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Investigation of Sheep Reproductive Tract as an Animal Model for Pelvic Organ Prolapse and Urogyencological Research

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Investigation of sheep reproductive tract as an animal model for pelvic organ prolapse and urogyencological research

By

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Investigation of sheep reproductive tract as an animal model for

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Pelvic organ prolapse is characterized by the failure of vaginal wall support and protrusion of the pelvic organs through the vaginal orifice. Exact etiology of pelvic organ prolapse is not completely understood. The surgical procedures for pelvic organ prolapse utilize various biomaterials for holding the organs in place. However, the biomaterials used for restoring these organs have a high rate of failure in a complicated anatomical and biomechanical environment. With the given issues at hand, animal models are the best answer for understanding the pathophysiology of prolapse, and determining the cause of failure of these surgical interventions. For this study, we are investigating sheep as an animal model for human pelvic organ prolapse. We compared the anatomy of the sheep pelvic floor with humans. We found that anatomical parameters are a good measure/biomarker for estimating structural and anatomical changes in the body of the animal. As the anatomical measurements are applied to human vaginal prolapse, we can apply the same principles in sheep and further explore the feasibility of using sheep as an animal model for prolapse. Additionally, we evaluated location dependent biomechanical properties of the sheep vaginal tract. We have characterized the structure-property

relationship of sheep vaginal wall tissue in the top third and middle third regions. We found that in contrast to current published research, sheep vaginal tissues are anisotropic in nature. This anisotropic characteristic of the sheep vaginal wall tissue is a direct function of the microstructural arrangement of collagen, elastin, smooth muscle and other extracellular matrix components. We also developed decellularized scaffolds as potential biomaterials, which can be potentially utilized in prolapse surgeries. We developed three different types of vaginal tissue scaffolds using SDS, Triton X-100, and trypsin for reconstructive surgery applications. During the decellularization, all of the cellular components are removed, which leaves the acellular ECM behind. We analyzed the biomechanical properties and microstructural properties of these scaffolds and found that the SDS samples were better in all aspects of the preclinical evaluation. Future studies will aim at applying the anatomical and biomechanical techniques used in this study to prolapsed sheep vaginal wall tissues.

DEDICATION

I would like to dedicate this thesis to the following pillars of my strength:

My parents, Dr. Arun and Dr. Ranjita Patnaik, who have raised me to be the person I am today. Thank you for giving me a chance to prove and improve myself through all my walks of life. Thank you for believing in me and cheering for me throughout my journey. I'm truly honored to have you as my parents.

My wife, Tanmayee, who has been a great source of motivation and inspiration for me. Her co-operation, dedication and encouragement are the reason for what I am today. I learn a little more each day from her, in my tryst to be a better person.

My Brother, Sangeet, who is younger but has stood by me through thick and thin. You are the greatest friend and best brother, one could ask for. Thank you for considering me, your mentor and icon. I hope I'll continue to motivate and inspire you.

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CHAPTER I

INTRODUCTION

1.1 Pelvic Organ Prolapse (POP)[1]

Pelvic organ prolapse (POP) is a fast emerging epidemic affecting vast portions of the population (Subak, Waetjen et al. 2001, Hunskaar, Burgio et al. 2005, Jelovsek, Barber et al. 2005). POP can be defined as the descent of the anterior, posterior, and/or apical vaginal compartment(s) with protrusion of one or more pelvic organ(s) into the vagina (Fig. 1.1). POP occurs due to a loss of structural support to organs contained within the female pelvis (e.g. bladder, rectum, uterus, intestine), which results in anatomical changes that can cause a considerable reduction in a woman's quality of life (FitzGerald, Kenton et al. 2001, Epstein, Graham et al. 2007, Jelovsek, Maher et al. 2007).

[1] Pelvic organ prolapse can be either the prolapse of the vagina, uterus, urethra, bladder, intestine or rectum. For the remainder of the document, all references to the word "POP" or "pelvic organ prolapse" will indicate vaginal (vault) prolapse only (other types of prolapse are out of scope of this study).

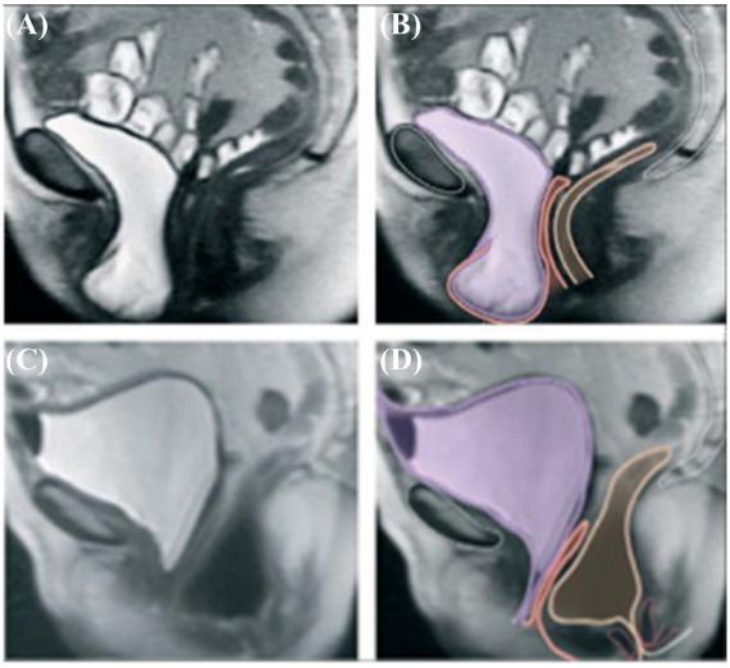

Figure 1.1 Photographs were taken in lithotomy position and sagittal MRI showing prolapse of the vaginal wall.

Types of prolapse can include (from top to bottom): bladder (cystocele) (A-B) or the rectum (rectocele) (C-D). Color codes for MRI images are: purple (bladder), orange (vagina), brown (colon and rectum), and green (peritoneum); adapted from (Jelovsek, Maher et al. 2007).

Pelvic organ prolapse can be either the prolapse of the vagina, uterus, urethra, bladder, intestine or rectum. The different types of pelvic organ prolapse are listed in Fig.1. 2. The three vaginal compartments involved in prolapse are the anterior, posterior and apex. Prolapse of the anterior compartment is the most common presentation, which usually involves the bladder (cystocele), the urethra (urethrocele), or both (Cystourethrocele). Prolapse of the posterior compartment involves the rectum (rectocele) and/or parts of the small intestine (enterocele). Prolapse of the apical compartment is the second most common form of prolapse, and it usually involves the protrusion of the uterus into the vaginal tract (uterine prolapse or vaginal vault prolapse[2] (Fig. 1.3)).

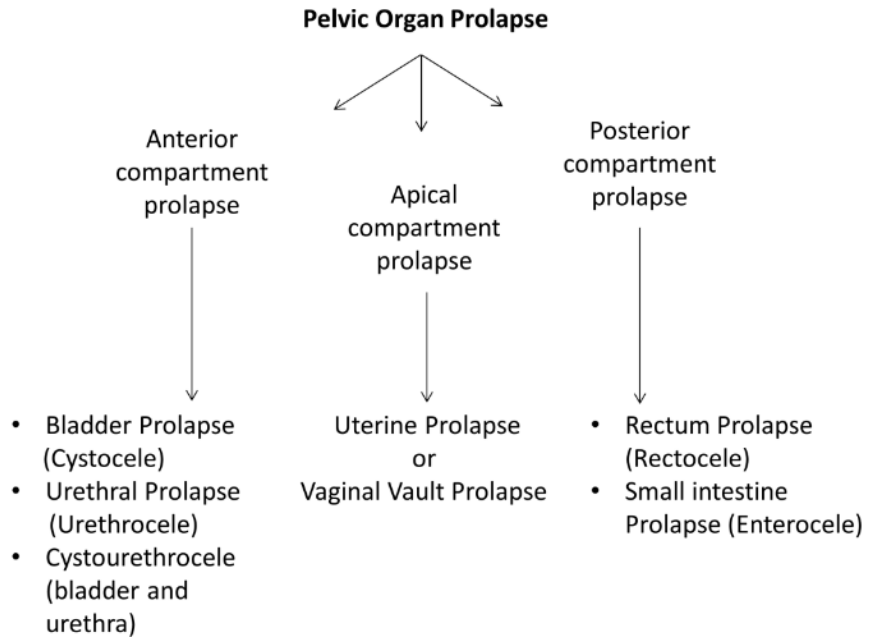

Figure 1.2 Types and onset of pelvic organ prolapse (POP).

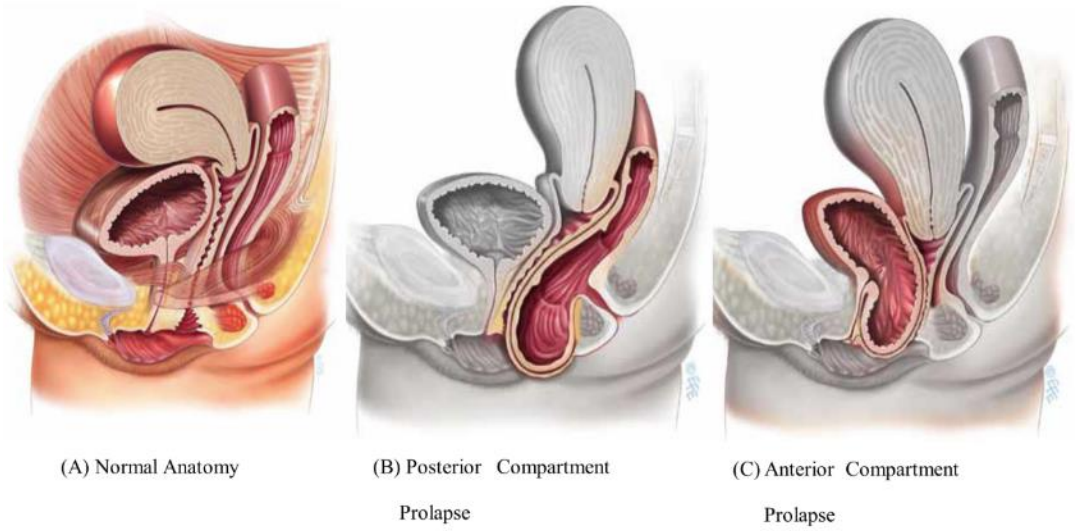

Figure 1.3 Types of vaginal prolapse.

(A) Normal female pelvic floor anatomy, (B) Prolapse of the posterior compartment, and (C) Prolapse of the anterior compartment (Image source - IUGA[2]).

