QUANTITATION OF ESTROGEN RECEPTORS AND RELAXIN BINDING IN HUMAN ANTERIOR CRUCIATE LIGAMENT FIBROBLASTS

Deborah A. Faryniarz, MD[1], Madhu Bhargava PhD[2], Claudette Lajam MD[3], Erik T. Attia BA[2], Jo A. Hannafin MD, PhD[2]

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[1]Department of Orthopedic Surgery and Sports Medicine, The Permanente Medical Group, Inc., Santa Clara, CA; [2]Sports Medicine and Shoulder Service, Hospital for Special Surgery, New York, NY; and [3]Department of Orthopaedic Surgery, Mayo Clinic, Rochester, MN.

Running Title: Estrogen and Relaxin Receptors in Human ACL

Corresponding Author and address for reprints: Deborah A. Faryniarz, MD, The Department of Orthopedic Surgery and Sports Medicine, The Permanente Medical Group, Inc., 900 Kiely Blvd., Santa Clara, CA 95050

Telephone: (408) 559-3888 Email: DrDeb@Shoulder2Knee.com

SUMMARY

The significantly higher incidence of anterior cruciate ligament (ACL) injuries in collegiate women compared to men may result from relative ligament laxity. Differences in estrogen and relaxin activity, similar to that seen in pregnancy, may account for this. We quantified estrogen receptors by flow cytometry and relaxin receptors by radio-ligand binding assay in human ACL cells, and compared the presence of these receptors in males and females.

ACL stumps were harvested from seven males and eight females with acute ACL injuries. The tissue was placed in M199 cell culture medium. Outgrowth cultures were obtained and passage two cells were used for all studies. Estrogen receptor determination was performed using flow cytometry. Relaxin binding was performed in ACL cells derived from 5 female and male patients using I^{125} labeled relaxin.

Estrogen receptors were identified by flow cytometry in 4 to 10 % of ACL cells. Mean fluorescence of cells expressing estrogen receptors was approximately twice that of controls, with no significant differences between males and females. Relaxin studies showed low-level binding of I^{125} relaxin-labeled ACL cells. Relaxin binding was present in four out of five female ACL cells versus one out of five male ACL cells.

KEYWORDS

Flow cytometry, gender, estrogen receptor, relaxin, anterior cruciate ligament, binding

INTRODUCTION

Recent studies have shown that in collegiate athletes (basketball, soccer, and volleyball) females have a two to eight times greater anterior cruciate ligament (ACL) injury rate than males (Arendt, et al., 1995; Traina, et al., 1997; Zelisko, et al., 1982). Seventy-eight percent of these injuries were non-contact injuries occurring during landing, cutting, or decelerating. Understanding the etiology of non-contact ACL injuries in females may have significant impact on potential morbidity (premature osteoarthritis, chronic instability) in the young and active female athlete.

The disparity in injury rates has been attributed to extrinsic factors such as muscular strength, neuromuscular coordination, skill, and experience level. Intrinsic factors such as limb alignment, intercondylar notch dimensions, ligament and joint laxity have also been implicated (Traina et al., 1997). Several studies have suggested that ligament and joint laxity may be ascribed to the actions of hormones, particularly estrogen and relaxin (Arnold, et al, 2002; Deie, et al., 2002, Karageanes, et al., 2000, Romani, 1995; Charlton, et al., 2001). Changes in sex hormone levels during the menstrual cycle have been hypothesized to increase the extensibility of soft tissues, increasing joint laxity and predisposing to ligament injury. It has been suggested that hormonal fluctuations may contribute to a monthly "window of potential injury" (McShane, et al., 2000; Moller-Nielsen and Hammar, 1989; Myklebust, et al., 2003; Slauterbeck, et al., 2002; Slauterbeck and Hardy, 2001; Wojtys, et al., 1998; Wojtys et al., 2002; Wolman, 1999).

The attribution of hormones to ligament laxity stems from studies that have associated increased extensibility of soft tissues in the pelvic region and increased peripheral joint laxity during pregnancy with concurrent elevated levels of estrogen and relaxin. The interosseous ligament of the pubic symphysis, cervical tissue, and uterus myometrium stretch or soften during normal human pregnancy and after direct administration of estrogen and relaxin in animal models (Min and Sherwood, 1996). Estrogen and relaxin are known to work synergistically in rat myometrium, with relaxin receptors up-

regulated after pretreatment with estrogen. Significant peripheral joint laxity occurs after the first trimester of pregnancy correlating with a peak in relaxin (Calguneri, et al., 1982; Petersen, et al., 1995). Relaxin levels then decrease steadily during the remainder of the pregnancy, while joint laxity continues to increase until two weeks post-partum (Schauberger, et al., 1996). Thus, relaxin may initiate ligament laxity, which is then propagated in an environment of high levels of estrogen during late pregnancy.

