdU. For this, sections were prepared on glass slides and stained for BrdU as described by earlier publication.¹² For BrdU immunostaining, antigen retrieval was performed using pressure cook. To reduce non-specific binding, sections were blocked in normal rabbit serum (NRS; 1:5 diluted with TBS containing freshly added 5% bovine serum albumin) for 30 min. Primary antibody, BrdU (mouse anti-BrdU; Roche Molecular Biochemicals, Essex, UK) was diluted 1:30 in TBS. Incubation was carried out overnight at 4°C. Incubation with the secondary antibody rabbit anti-mouse IgG (1:60 diluted in NRS: TBS; DAKO Corp.) was carried out for 40 min at room temperature followed by incubation with alkaline phosphatase-anti-alkaline phosphatase complex (1:100 dilution in TBS; DAKO Corp.) for 40 min at room temperature. Slides were visualized using 500 μ l nitro blue tetrazolium (NBT) solution containing NBT substrate (Roche Molecular Biochemicals), buffer, 5-bromo-3-chloro-3-indolyl-phosphate and levamisole. Sections were counterstained with haematoxylin. The positive immunostaining of BrdU within the granulosa cells of growing follicles was used as a positive control.

Quantification of immunohistochemistry. BrdU incorporation of the OSE showing proliferating cells was quantified. Proliferation of the OSE was detected all around the circumference and in all sections. At least 20 histological sections were chosen randomly from both ovaries (right and left) of each group to be examined. The labelling index was determined by counting the number of OSE cells (labelled and

unlabelled) in each section under 600X magnification, and expressed as the percentage of labelled OSE cells. The labelling index is expressed as a mean value for the number of OSE cells in each section.

Statistical analysis. The Kolmogorov-Smirnov test was used in order to test whether the data is normally distributed or not. Data for studies of GnRH antagonist treatment and the ovarian cycle influences on OSE cell proliferation was not normally distributed therefore; The Kruskal-Wallis test was selected in order to compare different groups. Data for BrdU labelling distribution over different areas of the ovary was not normally distributed, therefore Chi-square test was used to compare the total percentages between groups. Differences were considered to be significant at $p < 0.05$. Analysis was carried out using Minitab version 15.

3. Results

OSE proliferation at different stages of the ovarian cycle and during early pregnancy. Results of the present study revealed that the highest proliferation was obtained during the late follicular and early luteal phase ($p = 0.52$). There was a gradual increase in the proliferation activity as the follicular phase advanced, reaching the maximum value towards the end of this phase ($p = 0.03$). After ovulation, proliferation in early luteal phase ovaries was also high before declining markedly by the mid luteal phase ($p = 0.009$). This low level was maintained in late luteal phase ovaries. During all stages of pregnancy (2, 3 and 4 weeks), the OSE cells showed diminished or extremely low BrdU labelling, and correspondingly very low labelling index as compared to the non-pregnant cycling animals ($p = 0.96$). The OSE proliferation was discernable mostly at week 2 of pregnancy (Figure 1).

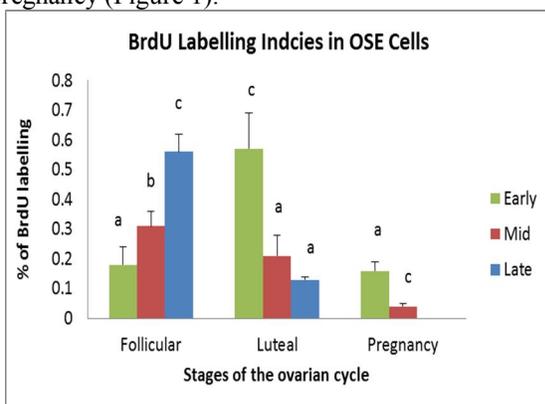


Figure 1. BrdU incorporation into the marmoset OSE cells. Over the total area of the ovary at early ($n = 4$), mid ($n = 5$) and late stages ($n = 6$) of the follicular phase, and early ($n = 5$), mid ($n = 5$) and late stages ($n = 5$) of the luteal phase and at week-1 ($n = 5$) and week-2 pregnancy ($n = 3$). Values are means \pm SEM. Different letters over the bars represent statistically significant differences.

