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The influence of ovarian steroids on ovine endometrial glycosaminoglycans

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The ovine endometrium is subjected to cyclic oscillations of estrogen and progesterone in preparation for implantation. One response to fluctuating hormonal levels is the degree of hydration of the tissue, suggesting cyclical alterations in glycosaminoglycan/proteoglycan content. The aim of the present study was to quantitate and characterize glycosaminoglycans in the ovine endometrium during estrogen and progesterone dominant stages. Endogenous endometrial glycosaminoglycan content was determined by chemical analysis and characterized by enzyme specific or chemical degradation. [^{35}S]-sulphate and [^{3}H]-glucosamine labeled proteoglycans/glycosaminoglycans were extracted by cell lysis or with 4M guanidine-HCl. Extracts were purified by anion exchange and gel chromatography and characterized as above. Estrogen and progesterone dominant endometrium contained 3.2 $\pm$ 0.1 and 2.1 $\pm$ 0.1 mg endogenous glycosaminoglycan/g dehydrated tissue, respectively. Characterization of endogenous glycosaminoglycan showed chondroitin sulphate and hyaluronan contributing over 80%. The major difference between hormonal dominant tissue was a higher estrogenic hyaluronan percentage and a higher progestational keratan sulphate percentage ($p < 0.001$). Estrogen dominant tissue incorporated 1.6–1.9 fold more radiolabeled proteoglycans/glycosaminoglycans ($p < 0.001$). Analysis of newly synthesized proteoglycans/glycosaminoglycans revealed a heparan/chondroitin sulphate ratio of 1:2.2–2.5. Keratan sulphate was not detected. Estrogenic hyaluronan was 1.6 fold greater in [^{3}H]-labeled tissue. Analysis of labeled proteoglycans/glycosaminoglycans revealed two size classes with apparent molecular weights $>2.0 \times 10^6$ and $0.8–1.1 \times 10^5$ and a charge class eluting between 0.1–0.5 M NaCl. The greater glycosaminoglycan content (particularly hyaluronan) and synthesis in estrogen dominant tissue supports a role for steroid hormones in endometrial glycosaminoglycan/proteoglycan regulation and consequent tissue hydration. It also suggests a role for these macromolecules in endometrial function and possibly the implantation process.
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Keywords: **endometrium, hyaluronan, estrogen, progesterone**

Introduction

Glycosaminoglycans (GAGs) are ubiquitous components of extracellular matrices (ECM) and cell surfaces. There are four classes of GAGs: chondroitin sulphate (CS)/dermatan sulphate (DS); heparan sulphate (HS)/heparin; keratan sulphate (KS) and hyaluronan (HA), each composed of different disaccharide repeat units containing a modified sugar and a uronic acid (or galactose). With the exception of HA, GAGs are found covalently attached to core proteins to form proteoglycans (PGs). PGs are involved in a diverse range of biological functions including immune response, and cell differentiation, adhesion and migration [1–3]. PGs also maintain structural organization and mechanical properties of connective tissue, which can be attributed to core protein and/or GAG interactions with other ECM macromolecules such as collagen [4]. Furthermore, the highly negatively charged GAG side chains attract osmotically active cations and an accompaniment of water-endowing PGs with the ability to contribute to tissue hydration and expansion [5].

Steroid hormones, such as estrogen (E) and progesterone (P) can influence ECM composition and organization [6]. The ECM of the uterine endometrium is subjected to cyclic oscillations of plasma E and P levels to prepare a favorable environment for blastocyst implantation and subsequent fetal development during pregnancy. In the ovine endometrium, the 17 day cycle proceeds through two phases: the luteal/secretory phase (days 2–13) and the peri-ovulatory/proliferative period (days 14–1) in which P and E respectively are the dominant hormones. One of the most obvious changes in response to fluctuating steroid hormone levels is the degree of hydration of the ovine

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endometrium and the appearance of oedema in the stroma during the E-dominant phase of the cycle [7]. The alterations to the hydration status of the tissue may be a consequence of quantitative changes in PG/GAG composition.

Investigations of uterine GAGs/PGs have focused on PG composition at times of dramatic structural change such as during pregnancy or in association with dilation of the cervix prior to delivery [8–13]. These studies indicate decorin as the main PG species present in uterine tissue. It is a member of the small leucine rich family of PGs and is glycosylated by a single DS chain on the 3.6×10^4 core protein. Decorin is relatively abundant in many connective tissues and has been found to interact with collagen fibers by inversely modifying the kinetics of collagen fibril formation and affecting the morphology of the fibrils particularly with respect to fibril diameter [14]. Biglycan, another member of the small leucine rich family of PGs is present in minor amounts in uterine tissue [11]. Biglycan contains two chains of CS or DS on a 3.8×10^4 core protein and is found associated with cell surface or pericellular areas. A large interstitial DSPG molecular weight (Mr) 2.0×10^6, with a structure analogous to versican, also accounts for a minor proportion of uterine PGs [14]. Versican core protein contains a HA binding domain; however the large DSPG found in female reproductive tissue does not exhibit the ability to interact with HA [15]. Small amounts of HSPG have also been observed in the uterus [16,17]. HSPG are predominantly present at cell surfaces and fall into either of two groups, syndecans and glypicans. These PG participate in cell-matrix interactions and growth factor binding suggesting an involvement in fundamental cell functions. Potter and Morris [16] positively identified syndecan 1 in mouse uterine tissue. Finally, HA has been confirmed as a major glycoconjugate synthesized and secreted by mouse uterine epithelial cells [18], particularly at the time of implantation [19,20]. Furthermore, there appears to be a positive correlation between HA expression and cell proliferation in uterine tissue [20].