The role of estrogen in the proliferation of human ACL fibroblasts and type I procollagen synthesis *in vitro* remains controversial. Yu (Yu, et al., 2001) demonstrated an inhibition of cell proliferation and collagen synthesis while Seneviratne et al. (Seneviratne, et al., 2004) demonstrated no effect. The effect of exogenous estrogen strength depends on the estrogen concentration and possibly on the species tested. The ultimate failure load of the ACL in ovariectomized rabbits decreased when subjected to pregnancy levels of estrogen (Slauterbeck, et al.; 1999). In contrast, ACL and MCL harvested from sheep subjected to physiologic estrogen levels or estrogen receptor agonists showed no difference in mechanical properties (Strickland, et al., 2003). Estrogen receptors have been identified in the human ACL fibroblast, providing evidence that the ACL is a target tissue for estrogen and may affect structure and composition (Liu, et al., 1996).

The affect of relaxin on collagen turnover has also been studied. Relaxin can cause alterations in collagen turnover by stimulating collagenase expression and by modulating collagen synthesis and secretion in human dermal fibroblasts (Unemori and Amento, 1990). In rats, relaxin reduces the density and organization of collagen fiber bundles as well as the length and degree of interdigitation of elastin fibers. The expression of relaxin receptor proteins in target cells is a prerequisite for hormone action. The relaxin receptor has yet to be characterized; however, relaxin-binding cells have been identified in smooth muscle and epithelial cells in the mammary glands, cervix, and skin of the pregnant pig and in multiple tissues of the rat, including the interosseous ligament (Unemori and Amento, 1990). Recent studies have identified relaxin receptors in the human ACL (Dragoo, et al.; 2003; Galey, et al., 2003).

The current study was designed to quantify human ACL relaxin receptors by radio-ligand binding assay and estrogen receptors by flow cytometry, and to compare the presence of these receptors in males and females.

MATERIALS AND METHODS

Cell culture medium, M199, Fetal Bovine Serum (FBS), 100x Antibiotics-Antimycotic (AbAm) solution containing 10,000U of penicillin/10,000 μg of streptomycin sulfate and 25 μg of fungizone/ ml, 10 x Phosphate buffered saline (PBS) were purchased from Gibco –BRL, Bethesda, MD. Monoclonal antibody to human estrogen receptor, saponin, sodium azide and bovine serum albumin were from Sigma-Aldrich, St. Louis, MO. Mouse IgG1 and Phycoerythrin(PE) linked Rabbit anti mouse IgG were from Caltag, Burlingame, CA. I^{125} labeled and unlabeled relaxin was kindly provided as a gift from Immundiagnostik AG, Bensheim,Germany.

Institutional Review Board approval (IRB# 97024) was obtained, and informed consent was given prior to harvesting the ACL stumps of 12 males and 14 females during reconstructive surgery for acute (<8 weeks) ACL injuries. Several samples were devoted to process development so final analysis was performed on eight females and seven males with mean age of 26.6 years and 32.3 years of age, respectively. Patients were excluded if they were pre-menarche or post-menopause. The average time from injury was 5.9 $\pm$ 2.2 weeks and 5.4 $\pm$ 1.8 weeks for female and male specimens, respectively. Neither age nor time from injury was significantly different using independent t-test for age ($p=0.2$) and Mann-Whitney analysis for injury (0.7).

The ACL stumps were cleaned free of synovium and minced so that only ACL fibroblasts would be cultured. The ACL tissue was placed in M199 cell culture medium containing 10% FBS and 1% AbAm and fibroblasts grown to confluence in a T-75 flasks in a cell culture incubator with 5% CO_2 and 100% humidity. Passage 2 cells were used for all studies to allow the cells to adapt to the culture media and to decrease any potential effects of local estrogen in vivo prior to tissue harvest. For estrogen receptor determination, single cell suspensions were prepared with 5 ml of trypsin-Edta (0.25%) for two minutes. When cells were detached, the trypsin was diluted with 10 ml of M199 and the suspension centrifuged at 400-x g for nine minutes. The cells were washed and centrifuged three times. Excess

M199 was decanted and the cells resuspended with 600 μl of FACS buffer (1x PBS containing 0.1% sodium azide and 0.1% bovine serum albumin) and filtered to remove debris. The solution was then pipetted (200 μl) into sample, negative, and positive flow cytometer tubes.