To ascertain which underlying tissue is contributing to the OSE proliferation, the BrdU staining was examined at different regions of the OSE layer. During the follicular phase the majority of the proliferating cells were detected over the large antral follicles which were very close to the surface (60-75 μm), and a lower number of proliferating cells were found distributed randomly over the stroma (Figures 2 and 3; A and B). During the luteal phase, most of the proliferating cells were located over the CL and little activity was observed over the stroma (Figures 2 and 3; C and D). During early pregnancy a variation in the distribution of the proliferating cells within the OSE was also detected. The highest number of proliferating cells was found over the CL, whereas slightly lower number of evenly distributed cells was observed over stroma. At this pregnancy stage, large antral follicles were not detected close to the ovarian surface (Figures 2 and 3; E and F).

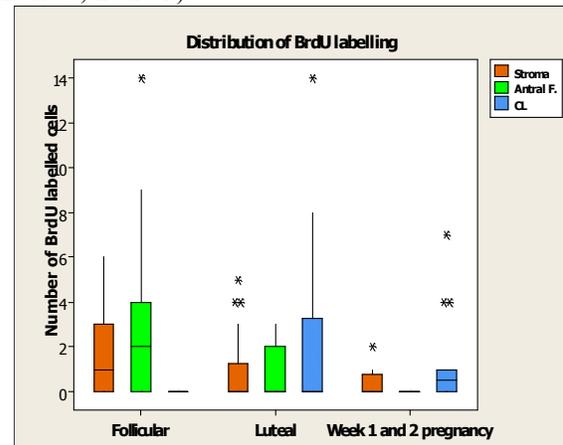


Figure 2: Box plots indicating the distribution of BrdU labelled cells in marmoset OSE through different regions. Overlying stroma, antral follicles and corpora lutea (CL) at the follicular phase ($n = 15$), the luteal phase ($n = 15$) and at week 1 and 2 pregnancy ($n = 8$). The lines of the boxes show the 25th, 50th (median) and 75th percentiles, the whiskers show the 10th and 90th percentiles and asterisks represent the outliers. Data are the number of BrdU labelled cells in OSE cells at each region for each section (2 sections/ animal).

Morphological changes in the OSE cells. The distribution of proliferation markers in OSE cells varied considerably during different reproductive stages (follicular, luteal and pregnancy). The OSE morphology and its adhesiveness to underlying tissues are also expected to alter considering the extent of multiplication capabilities within a limited surface area in which the cells can expand. The microscopic observation revealed considerable variation in the shape of the OSE cells ranging from squamous to cuboidal. Moreover, the association between the OSE cells was affected by the ovarian cycle and the ovarian compartments. OSE cells over the large antral follicles

which were located very close to surface were flattened in shape and disarrangement between these cells was noticed. There was no evidence of firm attachment with the underlying basement membrane (Figure 4; A). Over the CL, OSE cells appeared as squamous with very weak association between the cells, and it was seen that attachment of these cells

with the basement membrane was also loose (Figure 4; B). The OSE cells observed in a region away from the follicular development and the ovarian event over the stroma, displayed highly organized arrangement of cuboidal shape, and attachment in between the cells and of the cells with underlying basement membrane was also firm (Figure 4; C).

