The effects of hormone replacement on the biomechanical properties of the uterosacral and round ligaments in the monkey model

Michael D. Vardy, MD,a,b,c,* Thomas R. Gardner, MCE,a Felicia Cosman, MD,a,b Richard J. Scotti, MD,e Magdy S. Mikhail, MD,c A. Orahn Preiss-Bloom, BS,a J. Koudy Williams, DVM,d J. Mark Cline, DVM, PhD,d Robert Lindsay, MD, PhD,a,b

Columbia College of Physicians & Surgeons, New York, NY,a Helen Hayes Hospital, West Haverstraw, NY,b Albert Einstein College of Medicine, Bronx, NY,c Wake Forest University School of Medicine, Winston-Salem, NC,d and USC Keck School of Medicine, Los Angeles Calif,e

Received for publication April 21, 2004; revised October 11, 2004; accepted October 21, 2004

KEY WORDS
Estrogen
Ligament
Monkey
Biomechanics
Pelvic organ prolapse

Objective: The purpose of this study was to determine effects of ovariectomy (OVX) and conjugated equine estrogens plus medroxyprogesterone acetate (CEE/MPA), or ethinyl estradiol plus norethindrone acetate (EE/NA) on biomechanics of uterosacral (USL) and round (RL) ligaments in postmenopausal (PMP) monkeys.

Study design: This was a randomized, triple blind, placebo-controlled study. OVX monkeys received 12 months no treatment (Pbo) (n = 19), CEE/MPA (n = 19), or EE/NA (n = 21). USL and RL step strains and stress-relaxation data were curve-fitted, giving strain-dependent tensile modulus (TM) from 0% to 30%.

Results: (1) USL: TM for both treatment groups was greater than Pbo for strains from 0% to 12% (P < .04). (2) RL: TM for both treatment groups was smaller than Pbo for strains from 12% to 30% (P < .05). No differences were found between treatment regimens.

Conclusion: CEE/MPA and EE/NA both affect functional biomechanical properties by increasing tensile stiffness in the USL and decreasing it in the RL.

© 2005 Elsevier Inc. All rights reserved.
Estrogen receptor (ER) α and ERβ are known to be present in the major pelvic support structures in women, including the vaginal wall and uterosacral ligaments. Changes in the expression of these receptors have been shown with change in menopausal status, and these changes have been shown to be tissue dependent. Biomechanical studies with animal models have demonstrated that estrogen can affect change in ligament strength, although these have been largely limited to the orthopedic literature and to joint ligaments. The effect of estrogen on the biomechanical properties of pelvic supportive ligaments has never been evaluated. Several biomechanical principles important for the understanding of this work are defined in Table I.

Pelvic organ prolapse (POP) is an adverse event that has been associated with several selective estrogen receptor modulators (SERMs). Clinical trials with the SERM leovormeloxifene (Novo) were discontinued primarily for endometrial concerns; however, pelvic organ prolapse was reported as an adverse event associated with the drug. Double-blind placebo-controlled trials with idoxifene (Smith Klein Beecham) were stopped prematurely because of an increase in uterine prolapse and polyps. Recently, a double-blind randomized, placebo-controlled trial demonstrated an increase in POP with raloxifene and tamoxifen compared with CEE and placebo.

Cynomolgus monkeys have served as nonhuman primate models in several studies of aging, including monkey models of menopause. The pelvic anatomy of the Macaca species is almost identical to that of the human (Figure 1), providing a unique opportunity to study an analogous support system.

The objective of this study was to evaluate the effects of menopause and 2 common regimens of hormone replacement therapy on the biomechanical properties of pelvic supportive ligaments in the monkey model of menopause.

**Material and methods**

We carried out this randomized, triple-blind, placebo-controlled study on adult female feral cynomolgus macaques (Macaca fascicularis) ranging from 6 to 8 years of age obtained from the Primate Research Center of Bogor Agricultural University (Bogor, Indonesia). Animals of this age from this colony are typically multiparous. Information of the individual parity was not available.