A fluorescent label is needed to bind each ligament estrogen receptor (ER) in order for the flow cytometer to "count" each receptor. The ER present on the *nuclear* membrane has not been quantified. A technique had been developed to quantify nuclear receptors in breast cancer cells(3);however, this technique proved too robust for the ligament cells. Through a series of experiments with titration of permeabilizing agents and cell handling modifications, a new method was developed to quantify fibroblast nuclear receptor. The cell membranes were permeabilized using 1% saponin (200 μl of 2 % saponin added to flow tubes) for 10 minutes. The tubes were centrifuged for 10 minutes at 400-x g. The cells were resuspended with 200 μl FACS buffer and incubated with 10 μl of monoclonal antibody to human estrogen receptor (Sigma, St. Louis, Missouri) for one hour. Controls were incubated with 10 μl of mouse IgG1. The tubes were then placed in ice for the remainder of the experiment. The cells were again washed, centrifuged, resuspended with 200 μl of FACS buffer, and incubated with 10 μl goat anti-mouse secondary antibody linked to Phycoerythrin for one hour. After washing, the cells were resuspended with 500 μl of 2% paraformaldehyde (PFH) and examined by FACScan flow cytometer using CellQuest software. Ten thousand fibroblasts were analyzed and gated (**Figure 1**).

Breast cancer cells, HTB22 fibroblasts (ATCC, Monassas, Virginia, US) known to express ER were used as positive controls. JURKAT lymphocytes known to be void of ER were used as negative controls.

Relaxin radioligand binding studies were performed on ACL cells from five female and five male specimens as described by Palejwala et al. (Palejwala, et al., 1998) with minor modifications. Approximately $3x10^5$cells/well of 24 well plates were washed with M199 containing 10mM HEPES, pH

7.2, 0.1mg/ml BSA, 1 μg/ml leupeptin, and 0.05 mM phenylmethylsulfonylflouride (binding buffer) for 1hr at 4^{o}C and then incubated with increasing amounts of labeled relaxin. In some wells in addition to labeled relaxin, several fold excess of either unlabeled relaxin or insulin was added. Cells were then washed 5x with 2ml of the binding buffer for 10 min. Cells were then dissolved in 125 mM Tris-HCl buffer, pH 6.8, containing 4% sodium dodecyl sulfate and radioactivity determined using standard techniques.

RESULTS

The intracellular estrogen receptor in ACL cells was quantified using flow cytometry. Consistent cell populations of viable fibroblasts were confirmed by characteristic forward and side scatter (which measure cell size and cell complexity, respectively) (**Figure 1**). The negative control specimens were analyzed by flow cytometer to set up the gate for nonspecific staining, M1 (**Figure 2A**). The MFI above the M1 region (M2) was that due to phycoerythrin (fluorescence) bound to ER (**Figure 2B**).

MFI for the eight female specimens was 519 $\pm$ 332 and for the seven male specimens was 602 $\pm$ 504. Statistical analysis using a Mann-Whitney analysis showed no significant difference (~1) in MFI and thus no difference in the number of ER in males and females. Four to 10 % of ACL cells expressed estrogen receptors. Mean fluorescence of cell expressing estrogen receptors was approximately twice that of controls with no significant differences between males and females.

There was increased binding of I^{125} labeled relaxin to human female and male ACL cells with increasing amounts of added labeled relaxin. This binding appeared to be slightly more in female than male ACL fibroblasts. Most of this binding was non saturable and non specific. The binding was not done in the presence of 100 Molar excess of unlabeled relaxin; therefore, the extent of specific binding and number of receptors/cell could not be determined in these studies.

Approximately 10%, 25% and 50% relaxin bound by female ACL fibroblasts was replaced by 28, 280 and 2800 fold molar excess of unlabeled relaxin respectively, suggesting that this binding is specific. (**Figure 3A**). The data reported here is from 5 female samples with one of the samples showing less than 10% loss of bound relaxin even in presence of 2800 fold excess of unlabeled relaxin. In contrast, relaxin bound by male ACL fibroblasts was not replaced by 28, 280 or 2800 fold excess of unlabeled relaxin. (**Figure 3B**). The data reported here is from 5 male samples with one of the samples showing 20% loss of bound relaxin in presence of 2800-fold excess of unlabeled relaxin. The relaxin bound by either male or female ACL fibroblasts could not be replaced by 280- or 2800-fold excess of insulin suggesting that binding of relaxin observed with ACL fibroblasts is independent of insulin binding to its receptor or other proteins.

DISCUSSION

The studies indicate that estrogen receptor levels are similar in ACL cells from males and females. The functional implications of this finding are unclear, and may depend on other local factors. For instance, circulating estrogen in females is 20 to 100 times that in males, but the level found in synovial fluid is unknown. ERs are activated not only by estrogen binding but also by growth factors. Thus, estrogen-independent binding may occur when local concentrations of growth factors are high or possibly when estrogen concentrations are low (e.g., in men and postmenopausal women).

Our results concur with the more recent literature that estrogen alone may not play a role in gender differences in ACL injuries. Earlier studies that found significant differences due to estrogen looked at effects of *supraphysiologic* hormone doses on mechanical properties and cell synthesis. Subsequent studies hone in on *physiologic* hormone doses and appropriate models to evaluate these differences.