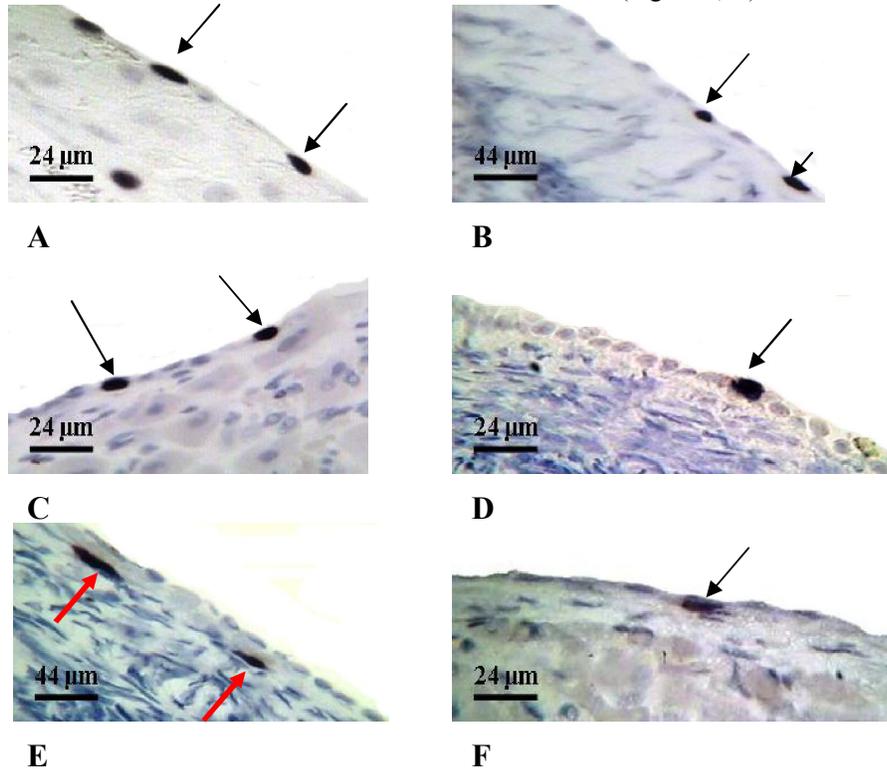


Figure 3: Photomicrographs showing BrdU labelling within the marmoset OSE cells. BrdU labelling within the OSE cells at follicular phase of the ovulatory cycle (A) over large antral follicle and (B) over stroma. C-D demonstrate the morphology of OSE cells at luteal phase (C) over mature CL and (D) over stroma. E-F OSE cells at pregnancy (E) over stroma and (F) over CL. Red arrows indicate labelled cells within the tunica albuginea layer (black stain).

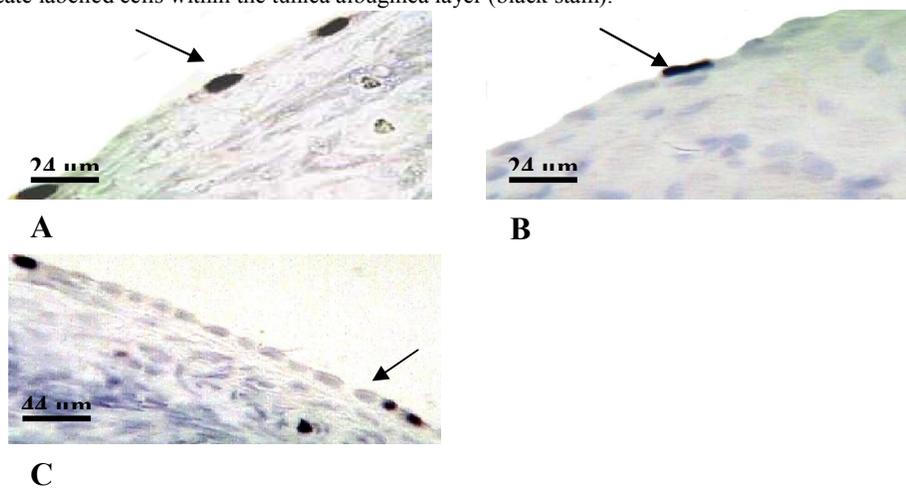


Figure 4: Photomicrographs representing the morphological changes of the OSE cells at different regions of the marmoset ovary. (A) OSE covering large antral follicle (arrow indicates flattened shaped cells), (B) over a mature CL (arrow indicates squamous shaped cells) and (C) over ovarian stroma (arrow indicates cuboidal shaped cells).

Effect of Antarelix treatment on OSE proliferation. Prior studies on Antarelix treatment in marmoset monkeys revealed significant reduction in BrdU labelling in ovarian endothelial cells and steroidogenesis in granulosa and theca cells of growing follicle. It was a matter of interest to study how these changes would affect the overlying OSE cells. A comparison of sections prepared from the ovaries of late follicular controls and the treatment

groups revealed that the OSE cells of GnRH antagonist-treated ovaries from day 0-10 and from day 5-10 exhibited a decrease in the proliferation after both treatment schedules. Quantitative analysis confirmed significant decrease in cell proliferation in the OSE cells in both treated groups, and the effect was more pronounced in the longer treatment schedule (day 0-10 treated group; $p = 0.001$) (Figure 5).