The typical life span for this species is 30 years, and the average age at menopause is approximately 20 years. All procedures were conducted in accordance with state and federal regulations and were approved by the Institutional Animal Care and Use Committee of the Wake Forest University School of Medicine, Winston-Salem, NC, which is accredited by the Association for the Advancement and Accreditation of Laboratory Animal Care. Animals were ovariectomized and randomized to receive 12 months of either no treatment (OVX) (control, n = 19), or conjugated equine estrogens plus continuous medroxyprogesterone acetate (CEE/MPA) (n = 19), or ethinyl estradiol plus norethindrone acetate (EE/NA) (n = 21) at doses that were scaled from those doses taken by an average women.

Drug doses were calculated based on the assumption that an average woman consumes approximately 1800 calories per day. Monkeys were fed 120 calories of diet per kilogram of body weight and, therefore, consumed 0.042 mg/kg body weight of CEE plus 0.169 mg/kg body weight of MPA and 0.339 mg/kg body weight of EE plus 0.068 mg/kg body weight of NA. At the end of this period, animals were humanely euthanized (100 mg/kg pentobarbital intravenously). Multiple organ systems were evaluated; other end points are reported by Suparto et al.

The USL and RL were harvested immediately after sacrifice, wrapped in gauze soaked in physiologic saline.
(0.15 mol/L), and stored at \(-25^\circ\)C until testing. The USLs were identified at necropsy with gentle dorsal traction of the uterus (Figure 2). A silk suture was placed at the uterine insertion, and a second suture 1.5 cm ventral to the first suture (original lengths of the ligaments in vivo were 1.5-2 cm total). The ligament in between the 2 sutures was dissected around the sutures (Figure 3A). Before testing, the USL specimens were defrosted and surrounding tissues that were clearly nonligamentous were trimmed from the ends and surface of the ligaments. The resulting tested lengths of the ligaments ranged from 5 to 15 mm. Small tabs of emery cloth were carefully attached to each end of the specimen with cyanoacrylate, taking care to avoid excess cyanoacrylate on the portions of the specimen not covered with emery cloth (Figure 3B). The main reasons for the dissection was to ensure a direct application of the emery tags to ligament rather than only the peritoneal sheath, which is of critical importance to ensure that no slippage of the material occurred during testing and accurate strain measurements were obtained. The specimen was then refrozen wrapped in gauze soaked in physiologic saline. On the day of testing the specimen was defrosted by placing the sealed specimen bag in a beaker of water at room temperature for 30 minutes. The specimen was then mounted in the testing device (Figure 4A-D). A 5-g tare load was applied to determine the initial test length of the specimen. The ligament was then mechanically tested by the same blinded tester using the technique of Akizuki et al\(^8\) with a computer-controlled custom made tensile testing apparatus (Figure 5). The device applied a series of step strains (Table I) ranging from 5% to 30% (Figure 6A) and measured the resultant force in the ligament (Figure 6B), which was continuously bathed in physiologic (0.15 mol/L) saline.

Digital photographs of the specimen were taken at the 5% and 30% strain levels (Figure 4), as well as during failure. A calibrated grid, mounted directly behind the test specimen, was captured in all photographs. The specimens were allowed to stress-relax at each strain level, and the resulting equilibrium strains were curve-fitted using an exponential function to obtain the strain-dependent tensile modulus from 0% to 30% strain. Upon completion of the step strains, the specimen was then tested to failure by applying a ramp strain of 0.1 mm/sec. Failed ligaments were then refrozen for future histologic and immunohistochemical analysis.

To compute the engineering stress, the cross-sectional area of the specimen was obtained from the average width and thickness of the specimen. The specimen width was measured from the digital photograph taken during the 5% strain stage of testing. The measurement function of Scion Image (Scion Corporation, Fredrick, Md) was used to obtain the width measurements, using the grid behind the ligament testing area as an accurate scale. Three width measurements were taken at different locations along the ligament, and the average was obtained. Specimen thickness was manually measured.
using a digital caliper with 10-μm resolution at 3 evenly spaced locations along the length of the specimen as a 3-g load was applied to provide a nominal tension. The average of these 3 width and 3 thickness measurements was used to calculate the cross-sectional area. The calipers were aligned by inspection and, therefore, were subject to a potential small examiner error. Errors in cross-sectional area would be expected to inversely affect the stress calculations. The experimental apparatus, which had a resolution of ± 1 microns, was used to calculate the original length and change in length and resulted in measurement errors in strain and overall length much less than 1%.