[2] http://www.iugastore.com/77-224-thickbox/pelvic-organ-prolapse-poster.jpg

1.1.1 Epidemiology of POP

POP has been termed "the silent epidemic" (Palm 2009), and it is estimated that 50% of all women who have given birth to one or more children have or will have some form of POP (Subak, Waetjen et al. 2001). Due to the varied cut-offs in diagnosis or the definition of POP symptoms, the epidemiological data reported to date varies between 25 and 97 % of the female population (Nygaard, Barber et al. 2008). Studies show that the lifetime risk for undergoing surgery for POP is between 11-19% in the USA, with an annual direct cost of over $100 million (Hendrix, Clark et al. 2002, Smith, Holman et al. 2010). Reoperation rates for recurrence of prolapse have been reported as 13% by 5 years (Jelovsek, Barber et al. 2005), 17% by 10 years (Denman, Gregory et al. 2008), and as high as 29.2% past 10 years (Olsen, Smith et al. 1997). Familial transmission (both paternal and maternal) of prolapse has also been reported with a relative risk of five times, in comparison to the general population (Jack, Nikolova et al. 2006, McLennan, Harris et al. 2008, Miedel, Tegerstedt et al. 2009, Lammers, Lince et al. 2012). Surgical repair of a pelvic floor defect is therefore one of the most common risk factors for the onset of POP in women (Jelovsek, Maher et al. 2007). Furthermore, female pelvic floor disorders (FPFDs), including POP, represent a considerable financial burden to the U.S. healthcare system at an annual societal cost of over $26.3 billion (1995 dollar) (Wagner and Hu 1998), with $1.5 billion for surgery for POP alone (Subak, Waetjen et al. 2001). Although the majority of POP surgical complications are associated with the elderly population, recent studies suggest that these numbers could even be higher in younger women (Shah, Kohli et al. 2008). Over the next two decades the demand for treatment, management, and research in FPFDs is expected to increase at twice the rate of the

affected demographic, in turn resulting in a 45% increase in demand by 2030 (Luber, Boero et al. 2001). Furthermore, the elderly population is estimated to almost double by 2050 and with advancing age being a risk factor for pelvic organ prolapse, additional healthcare measures will be necessary as will more extensive studies to narrow down the exact incidence of pelvic organ prolapse.

1.1.2 Risk Factors

Studies suggest that POP is a multifactorial disorder with risk factors that are modifiable and non-modifiable, as well as socioeconomic factors playing significant roles (Hendrix, Clark et al. 2002, Swift, Woodman et al. 2005, Jelovsek and Barber 2006, Woodman, Swift et al. 2006). The most common modifiable and non-modifiable risk factors for POP are listed in Table 1.1.

Table 1.1 Risk Factors for Pelvic Organ Prolapse

Non Modifiable Risk factors	**Modifiable Risk Factors**
Advancing age	Large infant size
Race (esp. Caucasian)	Vaginal parity
Menopause/Estrogen deficiency	Obesity
Connective tissue disorder	Cigarette Smoking
Neuropathy	Chronic strain (job related, severe coughing etc.)

(Adapted from Chow et al. (Chow and Rodríguez 2013))

Risk factors for POP include increasing age, obesity, diabetes, multiple vaginal births, gravidity, history of hysterectomy, smoking, chronic constipation, prior POP surgery, prior incontinence surgery, chronic cough conditions, as well as some genetic factors (Hendrix, Clark et al. 2002, Goh 2003, Nygaard, Bradley et al. 2004, Klingele, Bharucha et al. 2005, Vakili, Zheng et al. 2005, Bradley, Zimmerman et al. 2007, Brækken, Majida et al. 2009, Fritel, Varnoux et al. 2009, Miedel, Tegerstedt et al. 2009, Salvatore, Athanasiou et al. 2009, Tinelli, Malvasi et al. 2010, Durnea, Khashan et al. 2014). Certain job related activities or recreational activities, which lead to an increase in intra-abdominal pressure or intra-vaginal pressure (e.g. landing of paratroopers or females involved in squatting and frequent heavy lifting activities), are highly susceptible to develop pelvic floor defects over time and could possibly lead to pelvic organ prolapse (Gerten, Richter et al. 2008, Slieker-ten Hove, Pool-Goudzwaard et al. 2009, Nygaard, Shaw et al. 2014). As previously mentioned, POP results due to loss or damage of structural supports that support the pelvic organs (i.e. rectum, bowel, bladder, etc.) with vaginal wall prolapse (anterior and posterior) being the most common presentation. This can result from weakening of the levator ani muscle and other connective tissue structures which not only control the mechanical function, but also help support neurological and anatomical functions (DeLancey 1990). Prolapse occurs as the soft-tissue support structures in the female bony pelvis degrade (Handa, Pannu et al. 2003). This degradation ultimately reaches a point where the tissue can no longer support the pelvic organs, primarily the bladder, rectum, and uterus, at which point the organs shift and descend into the vaginal canal, and towards the vaginal opening.

1.1.3 Clinical Symptoms

Patients with mild prolapse may not exhibit any symptoms, while patients with severe POP commonly suffer functional consequences such as urinary and fecal incontinence, chronic pelvic pain or pelvic pain syndrome, sexual dysfunctions, de novo dyspareunia (painful intercourse) as well as social and psychological issues (Hendrix, Clark et al. 2002, Digesu, Chaliha et al. 2005, Digesu, Khullar et al. 2005, Jelovsek and Barber 2006, Epstein, Graham et al. 2008). Bowel movements, urinary dynamics and sexual activity are hindered in patients with pelvic organ prolapse (Coates, Harris et al. 1997, Rogers, Villarreal et al. 2001, Achtari and Dwyer 2005, Özel, White et al. 2006, Rogers, Kammerer-Doak et al. 2006, Mueller, Kenton et al. 2007, Schimpf, O'Sullivan et al. 2007, Araki, Haneda et al. 2009, Dain, Auslander et al. 2010, Serati, Salvatore et al. 2011). In addition to the soft tissue defects, women with POP have a reduced bone mineral density, and are highly susceptibility to bone fractures (Pal, Hailpern et al. 2008, Pal 2009, Pal, Hailpern et al. 2011). Most of the POP patients have shown altered urodynamic activity, gastrointestinal complications (Whitehead, Bradley et al. 2007, Jo, Kim et al. 2014), and post-void residual (PVR) urine volumes (> 30 mL) (Hamid and Losco 2014, Töz, Kurt et al. 2015). Even though urinary tract infections (UTI) are not a risk factor for POP, the high PVR volume in POP patients can potential lead to recurring UTIs (Hamid and Losco 2014). Several collagen associated disorders such as Ehlers-Danlos syndrome, Marfan syndrome, varicose veins and joint hyper mobility are highly prevalent in POP patients (McIntosh, Mallett et al. 1995, McIntosh, Stanitski et al. 1996, Carley and Schaffer 2000, Lammers, Lince et al. 2012). A small percentage of older POP patients (> 50 years) with grade 2 or grade 3 prolapse, and a higher number of grade 1

POP patients have shown regression of their symptoms; indicating that prolapse may not be progressive as previously established (Handa, Garrett et al. 2004, Miedel, Ek et al. 2011). With the given variation in the clinical presentation, some patients may be completely asymptomatic and the severity of prolapse may not be linked with any urogenital and bowel movement issues (Ellerkmann, Cundiff et al. 2001). Hence, women exhibiting prolapse symptoms are subjected to a thorough pelvic examination and POP-Q assessment (Pelvic Organ Prolapse Quantification) (Jelovsek, Maher et al. 2007).

1.1.4 Diagnosis

A complete assessment of the POP defect is necessary prior to any surgical intervention or surgical planning. In order to have a common terminology and a diagnostic tool for pelvic organ prolapse for both clinicians and researchers, a standardized evaluation system called the Pelvic Organ Prolapse Quantification (POP-Q) system was developed (Fig.1.4) (Bump, Mattiasson et al. 1996, Hall, Theofrastous et al. 1996, Bland, Earle et al. 1999, Abrams, Cardozo et al. 2002). Detailed explanations of the POP-Q examination procedure are explained elsewhere (Bump, Mattiasson et al. 1996, Persu, Chapple et al. 2011). The POP-Q system is used to assess and measure the different stages of prolapse by a pelvic exam. This system ranges from stage 0-IV; stage 0 being normal anatomy and stage IV being complete eversion of the vaginal wall (Bump, Mattiasson et al. 1996).

In addition to the POP-Q system , the Baden-Walker Halfway System (Baden and Walker 1992), the Beecham system (Beecham 1980), the revised New York grading system (Scotti, Flora et al. 2000), the Women's Health Initiative (WHI) grading systems (Hendrix, Clark et al. 2002), the maximum POP stage (M-POP-S) system (Espuña-Pons,

Fillol et al. 2014), and the Satisfaction–Anatomy–Continence–Safety (S.A.C.S) scoring system (Mearini, Zucchi et al. 2015), are also used by physicians to diagnose the severity of POP or outcome of POP after surgical repairs. Although POP-Q is widely accepted, it is still not used as a routine standardized clinical assessment due to the amount of time required to collect the data (Hall, Theofrastous et al. 1996, Muir, Stepp et al. 2003). POP-Q is also useful for assessment of the anatomical restoration after pelvic reconstructive surgery. Currently, a more simplified form of POP-Q is used which takes less time, is easily reproducible, and very reliable (Swift, Morris et al. 2006).

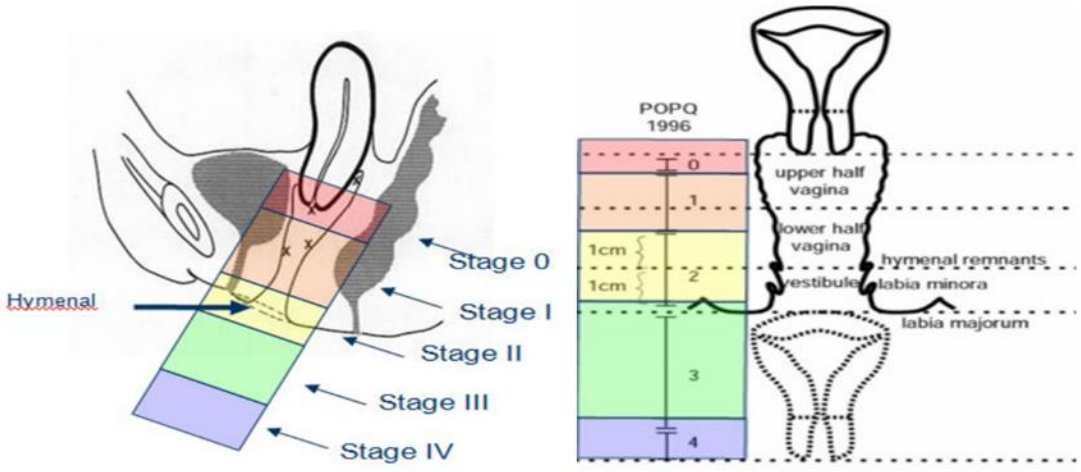

Figure 1.4 Pelvic organ prolapse quantification (POP-Q) system.