Although the main PG and GAG species have been identified in the uterus little is known of their relative abundance at different stages of the estrous cycle under different steroidal regimes. This study was undertaken to examine the possible relationship between the steroidal milieu and PG/GAG composition of ovine endometrium.

Materials and methods

Radioactive isotopes; [^{35}S]-sulfate and D-[6-^{3}H]-glucosamine HCl were purchased from New England Nuclear (Boston, MA, USA). The 650S DEAE Toyo-pearl ion exchanger, Sephadex G-50 (fine) and dextran standards were from Pharmacia Fine Chemicals (Uppsala, Sweden). Materials required for tissue culture including Dulbeco's Modified Eagle's Medium (DMEM) and the antibiotics; streptomycin, fungizone and penicillin were obtained from Trace Bioscience (Sydney, Australia). Papain (25 mg/ml), hyaluronidase (*Streptomyces hyalurolyticus*), chondroitinase ABC (*Proteus vulgaris*), keratanase (*Pseudomonas species*) and GAG stocks were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other reagent used were of analar grade and obtained from either the Sigma Chemical Company or Ajax Chemicals (Sydney, Australia).

Endometrial tissue collection

Corriedale ewes were run with vasectomized rams fitted with marking harnesses and checked daily for mating marks indicative of estrus (day 0). Eight animals were selected. Four ewes were on day 0 of the estrous cycle, the time of the lateinizing hormone surge, when E is the dominant steroid hormone (E-dominant). The remaining four ewes were on day 10 of the estrous cycle when P levels are maximal (P-dominant). The animals were anaesthetized, the reproductive tract exposed through a mid-line incision and surgically removed. The bicornuate uteri were cut in half to separate the two uterine horns and slit longitudinally to reveal the inner endometrial surface. The endometrium was dissected from the myometrium with fine scissors. Endometrium from one horn was snap frozen for subsequent analyses, while endometrium from the second horn was suspended in phosphate buffered saline (PBS), pH 7.4, with added antibiotics: penicillin 100 U, streptomycin 100 U and fungizone 0.25 μg/ml for immediate metabolic labeling. The Institutional Animal Ethics Committee approved all animal experimentation.

Tissue analysis

Endometrial GAGs were extracted and purified based on procedures described by Greiss and Wagner [21]. Endometrial samples were finely diced prior to dehydration and lipid extraction with 10 volumes of acetone:absolute ethanol (1:1 v/v) at 60°C for 1 h, 10 volumes acetone:absolute ethanol (1:1 v:v) at 20°C for 21 h and 10 volumes acetone at 20°C for 3 h. This dehydrated, lipid extracted tissue was dried over di-phosphorus pentoxide (P_2O_5) in a vacuum desiccator and multiple measurements made to determine the dry weight. The tissue was then powdered in a SPEX freezer mill (Spex Industries Inc., Metuchen, MA) for 30 s at full frequency during cooling with liquid nitrogen (N_2) and rehydrated in a buffer containing 0.1 M sodium acetate pH 5.5, 5 mM EDTA and 0.05 M cysteine hydrochloride. GAGs were solubilized from the tissue by enzymatic treatment with papain (10 μg/mg dry tissue) for 18 h at 60°C. Following digestion, the samples were centrifuged (3500 rpm, 10 min) and an aliquot of the supernatant assayed for DNA content using a fluorometric assay [22], which utilized the dye bisbenzimide, commercially known as Hoechst 33258. The remaining papain digested supernatants were treated with 5% cold trichloroacetic acid (TCA) for 15 min at 4°C to precipitate DNA and glycopeptides. After centrifugation (3500 rpm, 10 min), the supernatant was dialyzed against 0.05 M disodium sulphate and the GAGs were precipitated from solution with 0.5% (w/v) cetylpyridinium chloride (CPC) [21]. GAG-CPC

complex formations were established over 4 h at 37°C. Following centrifugation (3500 rpm, 10 min) the precipitated GAG-CPC complexes were dissociated with 2 M sodium chloride and GAGs re-precipitated with absolute ethanol at −20°C (1:3 volumes respectively). After incubating for 1.5 h at 4°C, precipitates were collected and the CPC-GAG dissociation/GAG precipitation procedure repeated on the supernatant component. GAGs were dissolved in distilled water, the two precipitates from the one supernatant combined, and GAG yield determined via two assays: uronic acid content (CS, HA and HS) and sulphated GAG content (CS, KS and HS). The uronic acid content was determined using an assay developed by Bitter and Muir [23], while the sulphated GAG content was determined based on a technique modified by Goldberg and Kolibas [24].

GAG composition of non-labeled material

After extraction and purification of endometrial GAGs with CPC, their composition was determined by treatment with GAG-specific enzymes or chemicals. Chondroitinase ABC digestion, which specifically degrades CS was for 18 h at 37°C in 0.1 M Tris-HCl/0.1 M sodium acetate buffer pH 7.0, using 0.125 U enzyme/mg GAG. Keratanase digestion (determines KS content) was for 18 h at 37°C in 0.1 M Tris-HCl/0.1 M sodium acetate buffer pH 7.0, using 1 U enzyme/mg GAG. Hyaluronidase digestion was for 18 h at 37°C in Sorensen's buffer (9 mM KH_2PO_4 and 7 mM Na_2 $HPO_4{\cdot}2H_2O$) using 400 U/mg GAG. HS content was determined by deaminative hydrolysis [25]. Samples were treated with 0.24 M $NaNO_2$ in 1.8 M acetic acid for 80 min at room temperature (1:1 v:v) and the reaction terminated by raising the pH with 5 M NaOH. In each case parallel incubations were conducted on the isolated GAGs in the absence of enzyme to later determine the degree of digestion. Furthermore, incubations were also performed using 100 μg GAG (standards)/ml in the presence and absence of enzyme to ensure enzyme viability. Undigested/digested GAG was separated via CPC precipitation and the undigested GAG yield was detected by uronic acid content or sulphated GAG content determination as above. The percentage of digestion was calculated for each GAG type and the composition of GAGs expressed in such a format.