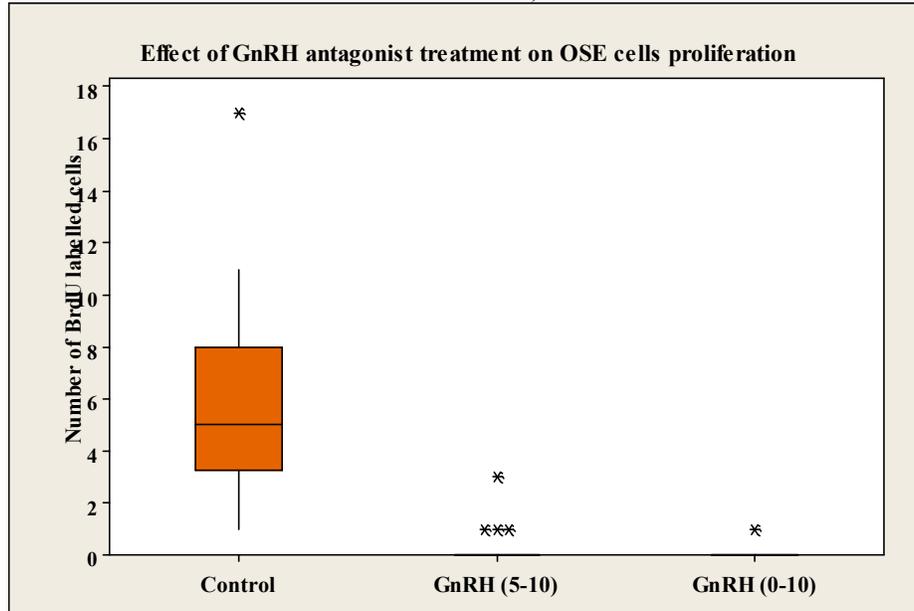


Figure 5: Box plots indicating BrdU incorporation into the marmoset OSE cells over the total surface area of the ovary after GnRH-antagonist treatment. From day 5-10 ($n = 5$; 5 sections/animal) and day 0-10 ($n = 5$; 3 sections/animal) follicular phase, ovaries from day 10 follicular phase were used as a control ($n = 5$; 4 sections/animal). The lines of the boxes show the 25th, 50th (median) and 75th percentiles, the whiskers show the 10th and 90th percentiles and asterisks represent the outliers. Data are the number of BrdU labelled cells in OSE cells for each section.

4. Discussion

Considering the fundamental role of OSE cells in ovarian tumourigenesis, the growth regulation of normal and neoplastic OSE cells by intraovarian regulators may play an important role in ovarian cancer development. In this regard, GnRH has been implicated as an autocrine regulator of normal OSE as well as a growth inhibitor of ovarian cancer cells. GnRH antagonists are widely used in the clinic during ovarian hyperstimulation programmes to block endogenous gonadotrophin secretion.¹³ GnRH regulates the biosynthesis and secretion of gonadotrophins. In this study we have demonstrated two significant findings regarding the OSE cell proliferation, under the effects of pregnancy and in response of GnRH antagonist treatment. We provide evidence that GnRH antagonist treatment and pregnancy both significantly reduce the proliferative activity of the OSE.

The present results indicates that during the ovulatory cycle, BrdU staining of OSE layer increases

towards end of the follicular phase, when it is in contact with large antral follicles, and decreases by the mid luteal phase. In fact the highest staining was seen in CL at early luteal phase. Low staining of OSE, overlying the stroma shows that the resting OSE cells at early follicle development or those which are not in the vicinity of the CL or antrum are not rapidly dividing. Since OSE proliferation was associated with large antral follicles and CL at all reproductive phases it can be predicted that events leading to luteogenesis contribute towards OSE proliferation whereas the reverse process, luteolysis, suppresses the proliferative response. Before luteogenesis remarkable changes take place in the granulosa and theca cells of growing follicles in late follicular phase, notably vascularization, angiogenesis and steroidogenesis. Interestingly, these changes are regulated by LH.¹⁴ Vascular endothelial growth factor (VEGF) is a pro-angiogenic factor that plays an important role in angiogenesis.¹⁵ In granulosa cells of growing follicles, VEGF expression is up-regulated most likely due to