Description of stages of prolapse in terms of stages – side view (A) and frontal view (B). Image Sources – Bladder Urogynelcology Prolapse (Australia).

1.1.5 Imaging Tools for Evaluation of POP

Higher stages of prolapse (i.e. beyond stage II) are visible outside the vaginal orifice but lower stages may require the assistance of diagnostic imaging techniques such as MRI, ultrasound, etc. Thorough assessment of the pelvic floor anatomy, including

development of 3D biomechanical models and tissue damage assessment by the use of magnetic resonance imaging (MRI), has been investigated by DeLancey and co-workers (DeLancey 1992, DeLancey 1994, DeLancey 1994, DeLancey, Morgan et al. 2007, Margulies, Lewicky-Gaupp et al. 2008, Ashton-Miller and DeLancey 2009, Chen, Ashton-Miller et al. 2009, Lewicky-Gaupp, Yousuf et al. 2010, Jing, Ashton-Miller et al. 2012, Luo, Chen et al. 2015). On the other hand, uses of ultrasound techniques are more convenient and cost-effective; moreover, quantitative POP-Q measurements are more often assed using ultrasound technique (Shek and Dietz 2009, Manonai, Rostaminia et al. 2015, Siafarikas, Stær-Jensen et al. 2015). Moroever, the superior quality imaging of MRI makes it ideal for visualization of mesh materials (Guillaume, Blanquer et al. 2012) and mesh-related changes in the pelvic floor of patients as well as animal models (Endo, Feola et al. 2014).

1.2 Management of POP – Surgical and Non-Surgical Options

1.2.1 Non-Surgical Management

Patients undergo either a non-surgical or a surgical option for vaginal vault prolapse repair. The non-surgical option involves pelvic floor training (Richardson and Hagen 2009, Braekken, Majida et al. 2010, Andersen, Bor et al. 2011, Stüpp, Resende et al. 2011) and the use of passive assistive mechanical devices and pressaries, which provide mechanical support and hold the prolapsed organs in place (Adams, Thomson et al. 2004, Alperin, Khan et al. 2013, Bugge, Adams et al. 2013). The use of pressaries dates back to Hippocrates (Shah, Sultan et al. 2006, Lamers, Broekman et al. 2011), and these notions are still applied in modern medicine today. The use of pressaries for POP patients is undertaken on a case to case scenario, i.e., based on preliminary POP-Q

evaluations, medical history and other anatomical factors. For example, patients who have undergone hysterectomy and/or are multiparous, or patients with shorter vaginal length or wider vaginal introitus, will have limited success using pressaries (Clemons, Aguilar et al. 2004, Fernando, Thakar et al. 2006). Although, the use of pressaries are considered a viable and "conservative" approach of POP treatment, issues such as anatomical changes (Jones, Yang et al. 2008), vaginal discharge (Collins, Beigi et al. 2014), vaginal lesions (Lopez-Olmos 2013) and fistula formation (Penrose, Yin et al. 2014) have also been reported. Furthermore, proper training for the use and handling of pressaries can also greatly improve the benefits of pressary use in prolapse patients (Bugge, Hagen et al. 2013). Pressaries offer a better alternative solution to surgeries, but for a limited time period (Jones and Harmanli 2010, Oliver, Thakar et al. 2011, Nemeth, Nagy et al. 2013); only a handful of studies have reported the long term effects of pressary usage (Lewthwaite, Staley et al. 2013) or weighed its attributes against surgical options (Lamers, Broekman et al. 2011). However, changes in lifestyle (reduction in body weight, avoiding heavy lifting, etc.), pelvic floor training and physiotherapy have provided a certain degree of relief to the symptoms in POP patients (Hagen, Stark et al. 2009, Hagen and Stark 2011).

1.2.2 Surgical Management

Surgical options for POP patients are tricky, and several factors such as location, type, and stage of the prolapse always dictates the type of mesh/graft (autologous, biologic, or polymeric), or the mode of surgery (vaginal route or abdominal route) to be used (Birch and Fynes 2002, Maher, Baessler et al. 2007, Bako and Dhar 2009, Cox and Herschorn 2012, Umoh and Arya 2012, Barber, Brubaker et al. 2014, Barski, Otto et al.

2014). In addition to the restoration of the pelvic floor anatomy, surgical procedures should also restructure the patient's urinary, bowel and sexual functions. Surgical options for pelvic organ prolapse surgeries can include colporrhaphy procedures, obliterative procedures (colpectomy/colpocleisis) and reconstructive surgeries (ligament suspension and sacrocolpopexy) (Fig. 1.5) (Siddiqui and Edenfield 2014).

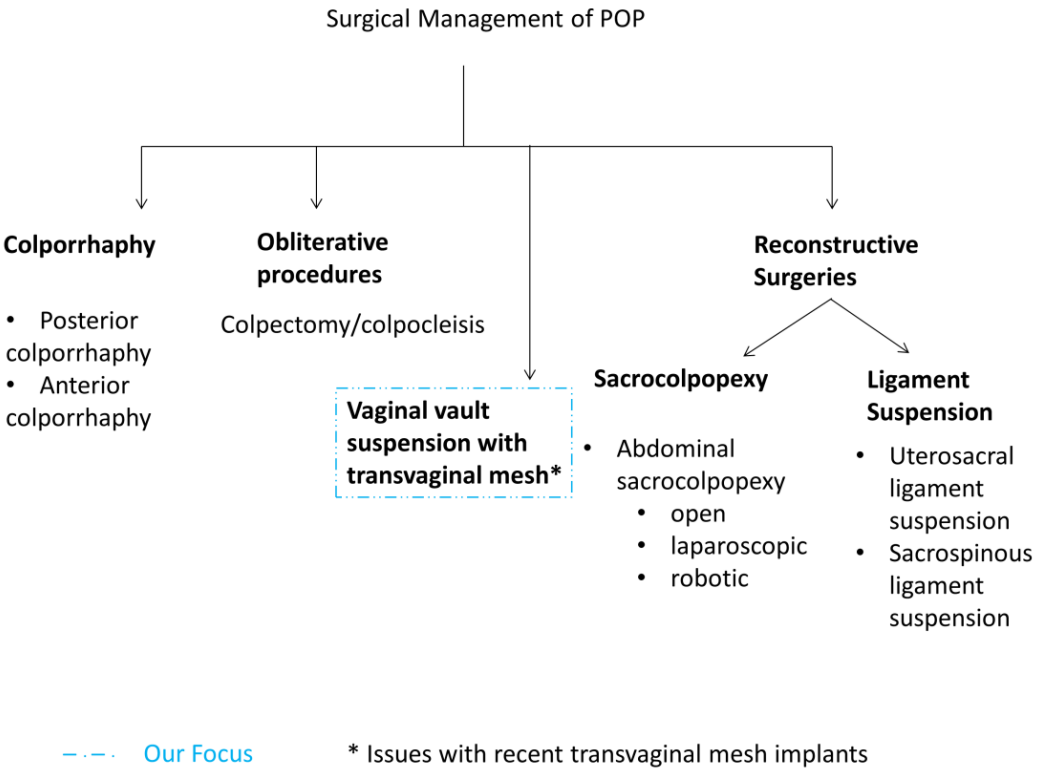

Figure 1.5 Surgical management of POP patients
(Adapted from (Siddiqui and Edenfield 2014))

Of the above mentioned surgical procedures, colporrhaphy is a minimally invasive procedure used for anterior vaginal repair i.e., cystocele (or dropped bladder) or posterior vaginal wall repair i.e., rectocele (or fallen rectum). For vaginal prolapse, surgical options are either obliterative or reconstructive. Obliterative procedures

(colpectomy/colpocleisis) involve shortening of the vagina, and are suited for elderly patients who are no longer sexually active. Obliterative procedures are rarely used as they do not provide the necessary support to the pelvic floor and could further lead to other complications such as incontinence, etc (Chaliha and Khullar 2006). On the other hand, reconstructive surgical procedures (ligament suspension and sacrocolpopexy) for pelvic organ prolapse are aimed for younger and sexually active patients. These reconstructive surgeries aims to fix the anterior and posterior vaginal wall defects, and ultimately resuspend the vaginal apex (Brubaker, Maher et al. 2010); either abdominal or vaginal route is chosen for these surgeries. Reconstructive surgeries for apical prolapse typically involve the use of native tissues and sutures, biological grafts or synthetic meshes. Vaginal and abdominal approaches are most often used for these reconstructive surgeries, but neither one is perfect as there is some degree of complication associated with the procedures. The repair of all types of pelvic floor defects or compartment defects can be achieved via the vaginal approach of prolapse surgery. Moreover, vaginal procedures have lower post-operative morbidity, reduced blood loss and fewer days of hospital stay (Chaliha and Khullar 2006). Commonly utilized vaginal mode of POP surgeries include sacrospinous ligament fixation and uterosacral ligament suspension (Abbasy and Kenton 2010, Koski, Chow et al. 2012, Umoh and Arya 2012, Barber, Brubaker et al. 2014, Siddiqui and Edenfield 2014); while colpopexy procedures take the abdominal approach (Nygaard, McCreery et al. 2004, Pollard, Eilber et al. 2013).

Colpopexy procedures are also called vaginopexy or vaginofixation, and usually involve attachment of the prolapsed vagina to a supporting tissue or organ in order to hold it in its original position. The two types of colpopexy procedures are abdominal

sacrocolpopexy and vaginal sacrospinous colpopexy. For vaginal vault prolapse corrections, sacral colpopexy procedures have shown to repair the pelvic floor tissues and recover most of its biomechanical properties (Epstein, Graham et al. 2008). Further, abdominal sacrocolpopexy procedures have a lower recurrence rate, as compared to vaginal procedures (sacrospinous colpopexy, uterosacral colpopexy and transvaginal mesh) (Nygaard, McCreery et al. 2004, Maher, Baessler et al. 2007, Maher, Feiner et al. 2013). Currently, the laparoscopic and robotic approach of sacrocolpopexy procedures have an added advantage of reduced cost, reduced bowel manipulation, less dyspareunia, reduced blood loss and even lower recurrence rate when compared to the abdominal approach (Hsiao, Latchamsetty et al. 2007, Geller, Siddiqui et al. 2008, Manodoro, Werbrouck et al. 2011, Maher, Feiner et al. 2013, Anand, Woelk et al. 2014, Li, Sammon et al. 2014). Prior surgical operations, such as hysterectomy, have been considered as a possible risk factor for onset of pelvic organ prolapse. After mesh implantation for a uterine prolapse surgery, the views of the surgical community stand divided - whether to spare the uterus (i.e. uterine sparing surgery) or, remove it (i.e. hysterectomy) (Huang, Chu et al. 2015). As a part of this study, we will focus on the issues relevant to vaginal mesh implants.