Metabolic labeling

Analyses of newly synthesized GAGs utilized biosynthetic radiolabeling methods. Fresh endometrial tissue was suspended in PBS with antibiotics as above, finely chopped and washed three times with PBS to remove any dead cells. 100 mg aliquots were incubated at 37°C for 8 h in 5 ml DMEM containing either 50 μCi [^{35}S]-sulphate or 50 μCi [^{3}H]-glucosamine. The [^{35}S]-sulphate selectively labels CS/KS/HS while the [^{3}H]-glucosamine labels all GAGs (CS/KS/HS/HA) along with the general pool of glycoproteins in the system. However, given CS does not contain glucosamine in its disaccharide backbone, the tritiated label is incorporated after glucosamine's conversion to galactosamine by a 4-epimerase. After labeling, the incubation medium was centrifuged (2000 rpm, 10 min). Protease inhibitors (0.1 M aminocaproic acid, 0.1 mM benzamide HCl, 0.2 mM iodoacetic acid and 0.0008% soybean trypsin inhibitor) were added to the supernatants which were then snap frozen in liquid N_2. The radiolabeled tissues were washed twice in DMEM, weighed and protease inhibitors as above added to tissue samples prior to snap freezing. An alternative extraction method from that used to investigate GAG yield and composition was employed so the intact PGs could be studied.

Characterization of labeled PG/GAG

(I) Size

Size based analyses of labeled PGs/GAGs utilized standard, well established procedures for cervical PGs [8,11]. PGs/GAGs were extracted from 100 mg of tissue for 48 h at 4°C with 1 ml of 4 M guanidium hydrochloride (GuHCl) containing 0.05 M acetate pH 5.8 and protease inhibitors. After centrifugation (3500 rpm, 10 min), the supernatant underwent lipid extraction by the addition of 3 volumes ethanol at −20°C and incubation for 2 h at 4°C. Following centrifugation (3500 rpm, 10 min), 3 volumes of chloroform/methanol (2:1 v:v) was added to the precipitates and incubated for 4 h at room temperature. The chloroform/methanol precipitate was then dried over P_2O_5 in a vacuum desiccator. Lipid extracted samples were resuspended in 4 M GuHCl as described above and applied to Sephadex G-50 (fine) columns 1.5 × 17 cm equilibrated with 0.05 M ammonium carbonate to remove unincorporated radioisotope. Samples from the void volume were lyophilized and taken for size based analysis using two alternative techniques; gel chromatography and gradient SDS-PAGE.

(A) Gel chromatography. Molecular exclusion liquid chromatography was performed at room temperature on a 1.2 × 95 cm column of Sepharose CL-6B equilibrated with 4 M GuHCl, 0.1 M sodium sulphate, 0.05 M sodium acetate pH 6.1 and 0.2% (w/v) Triton X-100 [8], and with a flow rate of 0.17 ml/min. The void volume was determined by the absorbance profile of eluted blue dextran (2.0×10^6) while the total volume of the column was determined with [^{3}H]-H_2O. Fluorescent dextrans (2.36×10^5 and 7.0×10^4) were used as Mr markers.

(B) SDS-PAGE. SDS-PAGE analyses was performed on 3.8–20% gradient gels containing boric acid and urea [26]. Radiolabeled PGs from 100 mg wet weight endometrial tissue were applied to each lane. A pre-stained broad range standard (Biorad Laboratories, Richmond, CA, USA) was used for Mr determination. Gels were stained in Coomassie brilliant blue, dried and subjected to phosphorimaging (STORM, Molecular Dynamics, CA, USA) prior to densitometric analysis (Image Quant, Molecular Dynamics, CA, USA) of radioactive bands present.

(II) GAG composition of labeled PGs

After extraction of PGs/GAGs with 4 M GuHCl as described above, the GAG side chain composition of endometrial PGs was determined by treatment of isolated GAGs with GAG specific enzymes or chemicals as described in "GAG composition of non-labeled material". Undigested/digested material was separated by chromatography on a Sephadex G-50 (fine) column (1.2 × 95 cm) equilibrated with 0.05 M ammonium carbonate at room temperature and with a flow rate of 1 ml/5 min. Results were expressed as dpm/100 mg wet weight endometrium.