The failed ligaments were refrozen to −25°C for future histologic and immunohistochemical analysis. A trichrome stain was applied to several ligaments after testing and failure. This identified a general pattern of collagen in the ligaments. The digital photos of the stained ligaments were compared with the longitudinal sections to confirm the methodology appropriately aligned the ligaments in the test device.

Individual, one-way analysis of variances (ANOVAs) with treatment as the factor were run for both ligament types to determine if there were statistically significant differences in the tensile moduli between treatments with tensile moduli at 0%, 6%, 12%, 18%, 24%, and 30% strain and failure as the variables. A Student-Newman–Keuls multiple comparisons test was used to discern differences between treatment regimens. A value of \( P < .05 \) was taken as representing a statistically significant difference.

Results

At necropsy, there were no significant differences in the mean body weights of the groups. The mean body weights, adjusted for baseline body weights for the groups, were 3.00 ± 0.11 kg for the nontreated OVX group, 3.36 ± 0.12 kg for the CEE/MPA group, and 3.04 ± 0.10 kg for the EE/NA group (ANCOVA, \( P = .19 \)).

The average stress strain curves for the uterosacral ligaments and round ligaments are provided in Figure 7A.
and B, respectively. For the uterosacral ligament, the tensile moduli for both treatment groups were statistically larger than that of the nontreated OVX monkeys for strains ranging from 0% through 12% (eg, $0.08 \pm 0.05$ MegaPascals [MPa] OVX, $0.32 \pm 0.28$ MPa EE/NA, $0.35 \pm 0.47$ MPa CEE/MPA at 12% strain [$P < 0.04$]) as shown in Figure 8. This trend continued for strains 18% through 30%, but was not statistically significant. No differences were found between treatment regimens for the uterosacral ligament. For the round ligament the tensile moduli for both treatment types were found to be statistically smaller than that of the nontreated OVX monkeys for strains ranging from 12% through 30% (eg, $3.19 \pm 2.62$ MPa OVX, $1.33 \pm 1.07$ MPa EE/NA, $1.28 \pm 0.88$ MPa CEE/MPA at 12% strain [$P < 0.05$]). Once again, while statistic significance was not achieved at the lower percentages, the trend clearly continued as shown in Figure 9. No differences were found between treatment regimens for the round ligament.

Mechanical and load limitations in the testing device, as discussed below, precluded obtaining a clearly defined failure load for a number of specimens. Given this uncertainty, failure stress was found to be $2.1 \pm 1.1$ MPa for the round ligaments ($n = 40$) and $0.6 \pm 0.4$ MPa for the uterosacral ligaments ($n = 53$). Failure stress of the round ligament was found to be statistically higher ($P = 0.024$) for the OVX monkeys than for either treatment. No statistic difference in failure stress was found between OVX monkeys and either treatment for the uterosacral ligament. The inability to obtain failure load did not preclude the calculation of tensile modulus. These ligaments still were evaluated, but only the failure load data (the end of the stress-strain curve) was not included in a small number on tests.

Comment

Both CEE/MPA and EE/NA affect the functional biomechanical properties of both the USL and RL in the monkey model, but with opposite effects. These results suggest that supportive ligaments may be end organs for hormonal effect. It may be suggested then that the diminished hormonal levels in the PMP state and/or hormone replacement therapy may impact on ligament support function and pelvic organ prolapse. The implications of this effect may be considerable, with pelvic organ prolapse contributing significantly to morbidity, including incontinence in pre- and postmenopausal women. Two hundred thousand women have surgery for prolapse, and 135,000 for urinary incontinence annually in the US. Adding to the long list of health considerations that need to be considered when contemplating hormone replacement therapy, women may now include pelvic support.