1.2.3 Issues with Gynecological Meshes

For vaginal vault prolapse correction and pelvic reconstructive surgeries, clinicians utilize commercially available synthetic meshes and biological grafts, or native tissues. The list of commonly used biological grafts and synthetic gynecological meshes are listed in Table 2.1. As there are no established “gold standard” procedures, a variety of surgical techniques and materials (either non-absorbable meshes or absorbable grafts)

are used for correcting pelvic floor defects (Le, Kon et al. 2007, Ridgeway, Chen et al. 2008, Peppas, Gkegkes et al. 2010). Native tissue repairs are used frequently for pelvic floor reconstructions, but these procedures have a higher rate of complication (recurrent prolapse, etc.) as compared to biological grafts or synthetic meshes (Maher, Feiner et al. 2013). On the other hand, biological grafts (absorbable materials such as xenografts, autografts and allografts) are the preferred over synthetic materials owing to their lower complication, infections and mesh exposure rates; however, the rate of recurrent prolapse was higher in these biological grafts than synthetic materials (Jeon and Bai 2007, Le, Kon et al. 2007, Peppas, Gkegkes et al. 2010, Wong, Nguyen et al. 2013). Between 2005 and 2010, over a 1000 complaints were received by the Food and Drug Administration (FDA) with regards to transvaginal meshes issues (FDA 2011). Nonetheless, as per the 2010 Cochrane review, the trend in the use of prosthetic transvaginal mesh for vaginal vault suspension surgeries did not change considerably (Maher, Feiner et al. 2010). The current updated Cochrane review and other scientific reviews have reported that much of the observed scientific findings may have been generalized to a certain extent, which could have led to the untimely retraction of several anterior compartment transvaginal mesh kits from the market (e.g. Prolift ®) (Jia, Glazener et al. 2008, Maher, Feiner et al. 2013).

Table 1.2 Commonly utilized surgical meshes and biological grafts in pelvic reconstructive surgeries

Trade Name	**Material**	**Company**
Biological Grafts		
Veritas	Bovine pericardium	Synovis Life Technologies
Xenform Matrix	Fetal bovine dermis	Boston Scientific Corp.
SurgiMend	Fetal bovine dermis	TEI Biosciences Inc.
Repliform Matrix	Human dermis	Boston Scientific Corp.
Axis Tutoplast	Human dermis	Mentor Corp.
AlloMax	Human dermis	C.R. Bard Inc./Bard Nordic
AlloDerm	Human dermis	LifeCell Corp.
Flex HD	Human dermis	Ethicon
Duraderm	Human dermis (irradiated or non-irradiated)	C.R. Bard Inc./Bard Nordic
Suspend Tutoplast	Human fascia lata	Mentor Corp.
Surgisis	Porcine collagen	Cook Inc.
InteXen LP	Porcine dermis	American Medical Systems Inc.
Pelvicol	Porcine dermis	C.R. Bard Inc./Bard Nordic
Strattice Pliable	Porcine dermis	LifeCell Corp.
Strattice Firm	Porcine dermis	LifeCell Corp.
Avaulta Plus	Porcine dermis (cross-linked)	C.R. Bard Inc./Bard Nordic.

(Adapted.(Kohli and Miklos 2001), (Brown, Londono et al. 2012))

Table 1.2 (Continued)

CollaMend	Porcine dermis (cross-linked)	C.R. Bard Inc./Bard Nordic
Pelvisoft	Porcine dermis (cross-linked)	C.R. Bard Inc./Bard Nordic
MatriStem	Porcine urinary bladder (4 layer)	Acell Inc.
Synthetic Meshes		
Gore-Tex	Expanded Polytetrafluoroethylene – Multifilament	WL Gore
Mersilene	Polyethylene terephthalate – Multifilament	Ethicon
Vicryl	Polyglactin 910 (absorbable) – Multifilament	Ethicon
Vypro II	Polyglactin 910 and polypropylene – Multifilament	Ethicon
Dexon	Polyglycolic acid (absorbable) – Multifilament	Davis and Geck
IntePro	Polypropylene	American Medical Systems Inc.
Polyform	Polypropylene	Boston Scientific Corp.
Gynemesh	Polypropylene	Gynecare
Novasilk	Polypropylene	Mentor Corp.

Table 1.2 (Continued)

Pelvitex	Polypropylene/porcine collagen	C.R. Bard Inc./Bard Nordic
Teflon	Polytetrafluoroethylene (PTFE) – Monofilament	C.R. Bard Inc.
Ascend	Polypropylene - Monofilament Knitted	Caldera Medical
Smartmesh	Polypropylene - Monofilament Knitted	Mpathy Medical
Surgipro	Polypropylene - Monofilament Knitted	Synecture/US Surgical
Trelex	Polypropylene - Monofilament Knitted	Boston Scientific Corp.
Marlex	Polypropylene - Monofilament	C.R. Bard Inc.
Prolene	Polypropylene - Monofilament	Ethicon
Atrium	Polypropylene - Monofilament	Atrium

Long term effects of transvaginal meshes in POP surgeries are controversial, and have shown to cause mesh erosion, pain, de novo dyspareunia (painful intercourse), mesh shrinkage, infection, protrusion of mesh into vaginal cavity, fistula formation, vaginal adhesion and inflammation (Boublil, Ciofu et al. 2002, Baessler, Hewson et al. 2005, Falagas, Velakoulis et al. 2007, De Ridder 2008, Margulies, Lewicky-Gaupp et al. 2008, Bako and Dhar 2009, M Muffly and Barber 2010, García, Ramírez et al. 2011, Bot-Robin, Lucot et al. 2012, Deffieux, Letouzey et al. 2012, Ellington and Richter 2013).

The lack of Level I evidence showing a comparative analysis of synthetic transvaginal meshes and native tissue repairs could be one of the possible causes of transvaginal mesh failure (Ellington and Richter 2013). The data concerning material characterization, mechanical properties, animal studies and translational clinical studies of these transvaginal meshes are clearly inadequate (Kohli 2012). Complications in vaginal meshes can arise from numerous factors even before the surgery, such as surgical instrument handling (Azadi, Jasinski et al. 2014), heat exposure during the manufacturing process (Ostergard 2011), bacterial colonization or degradation of mesh material (Vollebregt, Troelstra et al. 2009, Ostergard 2011), size or geometry of the graft (Falagas, Velakoulis et al. 2007), etc.

1.2.4 Most Commonly Observed Post-operative Issues

Prolift mesh was recently retracted from the market due to issues such as mesh erosions, pain and dyspareunia, mesh shrinkage, urine retention, pelvic abscess, voiding issues, symptomatic vaginal synechiae, protrusion into the bladder, fistulas with mesh, urinary tract infections, and pelvic hematoma (Frankman, Alperin et al. 2013, McLennan, Sirls et al. 2013, Kasyan, Abramyan et al. 2014, Huang, Chu et al. 2015, Zhang, Zhu et al. 2015). Furthermore, Frankman et al. found that prolapse patients with diabetes have seven fold chance of mesh exposure in a vaginal mesh placement surgery as compared to non-diabetic ones (Frankman, Alperin et al. 2013). This study also found that surgical skill of the surgeon is also a contributing factor in mesh exposure. On the patient side, women with higher body weight are highly susceptible to recurrent prolapse (Diez-Itza, Aizpitarte et al. 2007). Of the various mentioned issues with gynecological meshes, we

will focus on the mesh-suture mismatch problem i.e. suture retention strength of the biological grafts (Chapter 4).

1.2.5 Need for Preclinical Evaluation and Basic Science Research for Gynecological Meshes

Preclinical evaluation of these meshes should be thoroughly performed from the basic material level up to the systemic level (animal model). Current developments in vaginal mesh include coating of a wider range of biomaterials such as ECM hydrogel (Faulk, Londono et al. 2014), collagen, etc., for the improvement of its biocompatibility and integration with the host as well. Experts believe that in addition to the biocompatibility, the change in the biomechanical characteristics play an important role in the outcome of an implanted vaginal mesh (da Silva-Filho, Martins et al. 2010, Feola, Pal et al. 2014). Uniaxial tensile testing and biaxial puncture testing methods have been widely used for characterization of *in vitro* and *ex vivo* biomechanical properties of vaginal meshes (Zheng, Lin et al. 2004, Jones, Feola et al. 2009, Pierce, Grunlan et al. 2009, Feola, Abramowitch et al. 2010, Feola, Pal et al. 2014). In POP patients, loading conditions, or the ability of the tissues to bear load, may change internally in the body due to weakness in supporting tissues, increased intra-abdominal pressure and pelvic tissue injuries. Similarly, in case of transvaginal implants the altercation of the loads due to weak tissue integration and stress-shielding (material-tissue property mismatch) may lead to failure of the surgical repair. Clearly, the problem is a mechanical one and hence, biomechanical studies of these meshes can help us evaluate the issues leading to failure of the transvaginal implants. Due to this wide array of factors and extensive list of complications caused in transvaginal mesh repairs, it is difficult to narrow down the

problem to a single entity. Thus, no single surgical approach or surgical mesh can be regarded as the "best" one for POP patients. Hence, further studies with animal models can reveal the exact cause of mesh failure in POP patients.