By way of reference, the typical 28 day menstrual cycle consists of a menstruation period, followed by the follicular phase (**Figure 4**). Circulating estrogen levels during this proliferative phase are approximately 100 pg/ml. Ovulation occurs between day 10 and 14 when the estrogen levels peak (600 pg/ml). The cycle ends with the luteal phase when progesterone and relaxin levels peak. Estrogen levels in men and postmenopausal women are between 5 and 20 pg/ml (Schauberger, et al., 1996).

Lui et al were the first to show the human ACL as a target tissue for estrogen and concluded that sex hormones may affect its composition (Lui et al., 1996). ACL cells were obtained from an older population (average age 57 years) with varying degrees of pathology (osteoarthritis, tumor and ACL tears for which they underwent arthroplasty, amputation, and ACL reconstruction). These were collected from 13 women and 4 men. Most interesting is that 23% of the female specimens did not stain for estrogen receptors. In a subsequent study, Lui et al reported that rabbit ACL cultured fibroblasts exposed to supraphysiologic levels of 17B-estradiol had a 40-50% reduction in collagen synthesis as evidenced by incorporation of radio-labeled hydroxy proline and reduced fibroblast proliferation as determined by H^3 –thymidine incorporation) (Liu, et al., 1997). A more recent study by Seneviratne et al exposing cultured ovine ACL fibroblasts to physiologic levels (2.2 to 250 pg/ml) of 17B- estradiol demonstrated no effect on collagen synthesis and fibroblast proliferation (Seneviratne et al., 2004). In support of both studies, Yu et al presented dose and time dependent effects of 17B-estradiol on ACL cells derived from a 32-year-old woman undergoing a total knee arthroplasty for traumatic arthritis (Yu, et al., 1999). They saw a decrease in fibroblast proliferation with increasing 17B-estradiol concentration on day one and three of exposure but the dose-dependent effect diminished by days seven and fourteen. Procollagen I synthesis (as measured by I^{125} labeled monoclonal antibody to procollagen I) revealed a similar pattern. He concluded that rhythmic variations in estrogen during a menstrual cycle might have an effect on ACL fibroblast metabolism.

Multiple studies have reported on ACL injuries during the different phases of the menstrual cycle. Wojtys et al showed a higher than expected injury rate during ovulation (when estrogen peaks) in a retrospective study of 28 females with non-contact ACL injuries (Wojtys, et al., 2002). This effect was negated with oral contraceptive use. In contrast, Myklbust, et al. found significantly fewer injuries during ovulation in 17 handball players (Myklbust, et al., 2003); however, more of these women were on oral contraceptives (OCP). This was confirmed by a Swedish study looking at all injuries (not just ACL) in 86 soccer players over a 12-month period (1008 cycles). Injuries occurred most often in the premenstrual and menstrual phases. Women taking OCPs had a lower overall injury rate (Moller-Nielsen and Hammar, 1989). These studies have relied on the athletes' history of when in the cycle their injury occurred. A more recent study confirms validity of self-reported menstrual history (taken within three days of injury) with salivary sex-hormone levels. Slauterbeck et al support the results of the previous two studies that ACL injuries correlate with the follicular phase of the menstrual cycle (Slauterbeck, et al., 2002). Karageanes, et al. examined the proposition that hormonal laxity predisposes to injury. They found no significant difference in KT-1000 measurements between the luteal, follicular, and ovulatory phases in 26 female athletes over an 8 week period (Karageanes, et al., 2000).

This would suggest that macroscopic mechanical properties remain intact while, possibly, microscopic hormonal changes are more contributory. Early work suggested estrogen exposure did decrease ACL mechanical strength in ovariectomized New Zealand white rabbits. However, this 30-day exposure was supraphysiologic (Slauterbeck, et al., 1999). In a subsequent study, Strickland et al found no difference in ACL strength, stiffness, or load to failure between control, ovariectomized, and ovariectomized ovine with physiologic estradiol implants (Strickland, et al., 2003).

These studies suggest that significant "measurable" laxity is not contributing to increased ACL injury. Most published studies have concentrated on estrogen as the key to gender differences in ACL

injuries. Estrogen may very well play a role in this injury mechanism but require other sex hormones (such as relaxin) or growth factors. Estrogen receptor density may be the same between genders, but a specific hormonal milieu may be necessary to activate them.

Given the role relaxin plays in providing laxity to tissues during pregnancy, we hypothesized that relaxin is that necessary component. In our preliminary study, we found a difference in the concentration of relaxin binding sites between males and females. This result has recently been confirmed by another group using immunohistochemistry techniques (Dragoo, et al., 2003). The significance of differences in binding of relaxin to ACL cells from males and females is not clear. Interestingly, relaxin peaks during the luteal phase. Many clinical studies found increased ACL injuries in the follicular phase (Moller-Nielsen and Hammar, 1989; Myklebust, et al., 1998). It may be that estrogen priming occurs during the ovulatory phase, followed by a peak in relaxin during the luteal phase causing an increase in collagenase production and, subsequent predisposition to injury during the follicular phase. Further studies are needed to determine the role of estrogen and relaxin in acute ACL injury.