(III) Charge

Charge analyses of labeled PGs/GAGs were based on procedures previously described by Nicollier et al. [27] who examined intact PGs in glandular epithelial cells of guinea-pig endometrium. Little is known about charged based analyses of endometrial PGs. Techniques employed by Nicollier et al. [27] were used so direct comparison between studies could be made. Homogenized tissue (100 mg) was incubated for 2 h at room temperature with cell lysis solution (9 M urea, 3% (v/v) Igepal CA-630 and a mixture of protease inhibitors) [27]. After centrifugation (3500 rpm, 10 min) the supernatants from the cell lysed tissue and the corresponding medium fractions underwent lipid extraction as described above. The pellet was resuspended in 6 M urea, 0.2% (v/v) Triton X-100 and 0.02% (w/v) sodium azide in 20 mM Tris-acetate pH 7.0 and applied to a 1.5 × 17 cm column of Sephadex G-50 (fine) equilibrated in the same buffer. Radioactive fractions contributing to the void volume were pooled and analyzed on the basis of charge by anion exchange chromatography. The FPLC system used contained a 0.5 × 5 cm column of 650S DEAE Toyo-pearl equilibrated with 6 M urea, 0.2% (v/v) Triton X-100 and 0.02% (w/v) sodium azide in 20 mM Tris-acetate pH 7.0 and developed with a gradient of 0–2 M sodium chloride in the same buffer. The column was pumped at a flow rate of 1 ml/min at room temperature and fractions were collected every 30 s. Recoveries of radioactivity typically ranged from 85–100%.

Statistical analysis

Results were represented as mean ± standard deviation (SD) and were analyzed by analysis of variance and Student's *t* test.

Results

Water and DNA content of ovine endometrium

Endometrium at both E- and P-dominant stages of the cycle were dehydrated and the water content determined. The water content of E-dominant tissue was 90 ± 1% and 86 ± 0.5% for its P-dominant counterpart. The hydration status was statistically different with E-dominant tissue showing a 4% greater water content than the P-dominant tissue ($p < 0.001$). There was a 1.6 fold greater DNA content in P-dominant tissue (164 ± 12 ng DNA/mg endometrium) compared with E-dominant tissue (102 ± 9 ng DNA/mg endometrium) when DNA yield was expressed in terms of hydrated or wet weight tissue ($p < 0.001$). However, when DNA content was expressed in terms of dehydrated tissue there was no statistical difference with 1011 ± 89 and 1147 ± 83 ng DNA/mg endometrium for E- and P-dominant tissue respectively. Thus, the difference between the tissues is due to water of hydration and not to differences in cell numbers.

GAG levels and composition in the ovine endometrium

GAG content was measured in E- and P-dominant endometrium after dehydration/delipidation, papain digestion and CPC precipitation. Isolated GAGs were detected with either of two techniques: the uronic acid assay [23] which detects all GAGs with the exception of KS and the sulphated GAG assay [24] which excludes HA due to its non-sulphated nature. Irrespective of which method was employed, the E-dominant tissue consistently produced a greater GAG yield (approximately 1.5 fold higher) (Figure 1). Actual GAG content was within the range 3.1–3.3 mg GAG/g E-dominant tissue and 2.0–2.2 mg GAG/g P-dominant endometrium depending on the assay utilized. Collectively, these results indicate a possible relationship between GAG yield and tissue hydration. The composition of endometrial GAGs was investigated by examining the sensitivity to digestion with GAG-specific enzymatic or chemical treatments (Figure 2). Undigested endometrial GAGs were isolated from digested GAGs by CPC precipitation and the GAG yield determined with either the uronic acid assay or the sulphated GAG assay. Endometrial GAG composition was then expressed as percentage of total GAG as shown in Figure 2. CS was found

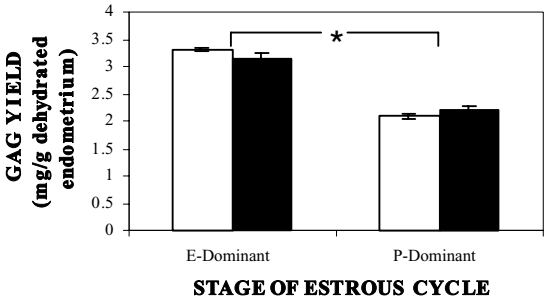

Figure 1. Yields of ovine endometrial GAGs during an E- ($n = 4$) and a P- ($n = 4$) dominant phase of the estrous cycle. GAGs were isolated from endometrial tissue by dehydration/delipidation, papain digestion and CPC precipitation. GAG content was determined by chemical analysis using the uronic acid assay which detects CS, HS and HA (□) and the sulphated GAG assay which determines CS, HS and KS (■). The data shows GAG concentration as mean mg GAG/g dehydrated endometrium ± standard deviation. The values obtained are considered significantly different ($p < 0.001$) between E- and P-dominant phases for both assays (*).

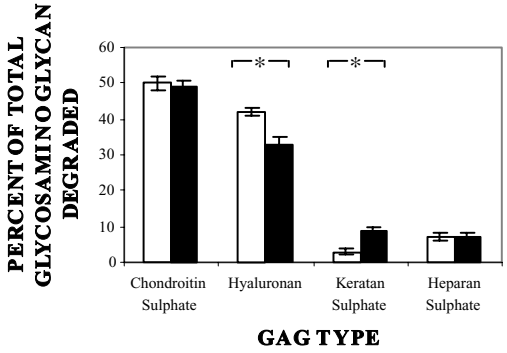

Figure 2. Composition of endometrial GAGs. GAGs were extracted from E- (□) and P- (■) dominant tissue ($n = 4$) and purified GAGs subjected to degradation with chondroitinase ABC, hyaluronidase, keratanase and nitrous acid deamination which selectively target the CS, HA, KS and HS GAGs respectively. Digested GAGs were separated from undigested material by CPC precipitation and the results expressed as a percentage of total GAG content. GAG types considered significantly different ($p < 0.001$) between E- and P-dominant phases are highlighted (*).