Treatment greatly increases stiffness in the USL while decreasing stiffness in the RL. The significant but opposite effect seen by both regimens on these ligaments, which are functionally very different, is particularly compelling. We might surmise that the uterosacral ligament, which serves as the main anchor of the uterus to the sacrum, needs to maintain this support as the mass of the average human uterus increases from 80 g to about 6000 g in the pregnant state, including a full-term fetus, amniotic fluid, and placenta. An increase in the biomechanical stiffness of the USL, when subjected to the increased stresses posed by pregnancy because of the much heavier uterus, may help prevent prolapse of the uterus. This increase in stiffness under hormonal influence provides an attractive teleologic explanation of how the body may adapt to tolerate the vast increase in loading imposed by pregnancy without concomitant excessive motion (laxity) of the uterus at the uterosacral junction. Unfortunately, we were unable to test the cardinal ligaments due to budgetary and storage limitations. We would expect the USL and cardinal ligaments to be functionally similar and, therefore, we would hypothesize that they may be similar in their response to hormonal influence. Our hope with this and future works is to systematically standardize and test all the supportive structures of the pelvis in this model.

It is noteworthy that these animals are typically infected with a herpes virus (70% of the colony). While the virus is not dangerous for the monkeys, it can be rapidly fatal for humans. Fixation deactivates the virus, but would render the tissue unsuitable for biomechanical testing. Hence, only fresh frozen tissue was biomechanically tested for this study, with appropriate safety precautions and biosafety level. Pending future funding, we plan to evaluate cardinal, paravaginal, and vaginal tissues important for pelvic support. The ligaments tested were refrozen after failure and are to be evaluated for collagen type, ER/PR, and trichrome stain.

Conversely, the round ligaments go through very different changes with pregnancy. They typically triple in length by full term, and rapidly return to their original length after delivery. The decrease in stiffness seen in the round ligaments would facilitate this large increase in length without inducing excessive stress in
the ligaments because of the large strains. As can be seen from Figure 9, this softening effect becomes even more pronounced at higher strain levels, further supporting the concept that the influence of estrogen and progesterone on the round ligament enhances its ability to undergo extensive stretching as the uterus grows. The decrease in stiffness observed in the round ligaments in this study under the influence of hormonal treatment again teleologically fits well with what is seen in nature throughout pregnancy and postpartum.

This study supports the hypothesis that hormonal status plays a role in pelvic support, and that menopausal status is a risk factor for prolapse. Application of these methodologies in the evaluation of SERMs might also help test the hypothesis we have previously proposed based on clinical observations, that various SERMs might also uniquely alter the biomechanical properties of pelvic supportive tissues.4

It has been proposed that the uterosacral ligament fails typically at the insertion to the uterus. This hypothesis might be tested by including the insertion site in the test setup with uterine tissue included with dissection and mounting. Unfortunately, this study was not designed to answer that important clinical question. Nor was this study designed to answer whether various portions of the USL have differing viscoelastic properties.

Figure 6  (A) A series of applied step strains as a function of time and (B) the resultant force in a uterosacral ligament.
These viscoelastic properties are the result of the widely differing histologies of supportive structures. All stress-strain curves were monotonically increasing; that is, as the level of strain increased, the stress required to achieve the higher level of strain also increased. The peak in force at each step strain followed by relaxation (Figure 6B) is believed to be a result of the inability of the material to instantaneously respond to the suddenly applied load, momentarily acting as an almost incompressible material, followed by the more gradual ‘squeezing’ of water out of the ligament in conjunction with relaxation of the polymeric collagen chains that occurs during the prolonged relaxation phase. Failure of the ligament commences once loads are applied at a high enough level that all the collagen fibers are loaded to various degrees and the most highly loaded fibers begin to fail.