The spectrum of tissue extensibility from extreme laxity to fibrosis or contracture comprises a wide area of disease processes across medicine (multidirectional instability, scleroderma, Dupuytren's, adhesive capsulitis, arthrofibrosis). Despite the limited understanding of how hormones affect tissues, they are being used to treat extreme disease states. Relaxin is FDA approved for use in extreme cases of fibrosis secondary to scleroderma (Sapadin and Fleischmajer, 2002). Animal models have been developed to look at the effects of hormones on joint contractures during pregnancy and show a trend toward decreased severity of contractures (Ohtera, et al., 2002). Further study of hormonal effects on tissues may help us better understand disparate disease processes and lead to methods of preventing the initial triggers.

There are several limitations to our study. First, our ACL samples were derived from post-injury specimens (several weeks after injury). It is unclear what effect inflammatory cells may have on receptors. In this injury model, receptors more appropriate for healing may be up-regulated, whereas those for homeostasis may be downregulated. Similarly, receptors in a rapidly growing population of fibroblasts may have a different receptor profile than those at homeostasis. Second, although flow cytometry is an excellent method to quantify receptors, it provides no data on the affinity or function of the receptor. Binding studies would be necessary to study this important concept. Moreover, function and affinity may change depending on the hormonal milieu, further complicating the validity of comparing gender differences.

We did try changing the "milieu" of the medium when we initially found no difference in receptor level between males and females. Since M199 medium contains eight picograms of estrogen whereas "estrogen-free" serum contains 2.2 picograms of estrogen, we hypothesized that the small amount of estrogen in our medium may have caused up-regulation in the male specimens. Five samples (3 female and 2 male) were run with both M199 and the low estrogen serum. No difference was found in estrogen receptor count when specimens were incubated in the two media.

In summary, estrogen receptor levels were similar in ACL cells from females and males, while relaxin receptor levels were significantly higher in female cells. Although ACL injuries in women is likely to be multifactorial, the differential effects of relaxin binding in female ACL cells and male ACL cells may play a role in modulation of cellular response to estrogen. Further studies are needed to determine the role of estrogen and relaxin in acute ACL injury.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Margaret Peterson, PhD for her statistical analysis of the data and Steve Merlin, PhD for his expertise on flow cytometry. We would also like to acknowledge

Immundiagnostik (Dr. Armbruster, CEO) for their generous supply of relaxin. As the sole supplier of labeled relaxin at that time, these studies would not have been possible without their collaboration. Grant support was graciously provided by The Soft Tissue Research Fund at the Hospital for Special Surgery.

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TABLE

Table 1: Specimen Demographics and Fluorescence

Female specimen	Age (yrs)	Injury (weeks)	Fluorescence (MFI)	Male specimen	Age (yrs)	Injury (weeks)	Fluorescence (MFI)
3	23	7	551	5	41	4	257
6	21	6	277	12	33	8	214
8	27	3	270	13	27	8	1350
10	34	8	924	17	36	5	180
18	24	2	411	22	36	4	643
21	42	7	168	28	26	5	310
25	14	8	1106	29	27	4	1259
27	28	6	444				

MFI, mean fluorescence index

FIGURE LEGENDS

Figure 1: Representative sample from FACScan cytometer. The y-axis is forward scatter, which measures cell size. The x-axis is side scatter, which measures cell complexity. This scan is characteristic of viable fibroblasts. 10,000 cells were gated and analyzed.

Figure 2A: MFI (Mean Fluorescence Intensity) set to exclude 99% non-specific staining
Region M1 is amount of fluorescence with negative control (background noise). Thus, any fluorescence above that (region M2) would account for fluorescence due to estrogen receptors. The y-axis is number of receptors and the x-axis is MFI.

Figure 2B: Representative ACL sample showing MFI output from FACScan cytometer
M1 fluorescence is excluded as non-specific staining; M2 fluorescence is due to binding of ERs.