LH, and high cellular level of this factor is maintained up to luteal phase controlled by LH/CG.¹⁶ In the CL the same granulosa cells transform to granulosa-lutein cells responsible for steroidogenesis. OSE cell morphology is believed to be another marker of proliferative activity.¹⁷ It has been suggested that squamous and cuboidal forms of cells represent the groups that have not or have undergone post-ovulatory proliferation respectively. OSE cells tend to assume columnar shapes when they form clefts and inclusion cysts.¹⁸ These cells are the primary targets for mitogenic and inflammatory action leading to tumourigenic growth.¹⁹

The OSE cells attach to the basement membrane that participates in a number of fundamental biological processes such as cell growth, differentiation tissue development and repair.^{20,21} It has been proposed that loss of ovarian surface basement membrane after preovulatory stimulation by gonadotrophins is a critical step in early tumourigenicity of the OSE.^{22,23} We found that non-proliferative cuboidal and tightly attached OSE cells were present away from the ovulation sites. The proliferative squamous cells with very loose attachment were discernible over CL and disarranged flattened cells were present in the OSE layer just before ovulation. Some of these cells might transform to columnar cells if inclusion cysts or clefts are formed after ovulation.

Although it was not expected to detect OSE proliferation during pregnancy, OSE cells during 2nd week of gestation displayed positive BrdU staining specifically in proximity to CL. These observations suggest that the OSE cells at this stage are still under the influences of the CL mitogenic factors or probably because the progesterone concentration has not been high enough to suppress any proliferative action.

So far the results indicate that, in marmosets, luteogenesis rather than folliculogenesis seems to influence OSE proliferation and luteolysis and pregnancy reverses this process. In primates LH/CG is necessary for CL development, which induces numerous paracrine regulators of luteal development including VEGF that controls angiogenesis and vascularization.^{24,25} As LH concentration starts to recede, the natural luteolysis advances starting from mid luteal phase. During luteolysis the LH level falls and all its stimulatory actions like angiogenesis and steroidogenesis decline.

In this investigation, the potential anti-proliferative response to treatment with a GnRH antagonist was examined on OSE cells in marmoset monkeys. Blocking FSH and LH/CG release at zero time by Antarelix (0-10 days) in this study would have arrested the development of antral follicles with consequent prevention of ovulation.⁹ Further, β -

oestradiol synthesis was decreased as Antarelix treatment inhibits steroidogenesis. At 5th day blockage (5-10 days) some gonadotrophin concentration would have built up in within first 5 days which apparently is sufficient to grow follicles up to pre-antral stage. Thereafter, LH "surge" which takes place at late follicular phase necessary for ovulation and CL formation must have been prevented. The depressive effect of Antarelix on OSE proliferative activity was clearly discernible regardless of the time of administration. It is proposed that pre-ovulatory large antral follicle and its derived CL may be responsible for OSE proliferation in the contact regions. This conclusion is derived from the observation that OSE proliferation was suppressed when drug was administered on 5th day of follicular cycle when pre-ovulatory follicles were not able to be differentiated. There could be several explanations of Antarelix's OSE anti-proliferative effect which may function in isolation or in combination: 1) reversal of LH/CG up-regulation of localized growth factors like IGF and HGF which are responsible for mitogenic action through their anti-apoptotic effect on OSE, 2) inhibition of gonadotrophin-induced steroidogenesis in antral follicles and CL would minimize production of mitogenic β -oestradiol in granulosa cells, and consequently OSE proliferation would be inhibited, 3) GnRH antagonists result in skew in frequency of antral follicles,⁹ and apparently this would reduce ovulation frequencies and resultant epithelial rupture, and thereby prevent the neoplastic growth of OSE, and 4) this compound may bind to GnRH-I/ GnRH-II receptors in OSE cells itself and inhibit all downstream pathways which involve mitogen activating protein (MAP), P38 and the extracellular signal regulate kinase (ERK-1/2) responsible for mitogenic proliferation.²⁶

In conclusion, BrdU uptake and immuoreactivity equating to proliferating cells was detected at the ovulation site before and after ovulation and lower number of BrdU-stained cells were found to be distributed randomly in OSE at other sites around the ovary, suggesting that the OSE proliferative activity takes place in a cyclic manner depending on the ovarian events that occur underlying the OSE during the ovarian cycle. GnRH antagonist treatment resulted in complete inhibition of OSE cells proliferation.

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