There were several limiting factors associated with this study. The most problematic was the difficulty in identifying, preparing, and mounting these ligaments. Unlike the ligaments typically evaluated by our orthopedic colleagues, these tissues were found to be quite nonhomogenous, containing loose areolar connective tissues with collagen, elastin, smooth muscle, as well as peritoneum and fat tissue. For the uterosacral specimens, a well-formed, clearly defined ligament could not reliably be identified without some level of strain, and early attempts to dissect out individual uterosacral ligament bundles in vitro proved futile. Individual ligamentous fibers did resolve when the tissues were mounted and subjected to stretch, but these fibers showed a varied and somewhat diffuse pattern, with regions of nonligamentous material, even at high strains. Also, the placement of the emery tags was done by hand, and the axis was determined by inspection and is subject to human error. The only control for this is the digital photodocumentation of each ligament during testing, which did confirm the proper longitudinal axis of the specimen. Misalignment could affect our findings. The method of collection and tagging of the ligaments allowed a clear identification of the longitudinal axis of the ligament sample with tension on the 2 sutures (Figure 3A) at the ends of the sample. With the ends under even a minimal amount of tension, ligamentous tissues coalesced into a band. When this tissue is dissected while not under tension, the clearly identifiable bands start to dissolve into strands, and isolation of nonligamentous tissues becomes problematic. For this reason, only the minimum amounts of tissue well away from the ligament bands were excised.

The most difficult part of testing these ligaments is reliably mounting them. The challenge is in avoiding failure at the clamp site, which can make the data suspect. Several different methodologies were attempted before arriving on the final procedure for testing the USL. A series of pictures from in vivo (Figure 2) through the mounting preparation process (Figure 3) should provide adequate ability to reproduce our methods. It should be noted that our method eliminated the problem of breakage at the clamp site.

When working with a larger clearly identifiable ligament, biomechanical testing may be done either by using a dumbbell shaped ‘cookie cutter’ to punch out a dumbbell-shaped section of isolated ligament for testing versus testing the entire undissected ligament. The latter is frequently done for the human anterior cruciate ligament, and was done for both ligaments in this study. The use of a dumbbell-shaped specimen allows one to control the test area, normally forcing failure to occur in the middle of the dumbbell. This is thought to provide a more accurate assessment of the true material properties of the ligament. This technique is frequently used in broad type ligaments, or tissues where the material is thought to have a multidirectional loading vector and, correspondingly, anisotropic material properties (different responses in different directions of loading). The use of the full ligament is thought to provide more of a structural property; however, when the cross-sectional area is used, an average stress and strain may be computed. The ligaments used in this study did not lend themselves to the dumbbell sectioning and seemed to have a clearly defined direction of loading. Therefore, they were considered to be better suited to a structural type of material testing, the results of which can be more directly attributable to how the ligament may perform in vivo, rather than testing
subcomponents of the ligament. A presumption based on the experience of our orthopedic colleagues was that the extraneous tissue trimmed had negligible effect on the measured parameters.

This was much less problematic for the round ligaments, though their mechanical properties were affected by the presence of a central artery, lymphatics, and nerves, enclosed in a duplicature of peritoneum, which ran through the RL specimen. Therefore, because of the somewhat heterogeneous nature of the specimens, with ligamentous fibers embedded throughout, it was decided to test both the uterosacral and round specimens as prepared, and then obtain the engineering material properties of the specimen by applying the initial cross-sectional area at a minimal tare load.

A question that should be addressed is what effect the freeze thaw cycles have on the ligaments tested. This has been studied in many different animal models, and effect of a freeze thaw cycle and duration of freeze is felt to be negligible. The duration of freezing was unfortunately not standardized, but was nominally the same for all specimens, with minor variations due to different harvest and testing times. But more importantly, the duration of the thaw cycles was standardized, as was the amount of time spent thawed. Nonetheless, all specimens underwent the same overall number of freeze cycles.

Figure 8  Tensile modulus of the uterosacral ligament as a function of strain. Error bars denote standard deviations, * denotes where CEE/MPA and EE/NA are significantly different from OVX ($P < .05$).
thaws (two) to control as well as possible for any effect. Unpublished studies by one of the authors on the effects of freezing on bovine articular cartilage have shown that freeze-thaw cycles may affect the permeability of soft tissues, but it does not seem to affect the modulus of the tissue. Most importantly, although a number of studies have looked at the effects of freeze-thaw cycles on the material properties of various soft tissues, and as far we know, no study has demonstrated a differential effect (a different effect for one treatment group than the other treatment group) of freeze-thaw cycles on any material property. Because this study, as most material property studies, is looking at the relative difference between material properties of 3 different treatment groups, all of which have undergone the same number of freeze-thaw cycles and approximate duration of freezing, it is reasonable to assume that basic conclusions of this study are unaffected by freezing and thawing.