1.3 Current Animal Models for Prolapse Research

Exact etiology of Pelvic Organ Prolapse (POP) remains elusive to date, and one of the primary hurdles is the non-availability of animal models for POP. In addition to understanding the mystery behind the weakening of the supportive tissues in prolapse patients, better animal models are required to understand the cause of failure of vaginal mesh implants. Non-human primates such as rhesus macaque (Otto, Slayden et al. 2002, Feola, Abramowitch et al. 2010, Liang, Zong et al. 2014), squirrel monkey (Coates, Galan et al. 1995, Coates, Gibson et al. 1995, Kramer, Gendron et al. 2006, Pierce, Baumann et al. 2007, Pierce, Coates et al. 2008), baboons (Mattson, Kuehl et al. 2005), chimpanzee (Asiimwe, Reynolds et al. 2014), etc., are ideal models for pelvic organ prolapse and vaginal mesh implantation studies, due to their ability to prolapse spontaneously and their similar genetic makeup. However, due to ethical, economic and logistical concerns, experts resort to other feasible options such as murine and rodent models (Zheng, Lin et al. 2004, Moalli, Howden et al. 2005, Alperin and Moalli 2006, Lowder, Debes et al. 2007, Alperin, Debes et al. 2008, Lee, Gustilo-Ashby et al. 2008, Moalli, Debes et al. 2008, Rahn, Ruff et al. 2008, Abramowitch, Feola et al. 2009, McNanley, Johnson et al. 2009, Wieslander, Rahn et al. 2009, Alperin, Feola et al. 2010, Budatha, Silva et al. 2013, Hung, Wen et al. 2014). Other than non-human primate and rodent models, vaginal prolapse is a common obstetric issue in farm animals (such as sheep, cows, buffalos, pigs, goats, etc.) (McLean 1956, Popovic 1970, Grommers, Elving

et al. 1985, Hosie 1989, Clarkson 1990, Hay 1991, Hosie, Low et al. 1991, Mitchell 1993, Schulz and Bostedt 1995, Kloss, Wehrend et al. 2002, Anthony, Scheaffer et al. 2003, Sah and Nakao 2003, Purohit 2005, Dhillon, Singh et al. 2006, Gyimesi, Linhart et al. 2008, Miesner and Anderson 2008, McNanley, Johnson et al. 2009, Akhtar, Lodhi et al. 2012), domestic animals (such as dogs, cats, rabbits, etc.) (van der Kolk 1984, Van Herck, Hesp et al. 1989, Biddle and Macintire 2000, Alan, Cetin et al. 2007, Sarrafzadeh-Rezaei, Saifzadeh et al. 2008, Gouletsou, Galatos et al. 2009, Pierce, Grunlan et al. 2009), and several exotic animals as well (e.g. camels (Gitao and Akabwai 1989), antelopes (Gyimesi, Linhart et al. 2008), elephants (Molel 1978), sea lions (Read, Reynolds et al. 1982), dolphins (Wallach and Boever 1983, Đuras Gomerčić, Gomerčić et al. 2010), etc.).

1.3.1 Non-human primate model

Non-human primates (including baboons, rhesus macaque, etc.) are considered to be the ideal animal models for POP (Coates, Gibson et al. 1995, Otto, Slayden et al. 2002, Abramowitch, Feola et al. 2009, Couri, Lenis et al. 2012). Like humans, primate models have similar hormonal system, upright posture, birthing method, pelvic floor tissues, and fetomaternal proportion; and due to their larger body size primate models are ideal for transvaginal surgery too (Rhodes 1962, Abitbol 1988, Otto, Slayden et al. 2002, Mattson, Kuehl et al. 2005, Schimpf and Tulikangas 2005, Wittman and Wall 2007). However, primates are more costly than other animal models (ranging from $7000 - $9000 or more) and have a lot more legal issues associated with them. Moreover, primates are not firmly bipedal and spend majority of their time in a quadruped orientation (Mattson, Kuehl et al. 2005, Couri, Lenis et al. 2012). Other disadvantages of

primate models include a shortened reproductive tract and microbial environment (microbiome) of the vaginal tract is very different from humans (Coates, Gibson et al. 1995, Mattson, Kuehl et al. 2005, Shively and Clarkson 2009).

1.3.2 Rodent and murine models

Rodent and murine models have been used as biocompatibility testing models (i.e. preclinical evaluation of mesh or biomaterial) as well as prolapse related research. These models are advantageous due to their availability, cost effectiveness, for ageing related studies, varied genetic models (knock out), etc. Disadvantages of these models are their ability to quickly repair an injury, their differences in pelvic floor support structure (horizontally oriented as compared to humans), muscles are located in a dorsal orientation to control their tail, their small size which restricts the use of surgical approaches and other similar techniques, and their lower fetal head-to-birth canal size (Abramowitch, Feola et al. 2009). Regardless, the FIB3 (McLaughlin, Bakall et al. 2007), FIB5 (Rahn, Acevedo et al. 2009), LOXL1-KO (Lee, Gustilo-Ashby et al. 2008) and HOXA11 (Connell, Guess et al. 2008) mice models exhibit pathophysiological characteristics similar to that of human prolapse.

1.3.3 Rabbit model

Rabbit models have previously been used for testing abdominal, hernia and gynecological meshes (Krause and Goh 2009, Pierce, Grunlan et al. 2009, Chung, Tu et al. 2014, Nazik, Narin et al. 2014). The advantages of rabbit models include their ease of handling, their relative low cost, and their larger size, which allows for more surgical flexibility (esp. laparoscopic procedures where implants can be performed in either

vagina or abdominal wall) and better imaging procedures. The disadvantage of rabbit models include location variation in the reproductive tract, a very different anatomy as compared to humans, the presence of two different part of vaginas (internal and external portions), the poorly understood immune system, the presence of external connective tissue layer in the anterior vaginal wall, and the high proteolytic activity of the reproductive tract (Rodriguez-Antolin, Xelhuantzi et al. 2009). Moreover, the incidence of vaginal prolapse occurring in rabbits are rare, and have been reported by Van Herck et al. as isolated incidents (Van Herck, Hesp et al. 1989).

1.3.4 Need for large animal model for prolapse research

Most explants from the above mentioned animal models are relatively smaller in geometry, which neither replicate the surgical conditions nor provide sufficient tissue to be used for further biomechanical testing (Ozog, Mazza et al. 2012, Manodoro, Endo et al. 2013).Therefore, a larger size of animal model will be beneficial for surgical and imaging purposes, that will enable the use of wide variety of meshes, and that will facilitate the availability of substantially larger explanted specimens for biomechanical testing (Mangera 2011, Deprest and Feola 2013). With the given issues and compromises between numerous animal models, we hypothesize that sheep are to be the ideal candidate for (i) understanding the mechanism of POP and (ii) understand the etiology behind failure of gynecological mesh implants.

Recently, scientists have projected sheep as an ideal large animal model for biomedical research due to their ease of handling, economical advantage, similar fetal size, comparative anatomy, and being best suited for surgical procedures (Kues and Niemann 2004, Mortell, Montedonico et al. 2006, Abramowitch, Feola et al. 2009, Bähr

and Wolf 2012). Chronologically, sheep were used as means of agricultural research, nutritional studies, wool production, pharmacological research, surgical research (orthopedic, catheterization, etc.) (An and Freidman 1998), circulatory studies (heart transplantation, hematology, vascular graft, etc.) (Ali, Kumar et al. 1996, Chow, Ng et al. 2008, Kónya, Wright et al. 2008, Trollope, Moxon et al. 2011), immunological (immunoassay, etc.) and pathological studies (diabetes, asthma, etc.) (Matute-Bello, Frevert et al. 2008, Psaltis, Carbone et al. 2008, Scheerlinck, Snibson et al. 2008, Srinivasan and Ramarao 2012). Pregnant sheep were also used for fetal development, pregnancy, and other reproductive studies (Liggins 1969, Pearson and Mellor 1975, Dreyfus, Becmeur et al. 1997, Mandon-Pépin, Oustry-Vaiman et al. 2003, Fabre, Pierre et al. 2006, Scaramuzzi, Campbell et al. 2006, Viñoles, Paganoni et al. 2010, Sandra, Mansouri-Attia et al. 2011, Morel, Laporte-Broux et al. 2012, Renard, Chavatte-Palmer et al. 2012, Fransolet, Labied et al. 2014). Sheep have been used as a large animal model for studying fetal implantation (Lee and DeMayo 2004), fetoplacental physiology (Morel, Laporte-Broux et al. 2012), invasive or non-invasive procedures at the time of pregnancy (Morel, Laporte-Broux et al. 2012), role of immune system in early stages of pregnancy (Sandra, Mansouri-Attia et al. 2011), placentation related studies (Carter 2007, Morel, Laporte-Broux et al. 2012), including various stem cell related and biomaterial studies as well (Casal and Haskins 2005, Abi-Nader, Boyd et al. 2012, Malaver-Ortega, Sumer et al. 2012, Harding, Roberts et al. 2013).

1.4 Sheep as a large animal model for POP research

On the other hand, sheep exhibit spontaneous vaginal prolapse, and have similar vaginal length, diameter and microstructural characteristics as humans (Barnhart,

Izquierdo et al. 2006, Krause and Goh 2009, Couri, Lenis et al. 2012). Moreover, sheep have three levels of pelvic floor support similar to humans (DeLancey 1994, DeLancey 1994, Abramowitch, Feola et al. 2009). Fetomaternal disproportions in sheep lead to dystocia (difficulty in giving birth) (McSporran and Fielden 1979), which is a common sequel to vaginal prolapse (Hosie 2008). Hence, as with humans, the anatomical proportions (genital hiatus, pelvic inlet area, etc.) of the pelvic floor are possibly associated with the development of vaginal prolapse. Additionally, due to its large size, sheep is now widely used for preclinical evaluation of gynecological meshes (De Tayrac, Alves et al. 2007, Rezapour, Novara et al. 2007, Krause and Goh 2009, Manodoro, Endo et al. 2013, Alcalay, Livneh et al. 2014, Endo, Feola et al. 2014, Feola, Endo et al. 2014).

1.5 Unmet Research Needs and Our Strategy

In order to establish a good large animal model for POP, it is important to have similar tissue biomechanical properties in addition to anatomical similarity with the human pelvic floor. However, detailed anatomical and biomechanical studies of female sheep pelvic floor tissues in relation to vaginal prolapse are limited. More studies have been performed on vaginal tissues obtained from cadavers, primates, and rats than from sheep (Abramowitch, Feola et al. 2009, Couri, Lenis et al. 2012). MRI studies are an established tool for pelvic floor research in humans, but the volume of MRI (or CT) studies done in humans is not analogous to animal research. The first reported MRI of a female sheep pelvic floor was published by Gauthier et.al only a year ago (Gauthier, Marquet et al. 2014). Only a handful of biomechanical studies have been conducted in terms of tissue structure-property characterization of the sheep pelvic floor. Rubod et.al reported one of the very first uniaxial tensile properties of sheep vaginal wall tissues

(Rubod, Boukerrou et al. 2007). Followed by Knight et al., who reported that parity negatively impacts sheep vaginal biomechanical properties (Knight, Moalli et al. 2013). It was also interesting to note that sheep vaginal tissues exhibited anisotropic as well as location dependent biomechanical behavior (Patnaik, Weed et al. 2012, Weed, Patnaik et al. 2012, Ulrich, Edwards et al. 2014, Ulrich, Edwards et al. 2014).