to be the major component of endometrial GAGs during both E- and P-dominant stages contributing 50.1 ± 1.8% and 49.2 ± 1.9% respectively, although the values were not considered statistically different. HA also contributed to a substantial proportion of total endometrial GAGs which altered depending on the hormonal milieu. HA was responsible for 42.1 ± 0.8% and 33.0 ± 1.7% of E- and P-dominant endometrial GAG respectively. Thus, a 1.3 fold greater HA content was established during the E-dominant stages of the cycle ($p < 0.001$). KS represents a minor proportion of total GAG content, however as in the case with HA, KS contribution alters depending on steroidal dominance. 3.2 ± 0.7% of E-dominant GAGs and 9.4 ± 0.4% of P-dominant GAGs were attributable to KS. Consequently, a 3 fold increase in KS composition was seen during the P-dominant stage ($p < 0.001$). Finally, HS proportions were equal for both stages with 7.1 ± 0.6% and 7.3 ± 0.8% of E- and P-dominant tissue respectively. Results suggest that not only total GAG content altered depending on the hormonal milieu but also the type of GAG. However, the ultimate PG structure formed may or may not change.

Rate of synthesis of GAGs in the ovine endometrium

To characterize newly synthesized GAGs and gain an insight into the type of PG formed, samples of endometrium from non-pregnant ewes at both an E- and P-dominant stage of the cycle were incubated with radioisotopes for 8 h. ^{35}S-sulphate selectively labels sulphate ester groups on the GAGs (CS/HS/KS), while ^{3}H-glucosamine labels all GAG types (CS/HS/KS/HA).

The results obtained for total GAG content in hormonally regulated ovine endometrium were reinforced when analyses of E-dominant tissue revealed a 1.9 and 1.6 fold greater radioisotope incorporation rate (/100 mg wet weight endometrium) for ^{35}S and ^{3}H, respectively, than the P-dominant counterpart (Table 1). Investigations into retention and release of PGs/GAGs between the tissue and the incubation medium showed that in both E- and P-dominant tissue the majority of ^{35}S labeled PGs/GAGs (73% and 71%, respectively) were retained in the tissue (Table 2). This fraction would include components of the ECM, cell surface and intracellular compartments. The remaining 27% to 29% of radioactive PGs/GAGs were released into the medium. Approximately 63% of the incorporated ^{3}H for both E- and

Table 1. Quantitative comparisons between radioisotope incorporation for E- ($n = 4$) and P- ($n = 4$) dominant endometrium. Endometrial ^{35}S and ^{3}H labeled PGs/GAGs were extracted with 4 M GuHCl and protease inhibitors. Radiolabeled PGs/GAGs were purified on 1.5 × 17 cm columns of Sephadex G-50 (fine). Elution was conducted under gravity with 0.05 M ammonium carbonate at room temperature. Radioactive incorporation was expressed as dpm $\times 10^3$/100 mg hydrated endometrium ± standard deviation. Incorporation expressed as dpm $\times 10^3$/100 mg dry weight endometrium is shown in parenthesis. Irrespective of which radioisotope was used, values for E- and P-dominant tissue were statistically different ($p < 0.001$). Recoveries of radioactivity were 95–100% in all cases

Hormonal dominance	*Radioisotope incorporation DPM ($\times 10^3$)/100 mg endometrium*	
	35-S	*3-H*
E-Dominant	12.8 ± 1.6 (128)	25.1 ± 1.8 (251)
P-Dominant	6.5 ± 1.0 (46)	15.7 ± 1.8 (112)
E/P	1.9 (2.8)	1.6 (2.2)

Table 2. Comparison of radioisotope PG/GAG incorporation retained within the tissue or released into the medium for E- and P-dominant endometrium. GuHCl extracted cell associated radiolabeled PGs/GAGs, and those present in the corresponding incubation medium were purified by application to Sephadex G-50 columns. When combined, the radioactivity present in the void volumes represented total radiolabeled PGs/GAGs. The proportions attributable to tissue and medium components were determined for both the ^{35}S ($n = 2$) and the ^{3}H ($n = 4$) PGs/GAGs, and expressed as percentage of total (±standard deviation for the ^{3}H PGs/GAGs). Retention and release values for E- and P-dominant tissue were not statistically different for the ^{3}H labeled material

		Hormonal dominance	
		E-Dominant	*P-Dominant*
35-S	Tissue	72.5%	70.9%
	Medium	27.5%	29.1%
3-H	Tissue	63.2 ± 2.7%	63.9 ± 6.2%
	Medium	36.8 ± 2.7%	36.8 ± 6.2%

P-dominant endometrium was retained in the tissue while 37% was released into the medium.

Characterization of GAGs in the ovine endometrium

(I) Size

Size exclusion chromatography of the GuHCl extracted tissue and medium fractions separated two size classes of ^{35}S and ^{3}H labeled PGs/GAGs consistent for both E- and P-dominant tissue (Figure 3). The first peak corresponded to a molecule whose hydrodynamic radius exceeded 2.0×10^6 relative to a dextran standard (void volume). The second peak revealed a smaller molecule covering a broad range of Mr. The major difference between size class profiles was the tail extension of the second peak and consequently, the proportion of low Mr PGs/GAGs which was most apparent in the ^{3}H labeled PGs/GAGs from the medium (Figure 3D). To further analyse the apparent size reduction, the second peak was classified into peaks 2A and 2B. Peak 2A represents PGs/GAGs with Kav's between 0.15–0.45 (Mr $>7.0 \times 10^4$) and peak 2B represents PGs with Kav's between 0.45–1.00 (Mr $<7.0 \times 10^4$).

