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Analysis of collagen status in premenopausal nulliparous women with genuine stress incontinence

***Declan P. Keane** *Research Registrar*, †**Trevor J. Sims** *Chief Laboratory Technician*,
***Paul Abrams** *Consultant (Urology)*, †**Allen J. Bailey** *Professor*

**Bristol Urological Institute, Southmead Hospital, Bristol; †Muscle and Collagen Research Group, University of Bristol, Langford*

Objective To determine if differences exist in the collagen status of premenopausal nulliparous women with genuine stress incontinence compared with continent controls.

Design Thirty-six premenopausal nulliparous women with urodynamically-proven genuine stress incontinence were compared with 25 controls. All the women studied had a periurethral vaginal biopsy taken of approximately 30–50 mg in wet weight. This biopsy was then analysed to determine the collagen content, the type I:III collagen ratio and the collagen cross-link content.

Setting A tertiary referral urodynamic unit.

Results The nulliparous women with genuine stress incontinence had significantly less collagen in their tissues ($P < 0{\cdot}0001$) compared with the continent controls. In addition, there was a decreased ratio of type I to type III collagen ($P = 0{\cdot}0008$), and the cross-link content was also significantly reduced in the women with genuine stress incontinence ($P < 0{\cdot}0001$).

Conclusion Genuine stress incontinence is present in a significant number of women before childbirth. The aetiology of their incontinence appears to be due to a defect in their connective tissue, with both a quantitative and qualitative reduction in their collagen.

INTRODUCTION

Although the prevalence of urinary incontinence is increased with age and parity, numerous studies have demonstrated that between 33% and 50% of premenopausal, nulliparous women may have episodes of stress incontinence[1–5]. Although the pathophysiology of genuine stress incontinence in parous women is well documented, related to neuromuscular damage of the pelvic floor[6–8], no studies have attempted to explain the aetiology in nulliparous women. Two reports have suggested that obesity may be an important factor responsible for incontinence in this group[9,10], although one of these reports included postmenopausal nulliparous women.

Recent reports[11–14] have shown an association between abnormalities in collagen and urinary incontinence and between collagen and utero-vaginal prolapse. Ulmsten *et al.*[11] showed that alterations in collagen may result in loose origins or insertions of the striated muscles of the pelvic floor; these in turn act on the closure mechanisms of the urethra, and thus deficiency in this connective tissue can prevent isometric contraction of these muscles and inhibit effective urethral closure. Versi *et al.*[12] showed a link between urethral pressure measurements and skin collagen content, although their study focused on women taken from a menopausal clinic. Sayer *et al.*[13] showed that collagen was the main component of the pubocervical (endocervical) fascia connecting the striated muscles of the pelvic floor to the urethra and that not only is this collagen different in women with stress incontinence compared with controls, but that there is also an increase in abdominal striae in these women[13]. This finding coupled with the study of Norton and *et al.*[14] which demonstrated an association between genitourinary prolapse and joint hypermobility, may indicate that stress incontinence and prolapse are part of an overall collagen disorder and not just one confined to the endocervical fascia around the bladder neck. The aim of this study was to determine if nulliparous women with genuine stress incontinence exhibited differences in the properties of their collagen compared with continent controls.

METHODS

Premenopausal nulliparous women with genuine stress incontinence do not represent the most commonly referred group to a urodynamic unit[15]. Therefore in an attempt to maximise the number of referrals for the study, other means of recruitment were required. In addition to the women referred to the urodynamic unit in the hospital, the study was made known to all urologists,

Correspondence: Dr D. Keane, The John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK.

gynaecologists and general practitioners in the Southwest region of England and Wales, as well as to select urogynaecologists in London. Posters were displayed in the Bristol hospitals and in Bristol University that sought to attract all young women with urinary incontinence. Finally, a visit was made to 35 sports and fitness centres in Bristol and Bath in an attempt to attract those young women who suffered from urinary incontinence during physical exercise. Over 70% of the women with genuine stress incontinence were direct referrals, the remainder came via the advertising campaign. Women who acted as controls were recruited from among hospital personnel and university students. Both groups underwent full urodynamic evaluation following a questionnaire on bladder function.

Urodynamic studies consisted of uroflowmetry, static and cough urethral pressure profiles, and filling and voiding cystometry combined with videourodynamics. All women with urodynamically-proven genuine stress incontinence, and all continent controls had a biopsy taken for collagen analysis. The biopsy was taken from the periurethral vagina following application of a prilocaine-based cream. The site of this biopsy was 1 cm lateral to the urethra in its lower third. The collagen from this area has previously been shown to be analogous to collagen in the endopelvic fascia[16]. The biopsy was taken by means of an Eppendorfer forceps and placed in a sterile container (without fixative) and stored at −80°C until it was taken to the laboratory for analysis. As 28/36 women (78%) with genuine stress incontinence stated there was no association of their symptoms with their menstrual cycle, no attempt was made to time the biopsies to the phase of their cycle.