Recent failures of transvaginal meshes remain unexplained, and studies have suggested a lack of proper mechanical characterization and biocompatibility evaluation of these meshes. Several studies have utilized sheep as animal model for assessment of various gynecological meshes (De Tayrac, Alves et al. 2007, Rezapour, Novara et al. 2007, Mangera, Bullock et al. 2013, Manodoro, Endo et al. 2013, Alcalay, Livneh et al. 2014, Feola, Endo et al. 2014) and have shown sheep to be better suited for prolapse research over other animal models (Abramowitch, Feola et al. 2009, Krause and Goh 2009, Feola 2011, Couri, Lenis et al. 2012). By utilizing sheep as an animal model, we can study both the etiology of POP, and understand the failure of these transvaginal meshes. In this study, we will cover the following:

1. Aim 1 – Perform a thorough characterization of the sheep pelvic floor anatomy and morphology.
2. Aim 2 – Quantify location dependent variation in mechanical and structural properties of sheep vaginal wall tissues
3. Aim 3 – Carry out bioengineering evaluation of decellularized vaginal wall tissue constructs obtained from three types of decellularization methods.

CHAPTER II

THOROUGH CHARACTERIZATION OF THE SHEEP PELVIC FLOOR ANATOMY AND MORHOLOGY

2.1 Comparison of human and sheep pelvic floor structure

2.1.1 Human pelvic floor structure

The anatomical details of the human female pelvic floor have been poorly understood until recently. In 1897, Hart and Barbour mentioned about the movable and fixed parts of the female pelvic floor (Hart and Barbour 1897). As per their observations, the movable structures include the bladder, urethra, peri-urethral connective tissue, fat peritoneum and uterus; and the fixed structures include the posterior vagina wall, rectum, levator ani muscle and its fascia. In 1927, Stevens clearly pointed out that in the case of prolapse the uterus is not the root cause of the issue, but merely part of the group of movable structures (or organs) that decent down (Stevens 1928). In their classical "integral theory", Petros et al. (Petros and Ulmsten 1990) described that the vagina is responsible for providing anatomical support to the pelvic floor and it also accommodates the stretching of bladder; any alteration in the connective tissue (collagen/elastin) of the vaginal wall and/its supporting ligaments can lead to pelvic floor relaxation (or laxity). These damaged ligaments weaken the force of the muscle contraction, which leads to prolapse and other urological issues in female patients (Petros 2011).

In 1994, DeLancey postulated the "hammock hypothesis" which proves that a sub-urethral layer of tissue (consisting of anterior vaginal wall and endopelvic fascia) acts as a supportive or hammock-like structural barrier to the pelvic organs, and this connective tissue layer was laterally attached to the arcus tendineus fascia pelvis and levator ani muscle (DeLancey 1994). With the aid of advanced imaging technologies and meticulous study of the pelvic floor anatomy, DeLancey's three levels of support of the vagina (DeLancey 1992) was devised, and is the most current and widely accepted description for female pelvic floor anatomy (shown in Fig. 2.1). The three levels of supporting structures are (i) Level I – (broad, road and uterosacral ligament), (ii) Level II – (arcus tendineus and fascia of the levator ani muscles) and (iii) Level III - (perineal membrane, levator ani muscles, and perineal body). Most of these anatomical structures are matrix dominated, consist chiefly of collagen and elastin, and provide mechanical support to the pelvic organs (Chen, Wen et al. 2002, Goh 2003, Wong, Harmanli et al. 2003, Söderberg, Falconer et al. 2004, Clark, Slayden et al. 2005, Moalli, Shand et al. 2005, Alperin and Moalli 2006, Phillips, Anthony et al. 2006, Alperin, Debes et al. 2008, Brækken, Majida et al. 2009, Kerkhof, Hendriks et al. 2009, Chen and Yeh 2011, Kannan, McConnell et al. 2011).

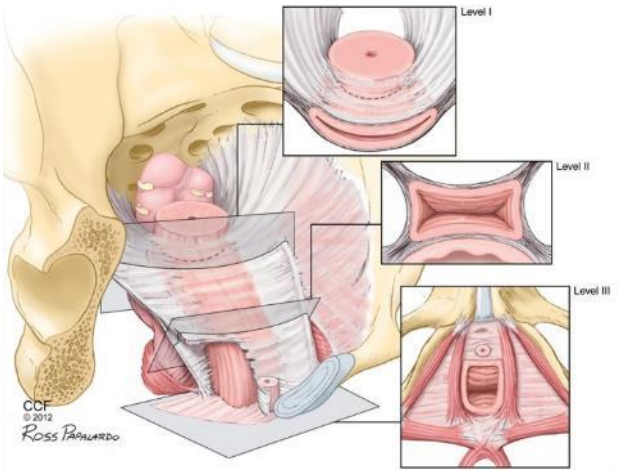

Figure 2.1 Three levels of support for vagina.

Three levels of support (I, II & II) are formed by ligaments, muscle and connective tissues as described by DeLancey (DeLancey 1992) (Image source- (Couri, Lenis et al. 2012)).

In addition to the levator ani muscles, the vagina and its' supportive tissues provide basic structural support to the pelvic organs (bladder, uterus and rectum) (DeLancey, Kearney et al. 2003). Human vaginal tissue consists of four layers – stratified squamous epithelium, subepithelium (lamina propria), layer of smooth muscle cells (muscularis) and an adventitia (Moalli, Shand et al. 2005). The vaginal lamina propria along with muscularis are the fibromuscular layer that is responsible for the basic structural support (DeLancey 1992). The changes in the extracellular matrix (ECM) of the pelvic floor tissues is postulated as one of the primary causes of pelvic organ prolapse (Kerkhof, Hendriks et al. 2009). This change in the ECM composition leads to alteration in their biomechanical properties and also leads to changes in the anatomical structures as well. Owing to biomechanical stresses from prolapse, collagen metabolism is altered in the sub-epithelial layer of vaginal tissue in prolapse patients (Moalli, Shand et al. 2005). This further is indicative of the fact that under the influence of biomechanical load, the

vaginal tissue undergoes active remodeling. Altercations in pelvic muscle mass and pelvic fascia, etc., are some of the most commonly observed anatomical defects in a prolapse patient (Boreham, Wai et al. 2002, Alperin and Moalli 2006, Badiou, Granier et al. 2008, Handa, Blomquist et al. 2011, Alperin, Tuttle et al. 2014). Even after decades of studies with prolapse patients, the etiology of POP is still elusive. Anatomical changes in prolapse patients hold the key to understanding the pathological mechanism behind the disease. Due to ethical concerns, regulatory issues and cost concerns, anatomical studies in prolapse patients are often limited. Hence, animal models are the key to improve our knowledge and understanding of this condition.

2.2 Female sheep pelvic floor structure

2.2.1 Arrangement of pelvic floor organs

Embryological origins of the human vagina can be traced to the fusion of Müllerian ducts and extension of urogenital sinus (Ulfelder and Robboy 1976, Patnaik, Brazile et al. 2015). Similarly, the sheep vagina derives its dorso-lateral segments from the Müllerian ducts and the lower vaginal segment is formed by a superior projection of the urogenital sinus (Bulmer 1952, Bulmer 1956). The reproductive track of sheep starts with the vaginal orifice at the posterior/caudal side and ends with uterine horns at the anterior/cranial side (Fig. 2.2). The structure of the vaginal tract is also similar to humans, and the narrowest portion is in the vaginal orifice, while the widest section was also located in the proximal/cranial vagina (Barnhart, Izquierdo et al. 2006). Moving from the vaginal orifice towards the cranial end of the sheep reproductive tract, the vulva is the first anatomical feature followed by the vagina, cervix, uterus, uterine horns, oviduct and finally the ovaries (Cox 1982). As we move cranially towards the uterus, the reproductive

tract takes almost an S-shaped curved and dives down to the cervix. As the uterus is bipartite or two horned, the tract is bifurcated in shape, and at the end of each horn the oviducts connect to the ovaries. From a vaginal orifice to the uterine horns, the reproductive tract can be divided into three regions – bottom third, middle third and top third; the human vagina is also categorized as lower third, middle third and top third (Baggish and Karram 2006). In the bottom third region of the reproductive tract, the tail section is ventral to the anus followed by the vaginal orifice. Similarly, in the middle third region, the rectum is ventral to the recto-genital pouch, vaginal canal, urethra (including urethral orifice) and pelvic symphysis. Finally, in the top third regions, bladder and ovaries (including oviduct) are ventral to vesico-genital pouch, body of uterus, and cranial sections of recto-genital pouch and rectum.

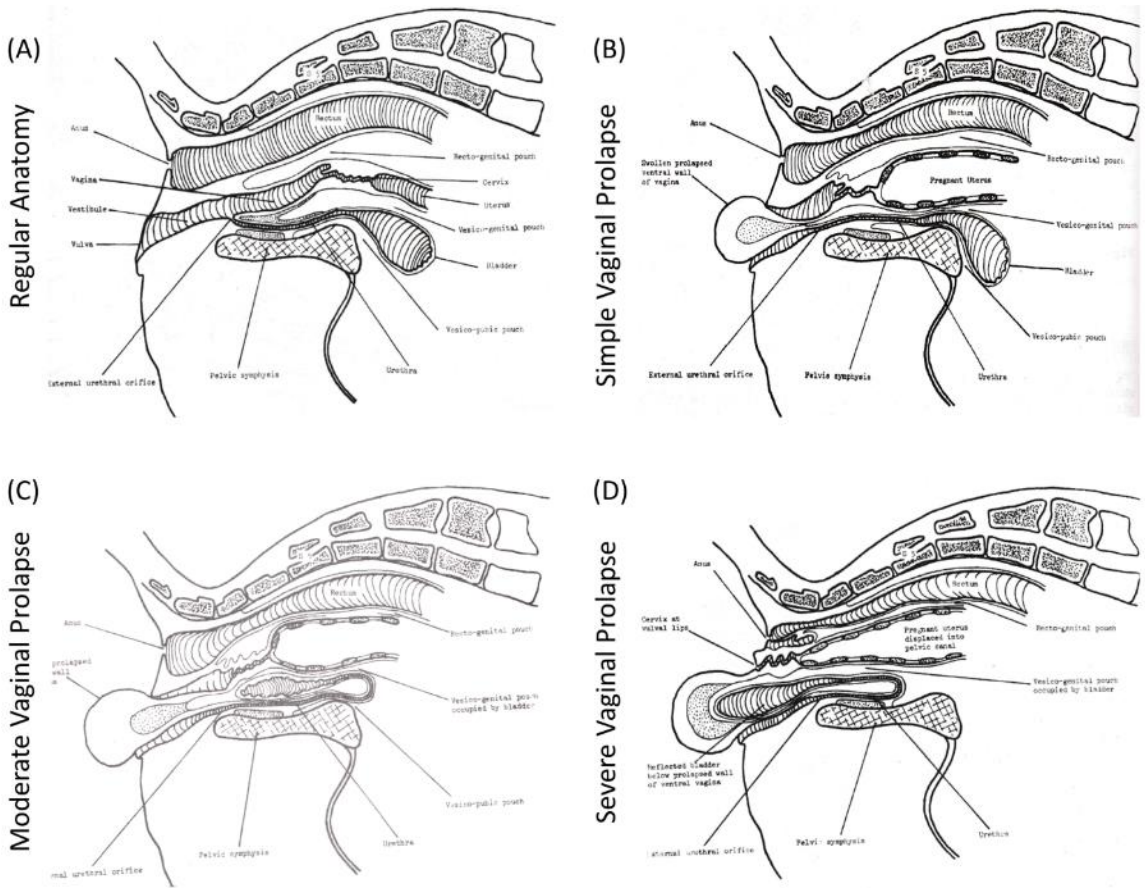

Figure 2.2 Sheep vaginal prolapse.