Figure 3A: Displacement of I^{125} ACL relaxin bound to human female fibroblasts by excess unlabeled relaxin

Figure 3B: Displacement of I^{125} relaxin bound to human make ACL fibroblasts by excess unlabeled relaxin

Figure 4: Typical ovulatory cycle

Figure 1

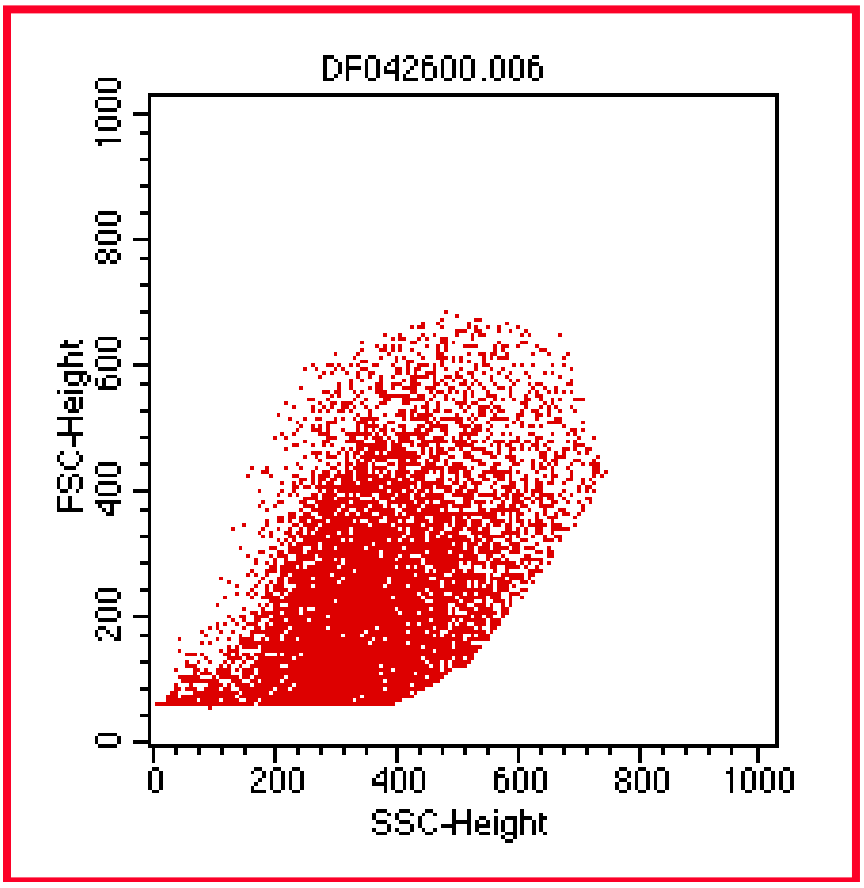
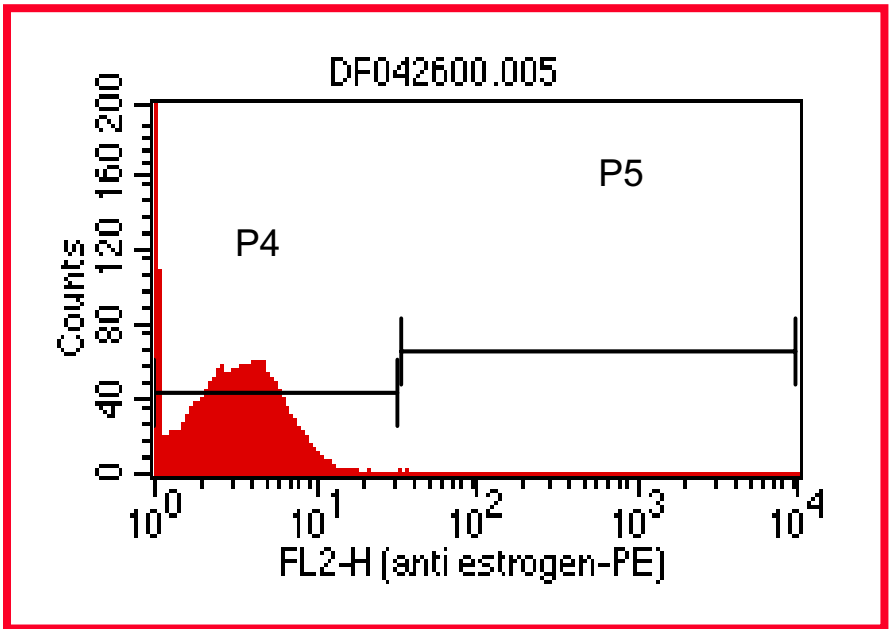


Figure 2A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P5
P4
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
FL2-H (anti estrogen-PE)