Although size exclusion chromatography consistently revealed two size classes, the proportions attributable to each varied depending not on hormonal milieu but on PG/GAG radioisotope label and location (tissue vs medium). The first peak contributed towards minor proportions of total ^{35}S and ^{3}H radiolabeled PGs/GAGs for both E- and P-dominant tissue and medium (10–26%). The majority of the ^{35}S radioactivity was present in Peak 2A for both tissue and medium irrespective of the hormonal milieu (62–72%). While the majority of ^{3}H labeled PGs/GAGs were also observed at peak 2A for the tissue derived fraction (62–64%), the medium fraction exhibited greater proportions of radioactivity in peak 2B (45–50%).

To more precisely determine the size of the various types of GAGs in their PG intact form, the ^{35}S labeled GuHCl extracted E- and P-dominant material was subjected to 3.8–20% SDS-PAGE (Figure 4). The large PGs (Mr $>2.3 \times 10^5$) barely entered the gel with retardation occurring at the base of the loading well (stacking gel) and at the stacking/resolving gel border. These PGs accounted for approximately $37 \pm 2.2\%$ of both E- and P-dominant radiolabeled material however a greater proportion of the PGs were retarded immediately upon entering the stacking gel for the P-dominant tissue while the E-dominant extract was retarded predominantly upon entering the resolving gel. Given that the density of the gel at both these points is 3.8% no qualitative difference between the two can be established. The smaller PG was separated with an approximate Mr

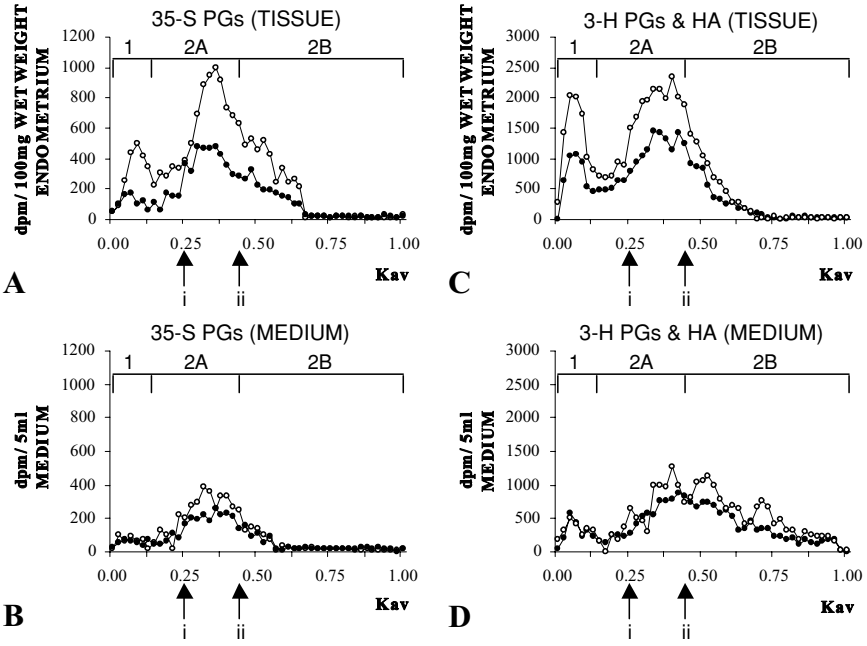

Figure 3. Sepharose CL-6B size exclusion chromatography of tissue retained and released PGs/GAGs. E- and P-dominant ovine endometrium were metabolically labeled with ^{3}H-glucosamine and ^{35}S-sulphate for 8 h prior to the extraction of PGs/GAGs with 4 M GuHCl and protease inhibitors. The tissue extracts and the corresponding tissue culture incubation media were applied to 1.2×95 cm columns of Sepharose CL-6B equilibrated with 4 M GuHCl, 0.1 M sodium sulphate, 0.05 M sodium acetate, pH 6.1, and 0.2% (v/v) Triton X-100. Columns were run at room temperature with a flow rate of -0.17 ml/min. Typical elution profiles of both the ^{35}S labeled (A and B) and ^{3}H labeled (C and D) PGs/GAGs from E- (o) and P- (•) dominant tissue are shown. Panels A and C are profile representations of the tissue extracted PGs/GAGs while panels B and D are reflections of the tissue released PGs/GAGs found within the incubation medium. The bands represent peak classifications; peak 1 (Kav 0.00, void volume), peak 2A (Kav 0.15–0.45) and peak 2B (Kav 0.45–1.00). The elution position of a 2.36×10^5 dextran (i) and a 7.0×10^4 dextran (ii) are also indicated.

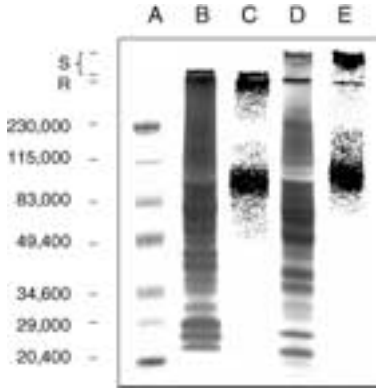

Figure 4. SDS-PAGE of ^{35}S radioactive PGs/GAGs from E- and P-dominant ovine endometrium. PGs/GAGs were extracted from 100 mg of tissue with 4 M GuHCl and subjected to 3.8–20% SDS-PAGE. S = stacking gel (3.8%); R = resolving gel (3.8–20%); (A) pre-stained broad range standard; (B) E-dominant macromolecules—coomassie stain; (C) E-dominant PGs—phosphor image; (D) P-dominant macromolecules—coomassie stain and (E) P-dominant PGs—phosphor image.

between 8.0×10^4 and 1.05×10^5 and represented $63 \pm 2.2\%$ of ^{35}S PGs.