The biopsy, which was approximately 30–50 mg in wet weight, was initially washed in phosphate buffered saline. The tissue was then divided into one-third and two-thirds, the smaller sample being used for cyanogen bromide (CNBr) digestion[17]. This tissue was then dried before being suspended in 70% formic acid at a concentration of 1 mL of formic acid per 10 mg of tissue dry weight. The CNBr was added as a solution in acetonitrile (1 μL CNBr/mg collagen). The vessel was sealed and incubated at 30°C for three hours. Digestion was stopped by diluting with distilled water. The CNBr peptides were then separated by SDS gel electrophoresis[18]. The gel was then scanned using a scanning densitometer and the ratios of type I to type III collagen were calculated, using the ratio of α(I)CB8 and α(III)CB5[19].

The remaining two-thirds of the biopsy was suspended in 0·9% NaCl to which sodium borohydride ($NaHB_4$) was added to give a substrate:reagent (collagen:$NaHB_4$) ratio of 30:1[20]. The reaction proceeded for two hours before the sample was washed and then centrifuged. The resultant pellet was freeze-dried and then hydrolysed in 6 mol/L HCl at 110°C for 24 hours.

Table 1. Comparison of general features of the two groups. No values were statistically significant. Values are given as median (range) or [mean]. GSI = genuine stress incontinence.

Variable	Nulliparous GSI ($n = 36$)	Nulliparous control ($n = 25$)
Age (years)	38 (13–49)	25 (16–50)
Mean	[33·6]	[30·7]
Body mass index	23·77 (19·2–33)	23·45 (18·8–41·5)
Sexually active	29	24
Frequency (voids/day)	7 (3–18)	7 (2–13)
Nocturia (voids/night)	0 (0–3)	0 (0–2)
Fluid intake (L/day)	1 (1–3)	2 (1–4)

The excess acid was removed by rotary evaporation and an aliquot was taken from the residue for hydroxyproline assay. This was run against standard hydroxyproline solutions on a continuous flow analyser. The concentration of hydroxyproline in the original sample was determined by comparison with the standard curve, and the collagen content calculated assuming 14% hydroxyproline in collagen.

The remaining part of the sample was used for quantitation of collagen cross-links. The sample was separated on a cellulose column to remove the noncross-link amino acids and then analysed on an LKB 4400 amino acid analyser to determine the collagen cross-links[21].

Statistical analysis

All statistical analyses were performed using SAS Statistical software (Version 6.08 for Windows). For all continuous variables group medians were compared with the Wilcoxon rank sum test. Differences in the medians were calculated by 95% confidence intervals (CI), and proportions were analysed with the χ^2 test with Yates' correction if required. Nonparametric tests were used in this study due to the nonnormality of urodynamic and collagen variables even in the control group. Analyses of covariance were performed to assess the relationship between collagen variables.

RESULTS

Table 1 compares data relating to the general features of the two groups. The results show that there was no difference between the groups in body mass index, and thus obesity was not the cause of incontinence in the nulliparous population in our study. Likewise, there was no significant differences in their fluid intake or voiding frequencies. This was determined by the women's urinary diary that was kept for the week before the urodynamic investigation. With regard to the severity of their leakage, 16 women in the study group were wet at least once a day, and a further ≥ 16 once a week. Nine in

Table 2. Comparison of collagen content, type I:III collagen ratios, and collagen cross-links in both groups. Values are given as median (range). Key as for Table 1; HLKNL = hydroxylysinoketonorleucine; HYL-PYR = hydroxylysylpyridinoline.

Variable	Nulliparous GSI ($n = 36$)	Nulliparous control ($n = 25$)	P	95% CI
Collagen content (%)	29 (14·1–53)	39·7 (19·6–54·2)	<0·0001	5·8–15·9
Type I:III ratio	65:35	71:29	0·0008	3–9
HLKNL (nmol/mg collagen)	0·19 (0·11–0·51)	0·31 (0·17–0·36)	< 0·0001	0·07–0·15
HYL-PYR (nmol/mg collagen)	0·25 (0·07–0·4)	0·3 (0·17–0·45)	0·0002	0·04–0·13

the study group (25%) had detrusor instability on urodynamics, compared with six in the control group (24%). None of the women with urodynamically-proven detrusor instability had symptoms of urgency.

The results of the collagen analysis for the two groups are shown in Table 2. . The table shows that there is a significantly reduced collagen content in the nulliparous women with genuine stress incontinence. Further analysis of this collagen shows that there is a reduced type I to type III collagen ratio and a significant reduction in both the immature and mature cross-link content in those women with genuine stress incontinence. Because of the wide range in age in both groups, we analysed age as a covariate for each of the four variables in Table 2. Age was statistically significant for collagen content (P = 0·016), and type I:III ratio (P = 0·003) but not for the other two variables.

An analysis of covariance was performed in order to investigate the relationships between the collagen variables. The results are summarised in Table 3. Because of skewness in both groups, the logarithm of the type I:III ratio was used in preference to untransformed data in order that the residuals from the analysis of covariance were normally distributed. All the P values from the analyses are presented, but only the P values for the interactions are of major interest. This table shows that the linear relation between HYL-PYR and HLKNL was different for the two groups. For the other four pairs of variables there was no statistical significant difference in the linear relations.