Figure 2B

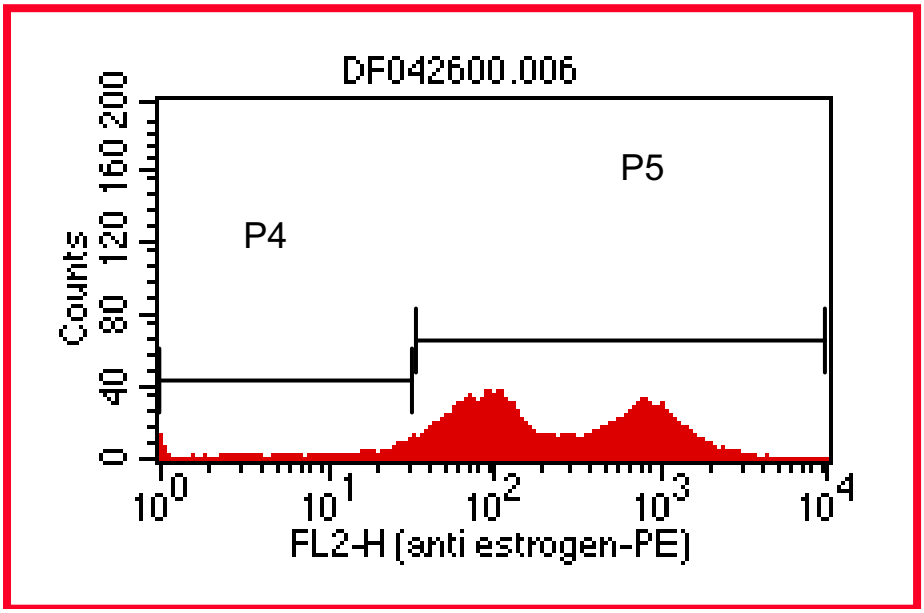


Figure 3A

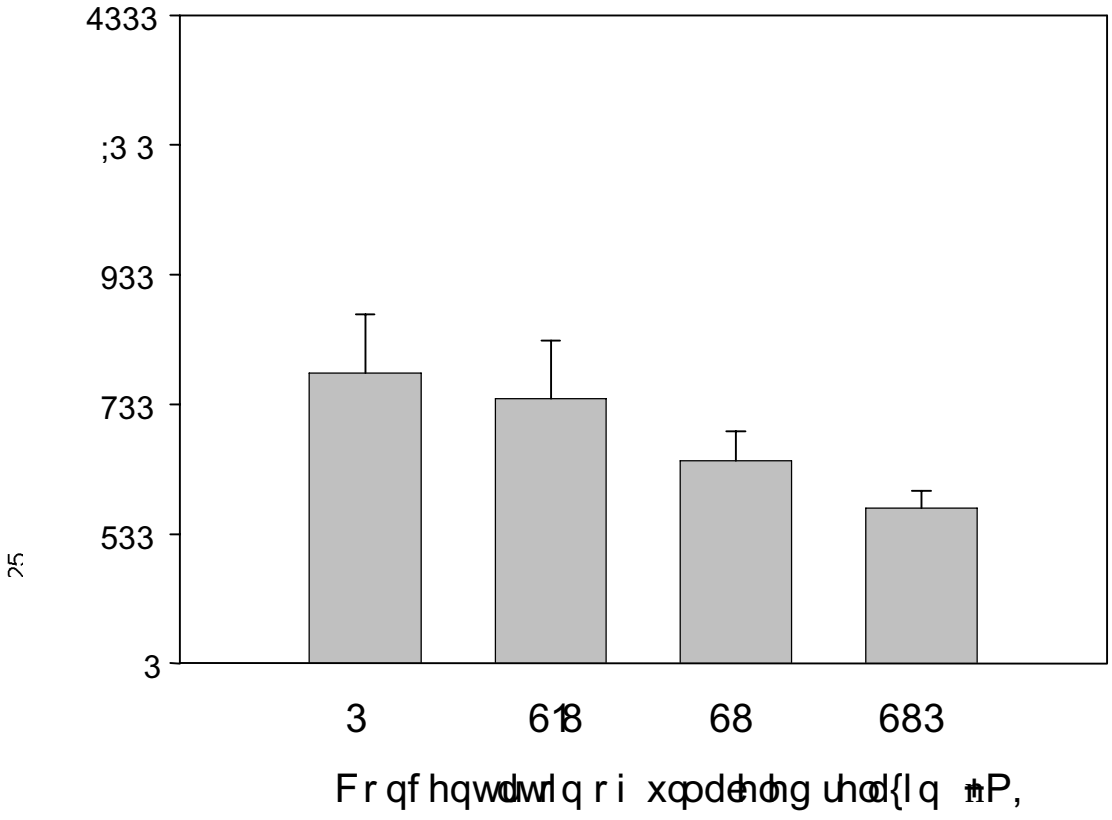

4333
;3 3
933
733
533
3
3
68
683

Figure 3B

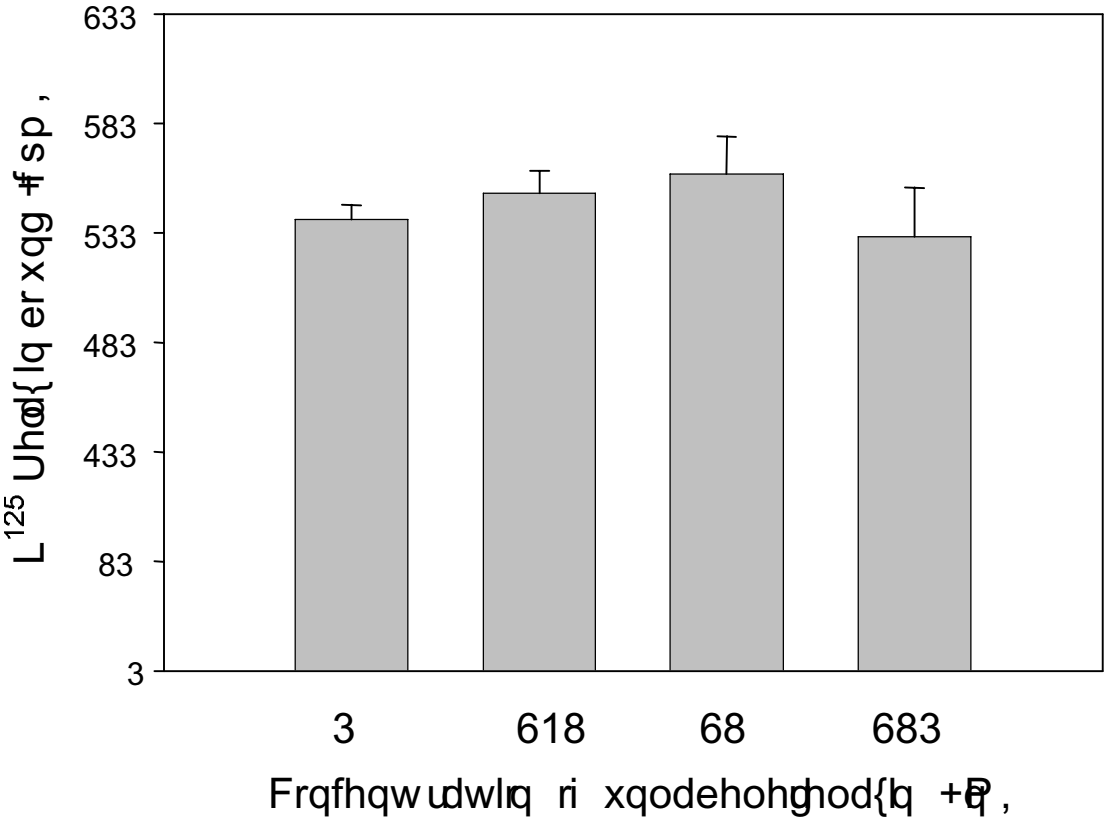
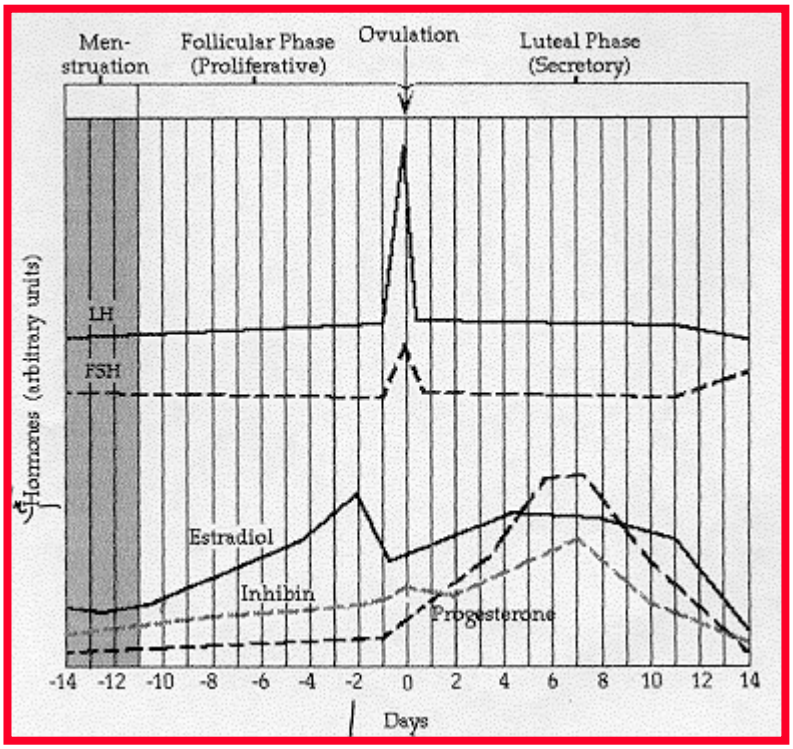


Figure 4


Men-
struation
Follicular Phase
(Proliferative)
Ovulation
Luteal Phase
(Secretory)
Hormones (arbitrary units)
LH
FSH
Estradiol
Inhibin
Progesterone
-14 -12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
Days