(II) GAG composition of labeled material

The GAG composition of radioactively labeled endometrial PGs was investigated by examining the sensitivity to digestion with GAG-specific enzymatic or chemical treatments (Figure 5). Samples were purified on Sephadex G-50 columns and compared with undigested PGs. Undigested E- and P-dominant endometrial ^{35}S labeled PGs revealed a single peak, corresponding to the void volume of the column, which indicated a lack of degradation products (results not shown). Following treatment with chondroitinase ABC, $66 \pm 8\%$ of E-dominant material was recovered as disaccharide products compared with $71 \pm 4\%$ of P-dominant material (Figure 5A). This indicates CS as the major GAG side chain for both E- and P-dominant tissue. There was no statistical difference between the two groups. Nitrous acid treatment (HS specific) of the tissue resulted in only minor signs of degradation with $26 \pm 2\%$ of the total ^{35}S labeled GAGs in the E-dominant tissue and $27 \pm 1\%$ in the P-dominant endometrium recovered as breakdown products (Figure 5A). Treatment with keratanase resulted in no products in either treatment group indicating a lack of newly synthesized KS in the non-pregnant ovine endometrium at either stage of the cycle.

Characterization of the GAG composition of ^{3}H endometrial PGs and HA supported results from the ^{35}S labeled counterparts. The ^{3}H radiolabeled components were primarily HA and CS. *Streptomyces* hyaluronidase resulted in 49% of E- and 29% of P-dominant material as degraded fragments while chondroitinase ABC digestion showed 37% and 43% respectively (Figure 5B). HS constituted 17% and 19% of E-

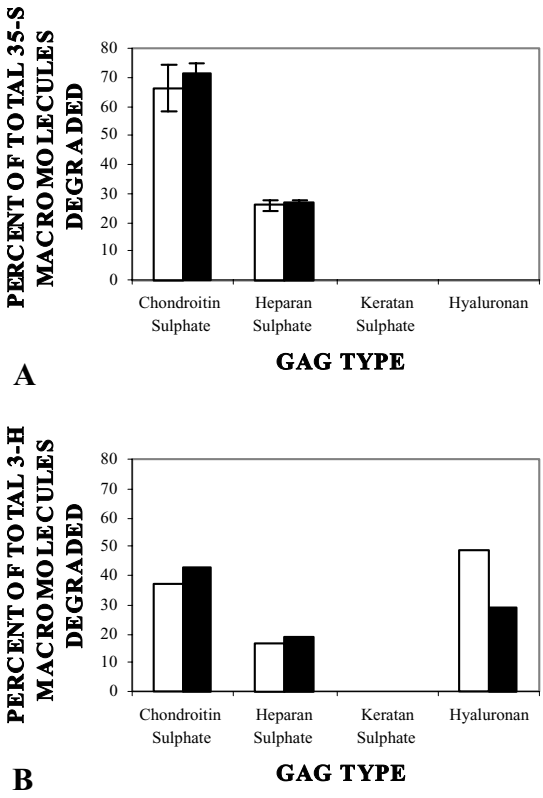

Figure 5. Structural characterization of newly synthesized GAG side chains of endometrial PGs and HA. Radiolabeled endometrial PGs/GAGs were extracted from E- and P-dominant tissue with 4 M GuHCl and protease inhibitors. PGs/GAGs were subjected to degradation with chondroitinase ABC, hyaluronidase, keratanase and nitrous acid deamination which selectively target the CS, HA, KS and HS GAGs respectively. Digested GAGs were separated from undigested PGs and HA on a 1.2×95 cm column of Sephadex G-50 (fine) equilibrated with 0.05 M ammonium carbonate. Panels A and B show the GAG compositions of ^{35}S ($n = 4$) and ^{3}H ($n = 1$, limited tissue) PGs (and HA) respectively. GAG compositions for both E- (□) and P- (■) dominant tissue are expressed as a percentage of total labeled PGs/GAGs (±standard deviation [^{35}S]).

and P-dominant ^{3}H PGs/GAGs, being converted to low Mr forms following degradation with nitrous acid (Figure 5B). Neither E- nor P-dominant tissue contained newly synthesized KS. Due to limited ^{3}H tissue availability, the alterations in HA proportion between E- and P-dominant endometrial GAG were confirmed to an extent by the results obtained when isolated non-labeled GAGs were digested with *Streptomyces* hyaluronidase (Figure 2). HA was responsible for $42 \pm 1\%$ of E-dominant non-labeled endometrial GAGs ($n = 4$) and $33 \pm 2\%$ of P-dominant GAGs ($n = 4$).

(III) Charge

Charge based characterization of purified, ^{35}S and ^{3}H labeled PGs/GAGs was achieved by anion exchange liquid

chromatography. The elution profile of both the ^{35}S and ^{3}H labeled PGs/GAGs extracted from E- and P-dominant endometrium revealed a charge class which was eluted from the resin between 0.1–0.5 M NaCl. ^{35}S labeled PGs/GAGs peaked at 0.25 M NaCl while that of the ^{3}H PGs/GAGs eluted with a slightly lower peak at 0.15 M NaCl.