A second factor was the low aspect ratio (length to width) of the uterosacral specimens due to the inherent geometry of these ligaments. The inhomogeneous structure of this ligament, as discussed above, precluded cutting a standard dumbbell shape test specimen. However, the mounting techniques discussed above securely held the ends of specimens, with minimal grip effects, and resulted in what appeared to be fairly
uniform strain in the body of the ligament throughout the loading procedure. This was not an issue for the round ligaments because of their relatively small and uniform cross-sectional area compared with their length.

Another limitation was the difficulty in obtaining failure loads for the uterosacral ligaments. Although failure loads were obtained for most of the round ligaments because of their much smaller size, only about half of the uterosacral ligaments could be taken to failure. However, the inability to take all of the specimens to failure was due solely to equipment limitations (either reaching the excursion limit of the grips before complete failure and a limited range of the associated load cell) and had no reflection on mounting techniques. No specimen, either round or uterosacral, had either slippage or failure at the grips. Therefore, a number of uterosacral ligament failure tests had to be stopped either because the limit of the load cell was reached, or because the ligament had started to fail, but maximum excursion of the grips was reached before a majority of the ligament fibers were stretched to the breaking point. We were therefore unable to evaluate failure of these specimens, and excluded them from the analysis of this parameter. It is important to note that valuable viscoelastic information (including the tensile modulus) was still obtainable without the failure data. Other valuable viscoelastic information about the tissue can be obtained with additional, more technical analysis of the methodology used and vast data collected, and we hope to present such analyses in the future. When failure was obtained, a typical failure occurred in a “snapping” fashion with the ligaments retracting to their opposing sides inside an intact peritoneal sheath of other tissues, which resulted in a small remaining resistance to continued loading. This has led the authors to wonder if the utilization of these ligaments (in some cases) in pelvic reconstruction may be using a failed ligament or a sheath devoid of a functional ligament.

Finally, although this species is our closest genetic cousin and the anatomy is almost identical to that of the human, there are some important differences. The change from semi-quadrupedal habits to biped with the resultant vector changes on the support system is likely to play some role in prolapse in humans. The vertical support needs are thought to be less for the monkeys. We believe that by identifying a change in the material properties of the ligaments in this close genetic cousin we provide a strong argument for generalizability of these findings to humans.

We believe that continued application of the applied methodologies to the supporting structures of the pelvic organs may provide a biomechanical understanding of the progression of pelvic organ prolapse. In addition, these methodologies could also be useful in studying the wide range of materials currently being used today in pelvic reconstructive surgery. Much focus has been given to ‘tissue ingrowth’ in various materials such as polypropylene mesh, a process that may very well also be under hormonal influence. Regardless of hormonal effect, a better understanding of the biomechanical properties of the variety of materials used in reconstructive surgery today, and how these properties change as tissue ingrowth progresses over time, may add science to an area driven largely by opinion and conjecture.

Much work is yet to be done and methodology needs to be standardized to allow comparison of the various heterogeneous tissues considered important in the function of pelvic organ support. Potential treatment regimens should also be evaluated with respect to their role in pelvic floor support or failure, as these mechanisms become further elucidated, refined, and utilized. Continued application of these methodologies to the supporting structures of the pelvic organs may help provide a better understanding of the progression of pelvic organ prolapse.

Acknowledgments

The authors would like to thank Dr Van C. Mow for the use of the tensile testing apparatus that was developed by his graduate students and postdoctoral fellows at the Biomechanics Research Laboratory at Rensselaer Polytechnic Institute and at the Orthopaedic Research Laboratory at Columbia University. We are grateful to Dr Thomas B. Clarkson for his facilitation of this work. We are indebted to Ms Dianna Swaim and Ms Jean Gardin for their valuable technical expertise.

References