The figure shows regular sheep anatomy (A), followed by simple vaginal prolapse (B), moderate vaginal prolapse (C) and severe vaginal prolapse (D). Adapted from Cox (Cox 1982).

2.2.2 Supporting structures

The perineal and pelvic region of female sheep are similar to that of humans, with the exception of dorsal and accessory (short and long) sacroiliac ligaments (Bassett 1965). Similar to humans the vagina and the uterus are held together by the help of endopelvic fascia and levator ani muscles (three levels of support). The broad ligament is a thin membranous sheet of connective tissue that covers pelvic organs (cervix, body of uterus and part of the bladder) and it also holds the ovaries in place (Bassett 1971). Any

damage to the fascia, broad ligaments or muscles will lead to either vaginal or uterine prolapse. The urinary bladder is commonly involved in sheep vaginal prolapse and it may involve the uterine horns, uterus, small intestine, etc. (Scott and Gessert 1998, Palmieri, Schiavi et al. 2011). Since sheep pelvic floor tissues, like humans, are composed of collagen, elastin and smooth muscle cells, we expect to observe similar anatomical and physiological changes in case of vaginal prolapse.

2.2.3 Sheep Vaginal Prolapse

As seen in human prolapse, female sheep also experience vaginal prolapse. Sheep depend on their pelvic viscera to support increased intra-abdominal pressures (McLean 1956). With the progression of pregnancy, the pelvic floor tissues of sheep become weaker and the pelvic organs become more mobile inside the pelvic cavity (Ayen and Noakes 1997, Noakes, Parkinson et al. 2001). Under the influence of hormones, there is a marked reduction of collagen content and increase of vaginal wall compliance of the pregnant sheep (Bassett and Phillips 1955, Ayen and Noakes 1997, Noakes, Parkinson et al. 2001). With increasing size of the fetus in the pelvic cavity, the the intra-abdominal pressure of pregnant sheep tends to become higher than normal. (Ayen 2002), and further, leads to straining of the vaginal wall. A chain of such physiological events, combined with other potential risk factors, leads to the onset of vaginal prolapse of the sheep vaginal wall prior to delivery. Vaginal prolapse in female sheep is characterized by a red ball shaped protrusion from the birth canal, which essentially is the vaginal tissue in "inside out" shape (Fig. 2.3). Based on severity of the condition, sheep vaginal prolapse can be categorized as (1) simple, (2) moderate, and (3) severe (Fig 2.2 B-D) (Cox 1982). Simple vaginal prolapse constitutes a swollen ventral vaginal wall of the sheep (Fig 2.2

B); other pelvic organs are not displaced. In moderate vaginal prolapse, either bladder or intestine is involved in the prolapse of the vaginal wall (Fig 2.2 C); here the edema of the vulva is prominent. In case of severe vaginal prolapse, uterus and cervix are caudal to their original positions, and protrusion of cervix is clearly visible (Fig 2.2 D); bladder and intestines could also be part of this condition.

Figure 2.3 Vaginal prolapse in sheep characterized by a swollen red ball of protruding vaginal mucosa.

It is usually 8-12 cm in diameter and may involve bladder, intestine or uterus (Bulgin 2007).

Similar to humans, the risk of vaginal prolapse in female sheep increases with age and parity; twins bearing female sheep and triplet bearing female sheep, are potentially at risk to prolapse five times and twelve times, respectively (Sharman 1973, Hosie 1989, Jackson, Hilson et al. 2003). Further, female sheep with history of vaginal prolapse are more likely to have a recurrent prolapse and ten times more likely to have a caesarean operation to correct the dystocia during subsequent lambing (Scott 1989). Similar to humans, vaginal wall eversion takes place due to the relaxation of the pelvic tissues and a

marked reduction in collagen and smooth muscle cells (Hosie 2008, Kerkhof, Hendriks et al. 2009). A higher displacement of the pelvic organs and a marked increase in compliance of the vaginal wall has been observed in female sheep during their estrus cycle and during their pregnancy as well (Bassett and Phillips 1954, Bassett and Phillips 1955, McLean 1956, Ayen, Noakes et al. 1998, Hosie 2008). Fat female sheep with high body condition score are highly likely to exhibit vaginal prolapse and hence, farmers are advised to keep the female sheep body condition score at 2 out of 5. High body conditioning scores (BCS; subjective scoring of muscle and fat in animals), typically 4 or greater (or extremely poor BCS (<2.5)), are another commonly noted risk factor for prolapse (Bulgin 2007). Description and detailed BCS of female sheep can be found elsewhere (Menzies 2007). Elevated BCS is equivalent to human obesity; it increases abdominal pressure which is distributed to the pelvic floor. Other factors such as less exercise or restricted movement, weight gain during the latter half of pregnancy, increased intake of roughage, excessive straining, vitamin D deficiency, hereditary attributes and poor quality feed have also been investigated as possible risk factor for female sheep vaginal prolapse (Zacharin 1969, Hosie, Low et al. 1991, Jackson, Hilson et al. 2003). To sum up, multiple risk factors can lead to vaginal prolapse in sheep; however, a lot of research needs to be done to better understand the sheep reproductive organ/system.

2.3 Knowledge gap and our objectives

In humans, anatomical parameters such as total vaginal length (TVL) and genital hiatus (GH) are measured via POP-Q system in (pregnant or prolapse) patients to check the anatomical alterations in the body, and asses the possible risk of trauma to the pelvic

floor (Vakili, Zheng et al. 2005, DeLancey, Morgan et al. 2007, Shek and Dietz 2009, Lewicky-Gaupp, Yousuf et al. 2010, Ghafar, Chesson et al. 2013, Siafarikas, Stær-Jensen et al. 2015). However, there are no standardized POP-Q systems for sheep. Cox described the three types of sheep vaginal prolapse but these were based on gross anatomical observations and not exact quantitative parameters (Cox 1982). Bosse et al. devised a measurement system for assessment of vaginal prolapse in sheep (Bosse, Grimand et al. 1989, Noakes, Parkinson et al. 2001), but it is not as descriptive as POP-Q. Changes in sheep pelvic floor anatomy and anatomical measurements with respect to vaginal prolapse were noted by Basset et al. (Bassett and Phillips 1955); however, no standard anatomical parameter was devised specifically for quantification of sheep vaginal prolapse. In this chapter, we will identify the anatomical measurements and morphological parameters, which are clinically relevant in humans and can be applied to sheep as well. Additionally, diffusion tensor-magnetic resonance imaging (DT-MRI) technique was used to quantify the 3D smooth muscle architecture of the vaginal tract at a microstructural level.

2.4 Materials and Methods

2.4.1 Identify anatomical parameters by literature study

To identify the anatomical parameters, a thorough literature search was performed using PubMed, MEDLINE, EBSCO and Google Scholar databases. Sources that were published in languages other than English, or before 1950, were acquired from recently published literature. The keywords used for the search were a combination of the following words – “human vagina”, “sheep vagina” “dimensions”, “ovine vagina”, “morphological variables”, “anatomical parameters”, “sheep pelvic floor”.

2.4.2 Quantitative measurements

2.4.2.1 Sheep pelvic floor tissue harvest

Intact reproductive tracts (vulva to uterine horns) of nulliparous female sheep (breed, age, and weights of the animals are provided in Table 2.1) were obtained from a local abattoir. Tracts were cleaned and excess fat was removed. Intact specimens were then further used for anatomical measurements or DT-MRI scanning. Explanted vaginal tracts were categorized, from caudal to cranial region, as top third (top 3rd), middle third (mid 3rd) and bottom third (bot 3rd) (Baggish and Karram 2006, Ulrich, Edwards et al. 2014); however, for this study we are focusing on the top third and middle third sections only. All anatomical parameters were recorded using an electronic caliper (Mitutoyo Instruments Inc.; sensitive to 0.001 mm) by a single examiner (Patnaik) as per previously published studies (Jelovsek, Sokol et al. 2005, Akbiyik and Kutlu 2010, Sathyanarayana, Beard et al. 2010, Alperin, Tuttle et al. 2014). For recording weights, specimens were patted dry using paper towel and placed on an electronic weighing machine (Brinkmann Instruments, Inc.). All measurements were repeated in triplicate for consistency. Density of vaginal tissue specimens were also calculated via Archimedes principle; for density measurements vaginal tissues were further divided as per anterior or posterior groups (total of four groups).

Table 2.1 Breed, age, live weight and animal number of sheep used in this study.

Breed	**Age (avg. months ± SD)**	**Live weight (avg. lbs ± SD)**	**Animal number (N)**
Rambouillet x Columbia Crossbreed (RC)	9.4 ± 0.516	160.800 ± 16.525	10
Rambouillet x Suffolk Crossbreed (RS)	10.0 ± 0.816	159.813 ± 9.551	16
Rambouillet x Columbia ewes with Suffolk buck (RCS)	10.0 ± 0.000	148.769 ± 3.113	13
Suffolk Western lamb (SW)	10.0 ± 0.000	155.000 ± 45.429	6

2.4.3 Diffusion Tensor Imaging (DT-MRI)

After all the anatomical measurements, four female sheep tracts (SW breed only) were used for DT- MRI imaging. These female sheep vaginal tracts were first sutured shut at the cranial end and the caudal end was kept open with the help of PDS*II sutures (Ethicon, NJ). A gelatin gel (Knox® Gelatine) solution was prepared by adding the gelatin powder to boiling water and rapidly cooled in the minus 80˚C freezer. This was poured inside the vaginal tract and then allowed to stand in the freezer for 20 minutes. After the gel hardened, the entire setup was then placed in the center of a polypropylene cylindrical tube (diameter - 3.5 inches) (McMaster-Carr, USA). With the help of the attached sutures, the vagina was suspended in the center of tube and care was taken to avoid any contact with the inner lining of the cylindrical tube. The tube was closed at one end with help of a stopper and more gel was poured into the tube via the open end. The gel was allowed to cool at room temperature and care was taken to avoid any bubble formation. The final sample setup was then used for DTI imaging studies.