Discussion

This study has demonstrated marked differences between the GAG content of ovine endometrium during E- and P-dominant phases of the estrous cycle, with E-dominant tissue containing significantly more total GAG. Characterization of endogenous GAGs showed all types present (CS, HA, HS, and KS) with CS and HA contributing over 80% of total GAG. The major differences between E- and P-dominant tissue were that estrogenic conditions favoured HA while progestational conditions exhibited a higher percentage of KS. The water attracting properties of the HA molecule would explain the higher level of hydration measured in the E-dominant tissue. Collectively, these results support: (1) a role for GAGs in facilitating water retention and therefore tissue hydration and (2) a role for steroid hormones in endometrial GAG regulation.

Studies on endometrial GAGs and PGs have been conducted in a number of species although little has been done in sheep. In human endometrium, accumulation of KS is maximal when P levels are at their peak [28]. KS was also identified as a major glycoconjugate secreted by rat uterine epithelial cells [17]. Jacobs and Carson found CS (80–90%) and HS (10–20%) to be the major side chains associated with mouse uterine PG (Mr $>2.0 \times 10^6$) [18]. However, HS contribution towards mouse uterine PGs varied from levels of 10% [18] to 50% [29] depending on the study. Discrepancies may have arisen from the animal strain tested, the homogeneity of the culture or the length of incubation [11]. In the present study, radiolabeling of newly synthesized GAGs also revealed a predominance of CS and HA ($>70\%$) while HS contributed only a minor proportion of total endometrial GAG (27%). However, unlike endogenous GAG, no newly synthesised KS was detected in either set of tissue suggesting either a low turnover rate for KS within the ovine endometrium, or the selective labeling of GAGs other than KS during the radioisotope incorporation process.

In the present study, size based analyses of newly synthesized ^{35}S labeled PGs by gel filtration identified two size classes. A minority ($<30\%$) represented a PG with a hydrodynamic radius $>2.0 \times 10^6$. The majority ($>70\%$) had a smaller Mr. The results were re-confirmed by SDS-PAGE which identified the smaller PG as having a Mr of 8.0×10^4–1.1×10^5. Similarly, two size classes were identified in the guinea-pig endometrium (Sepharose CL-4B) [27]. A minor proportion was eluted in the void volume indicating a molecule of Mr $>2.0 \times 10^7$, while the majority was eluted at Kav 0.55 representing a molecule with approximate Mr 1.0×10^5. While there are no previous studies on ovine endometrial PGs, ovine cervical PGs have been determined [8]. Density gradient centrifugation and analytical ultra-centrifugation identified a PG of Mr 6.2×10^4, somewhat smaller than that identified in this study. Size base analysis of cervical PGs has also been conducted in humans [11,30]. Combined, these studies showed three size classes present; $>5.0 \times 10^5$, 2.2×10^5 and the majority as 0.7–1.1×10^5 [11]. The PGs were largely CS/DS ($>70\%$) with less than 30% of radioactivity localized in HSPG for all size classes identified. The range of PGs exhibited similar size based characteristics as those found in the present study.

This study also identified two size classes for ^{3}H labeled PGs/GAGs; Mr $>2.0 \times 10^6$ and a smaller PG/GAG covering a broad range of Mr. The tissue extracted PGs/GAGs showed a size profile similar to that of the ^{35}S labeled material while the medium derived fraction showed a profile exhibiting a more extensive range of low Mr PGs/GAGs ($<7.0 \times 10^4$), suggesting possible depolymerization of GAG. This is in accordance with reports that HA depolymerization activity is induced by P in cultured human uterine cervix fibroblasts [31,32].

In the present study, the determination of charge on samples extracted via the technique used by Nicollier et al. revealed a class eluting between 0.1–0.5 M NaCl [27]. Analyses of charged PGs within the guinea-pig showed four charge classes; the majority failing to bind to the support column, a second eluting between 0.3–0.5 M NaCl (similar range to that observed in this study), a third minor peak at 0.8 M NaCl and the remaining class at 2 M NaCl [27]. However, it was postulated that the non-binding group from the guinea-pig may be lipid associated PGs. This study delipidated samples prior to analyses which could account for this discrepancy, as could the animal species involved [27]. Other studies, which utilized the more common GuHCl extraction procedure, showed mice uterine stromal cells generating PGs requiring higher NaCl concentrations for column elution [18]. While a minor proportion of ^{3}H material failed to bind to the support, the majority was eluted between 0.9–1.2 M NaCl. ^{35}S labeled PGs were eluted between 2.5–4.0 M NaCl. The higher NaCl concentration required for elution suggests more negatively charged PGs [18].

In the present study, there was a higher proportion of HA under estrogenic rather than progestational conditions. Such alterations may alter the endometrial environment and architecture necessary for tissue function at a given stage of the cycle. The greater HA proportions in E-dominant tissue may relate to a phase of cell proliferation. The hydrated matrix encourages the diffusion of growth factors such as TGF-β and promotes cytokine protection from proteolytic enzymes [31]. A recent study in sheep, showed that E alone stimulated cellular proliferation in all tissue compartments (glands, luminal epithelium and stroma) whereas a combination of E and P stimulated proliferation only in the luminal epithelium [33]. Furthermore, it is postulated that apoptosis must be involved in regulating uterine size since the dramatic increases in cell proliferation in response to E treatment did not result in increased DNA content. This was

reaffirmed in the present study which showed similar endometrial DNA yield (per unit dry weight) under both estrogenic and progestational conditions, despite E-dominant tissue exhibiting a greater overall wet weight compared to its P-dominant counterparts.

The alterations in total GAG content during the estrous cycle described here supports a role for GAGs, particularly HA, in endometrial function. Presently, little is known about how GAGs are regulated in the endometrium or their importance in endometrial tissue remodelling and embryo implantation. Identification of the PG core proteins may lead to a clearer understanding of their role in the endometrium.

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