Table 3. Analysis of covariance of collagen variables. Key as for Table 2.

Dependent variable	Explanatory variables	P
Collagen content	Group difference	0·45
	HLKNL	0·004
	Interaction between HLKNL & group	0·12
Collagen content	Group difference	0·34
	HYL-PYR	0·002
	Interaction between HYL-PYR & group	0·079
Type I:III ratio*	Group difference	0·49
	HLKNL	0·035
	Interaction between HLKNL & group	0·88
Type I:III ratio *	Group difference	0·67
	HYL-PYR	0·088
	Interaction between HYL-PYR & group	0·83
HYL-PYR	Group difference	0·059
	HLKNL	0·0001
	Interaction between HLKNL & group	0·024

*Logarithmically transformed.

DISCUSSION

The aim of this study was to determine if any pathological cause explained the finding of genuine stress incontinence in nulliparous women. Many theories exist to explain the aetiology of genuine stress incontinence in multiparous women. These include bladder neck descent, neuromuscular damage to the pelvic floor and urethra occurring at childbirth, and in some cases inherent sphincter weakness. The additional effect of the menopause appears to add even further insult to the urethral pressures in this group[22,23]. However, neither the neuromuscular damage of childbirth nor the oestrogen deficiency of the menopause can account for the finding of genuine stress incontinence in nulliparous, premenopausal women. Previous workers have focused on specific aspects of collagen in women with urinary incontinence such as collagen content[13], biomechanical properties[24] or collagen typing[14]. None have attempted to combine these parameters nor has the tissue of continent controls been studied. Collagen is the principal connective tissue linking the levator muscles to the urethra[25], and through it muscle forces are exerted on the urethra. Obtaining collagen from this paraurethral area is possible at open surgical procedures such as colposuspension, but is neither practical nor ethical in a nulliparous population. This necessitated finding an analogous, yet suitable, site to obtain our collagen for analysis. After several sites were tested, we found that the collagen in the periurethral vaginal tissue was the most similar to the collagen in the paraurethral tissue[16], and this formed the basis of the collagen compared in this study.

Our results have indicated that there is less collagen present in the nulliparous women with genuine stress incontinence compared with continent controls. The mean difference between the groups in collagen content was between 5·8% to 15·9%, which is a significant reduction. The analysis also suggests that the ratio of

type I to type III collagen is altered in the genuine stress incontinence group. Type I collagen is often regarded as a supportive collagen and is found in abundance in tissue where support is required, such as tendon, bone and dentine. Type III collagen, on the other hand, is found in tissue where less rigidity is required, e.g. the vascular system and the intestine. Therefore a reduction in the type I:III ratio in the genuine stress incontinence group suggests a less supportive collagen around the urethra in favour of a more pliable type. When this abnormality is added to an actual deficiency of collagen, it is not difficult to comprehend that the endopelvic (paraurethral) tissue is weaker in the genuine stress incontinence group.

The final aspect of collagen we studied was the cross-links content. As collagen matures and ages, it becomes stronger and more rigid due to a change in the number and nature of the cross-links, the immature reducible cross-links being converted to mature, nonreducible cross-links. The mechanism for the production of these mature multivalent cross-links joining three or more collagen molecules in the fibre has long been accepted as the most favourable theory to explain the observed increases in strength and rigidity on ageing[26]. Our results reveal that the mature collagen cross-link, hydroxylysinoketonorleucine and its maturation product hydroxylysylpyridinoline, are both significantly reduced in the women with genuine stress incontinence. This reduction further leads to a mechanically weaker fibre. Analysis of the cross-links and other collagen parameters revealed a strong association in the control group, but no such association in the genuine stress incontinence group. This further implies a general disorientation in the collagen matrix in these women.

Thus premenopausal women with genuine stress incontinence have 1. less collagen in their connective tissue; 2. a reduction in supporting type I collagen; and 3. a reduction in cross-link content. These findings have implications for this group of women and poses a therapeutic dilemma. As many of these women are too young to contemplate starting a family, the traditional approach to the treatment of these women is conservative, namely physiotherapy. Physiotherapy produced little benefit in this group, although their age and premenopausal status should have placed them in a category who improve with physiotherapy. We believe that the reason for the poor success lies in their collagen weakness, for despite increasing their pelvic floor strength, the weak connective tissue connecting these muscles to the urethra could not transmit the forces of muscular contraction.

Surgery may be more beneficial in this group, but as many of the supra-pubic procedures involve suspension of the paraurethral tissue to the ileo-pectineal ligament, these operations may be unsuccessful if one is elevating weakened paraurethral tissue. The answer may lie in either replacing or supplementing their collagen with an exogenous collagen; this is showing promise in some studies[27]. The short and intermediate term results from injected collagen are promising, and if fibroblasts encourage the deposition of endogenous collagen, this may be the treatment of choice in nulliparous women with genuine stress incontinence. In addition, its ease of performance and the fact it can easily be repeated may allow further injections if incontinence returns following childbirth.

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