Diffusion tensor imaging of the female sheep vaginal tracts were performed using previously established protocols (Zhang, Crow et al. 2010). A DT- MRI scanning

protocol was applied on the sheep tracts (n=4) with a 3T MRI scanner (GE Signa Excite HDx). Data volume of 96×96×36 was acquired with a voxel size of 1.17 × 1.17 × 2.6 mm. GE diffusion tensor protocol was used with two b values (0 and 1000 s/mm^2) and 55 gradient directions. The 3D muscle fiber orientation and fractional anisotropy (FA) of the smooth muscle tissue were analyzed to quantify the location based differences in the sheep vaginal tract. FA values reflect the differences between isotropic and linear diffusion patterns, and it ranges between 0 and 1 (0 = isotropic diffusion, 1 = highly directional).

2.4.4 Histology and Image Analysis

Routine histology was performed on sheep vaginal tissue samples (all breeds) using Movat's Pentachrome staining. Specimens were obtained from multiple locations of the reproductive tract and fixed in 10% phosphate buffered formalin (Sigma-Aldrich, MO) for at least 48 hours. Fixed samples were then embedded in paraffin wax and processed for sectioning. Five micron sections of the paraffin sections were further used for staining. Multiple adjacent images of the epithelial layer were taken using a light microscope (Leica Microsystems DB2500). These images were further combined into a single continuous image by Photostitcher® software. The obtained image was further imported into NIH ImageJ software for calculation of tortuosity (Eq. 2.1) (Borazjani, Weed et al. 2011). The step by step protocol for the tortuosity calculations are illustrated in the figure 2.4.

$$\text{Tortuosity} = (\text{Apparent length } (l_a)) \,/\, (\text{Straight line distance } (l_0)) \qquad \text{(Eq. 2.1)}$$

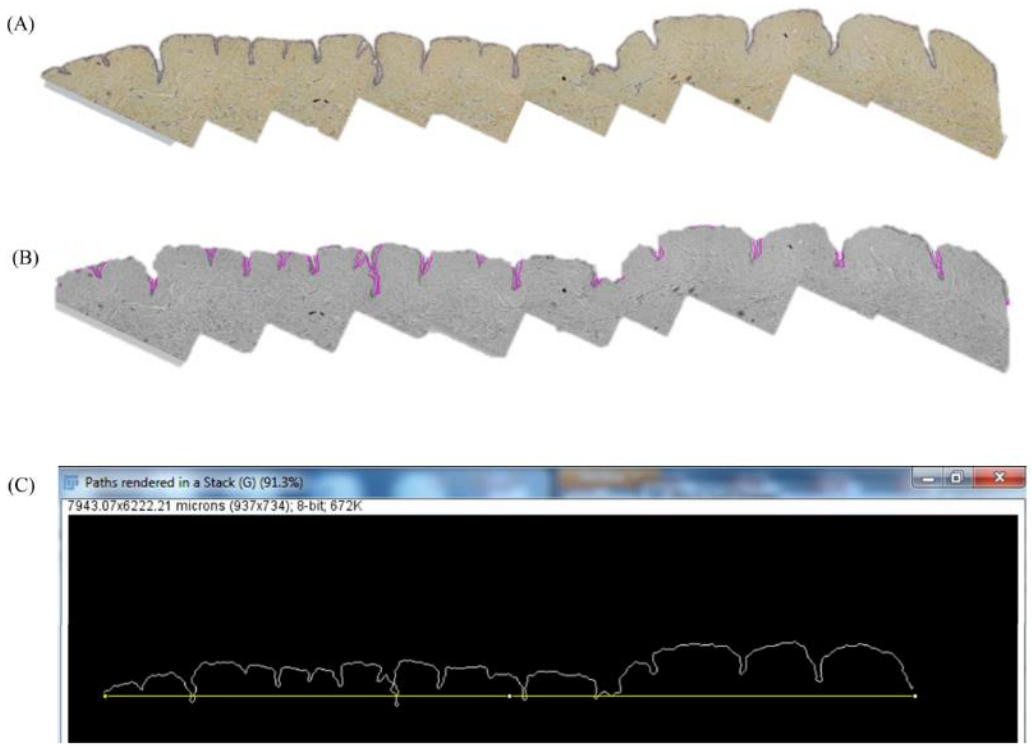

Figure 2.4 Protocol for calculation of histological image tortuosity.

(A) Multiple images of the epithelial layer of the sheep vaginal wall tissue were first captured using light microscope and then combined into a single continuous image. (B) This image was further imported into NIH ImageJ for further processing. (C) The epithelial layer was then traced and the apparent length (la) was calculated. Further, the straight line distance (l0) from the beginning to end of the traced image was then measured. Tortuosity value of the specimen was calculated by taking the ration of apparent length (la) to straight line distance (l0) (Eq.2.1) (Borazjani, Weed et al. 2011).

2.4.5 Statistical Analysis

Data was stored and analyzed using SPSS software (version 13; SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) was used to determine if the anatomical parameters were statistically different. Pair wise comparisons of breeds were performed using Tukey's test. Additionally, two sided t-test was performed for comparison of tortuosity and density data. The degree of association between continuous variables was calculated using Pearson's correlation test, which can be further utilized for multivariate

linear regression analyzes. The anatomical parameters were taken as the dependent variables, whereas age and weight were independent factors. Coefficient of regression was calculated for all significantly independent variables. Data was considered significant at $p < 0.05$.

2.5 Results

2.5.1 Analysis of anatomical parameters applicable to sheep

Similar to previous animal studies, necropsy was the single most feasible option to study to determine the various anatomical measurements. Noninvasive field methods rely on external genitalia, secondary sexual characteristics and behavior, which usually varies from species to species. From previously published studies, it was determined that perennial body (PB), total vaginal length (TVL) and genital hiatus (GH) were the most commonly observed anatomical biomarkers for vaginal prolapse (Vakili, Zheng et al. 2005, Shek and Dietz 2009, Schimpf, Harvie et al. 2010, Ghafar, Chesson et al. 2013). Moreover, these three measurements are part of the POP-Q system (Fig. 2.5) (Bump, Mattiasson et al. 1996).

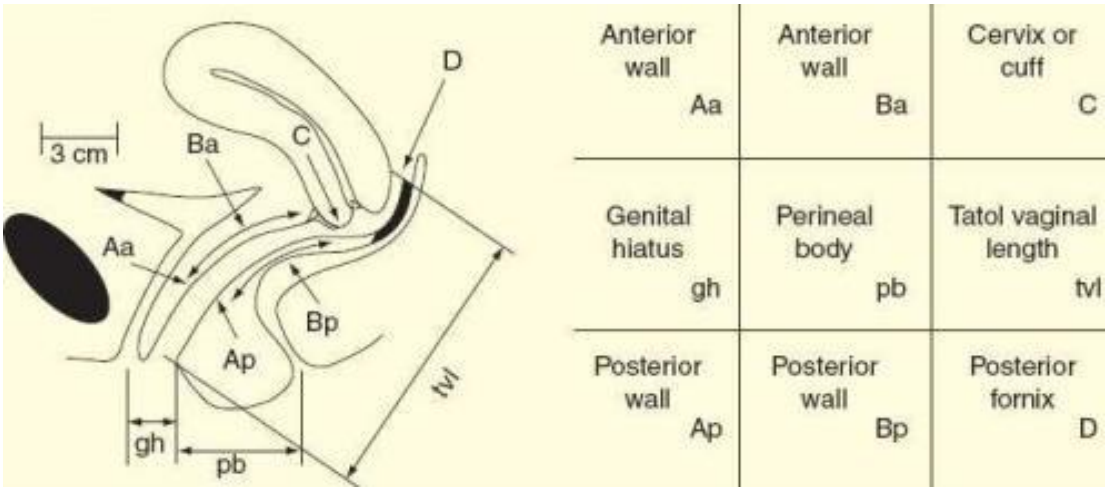

Figure 2.5 Standard POP-Q assessment system.

This system is used to assess anatomical changes in the female pelvic floor. This is widely applied to assess the risk of tissue trauma in pregnant and prolapse patients.

For this study, we will measure GH and further refer to it as vaginal hiatus long (VHL). The width of the vaginal orifice was also recorded and referred to as vaginal hiatus short (VHS). Similarly, PB was measured and will be referred to as anus-fourchette distance (AFD) i.e. distance from the center of the anus to fourchette. Anogenital distance (AGD), which is the sum of VH long and AFD, was also recorded in this study. Additionally, vaginal diameter (V.dia) and total vaginal length (TVL) were also calculated. Description and significance of the mentioned anatomical parameters are listed in Table 2.2. Illustrations of anatomical parameters are shown in Fig. 2.6 and 2.7. Along with these anatomical parameters, genital organ weights (bladder, cervix, uterus and vagina) were also recorded.

Table 2.2 Significance of anatomical measurements

Anatomical Measurements	Relevance	Source

• Vaginal hiatus
  ○ Short Axis (VH-Short)
  ○ Longitudinal axis (VH-Long)
	• Pregnancy and pelvic organ prolapse leads to increase in genital hiatus (GH) in female patients. For this study, we are referring genital hiatus as vaginal hiatus long (VH Long).
	• Additionally, width of the vaginal orifice (VH short) was also measured.
		VH – Short (current study)
		
		VH (Vakili, Zheng et al. 2005, Shek and Dietz 2009, Lewicky-Gaupp, Yousuf et al. 2010, Ghafar, Chesson et al. 2013, Siafarikas, Stær-Jensen et al. 2015)Long

• Anogenital Distance (AGD) and anus-fourchette distance (AFD)	• AFD is same as PB or perineum length/distance in humans. • AGD is sum of VH long and AFD. • PB is correlated positively with age and negatively with childbirth	(Kim, Jeon et al. 2007)

Table 2.2 (Continued)

• Vaginal Diameter (V. Dia)	• Mid-sagittal diameter is higher is prolapse patients. • Distention of the vagina takes place at the time of child birth and due to fetomaternal disproportion birth (both commonly observed in sheep and human prolapse cases).	(McSporran and Fielden 1979, Hsu, Chen et al. 2005, Lewicky-Gaupp, Yousuf et al. 2010)
• Total Vaginal Length	• Increased TVL is observed after pregnancy is nulliparous women and patients with POP.	(Bland, Earle et al. 1999, O'Boyle, Woodman et al. 2002)

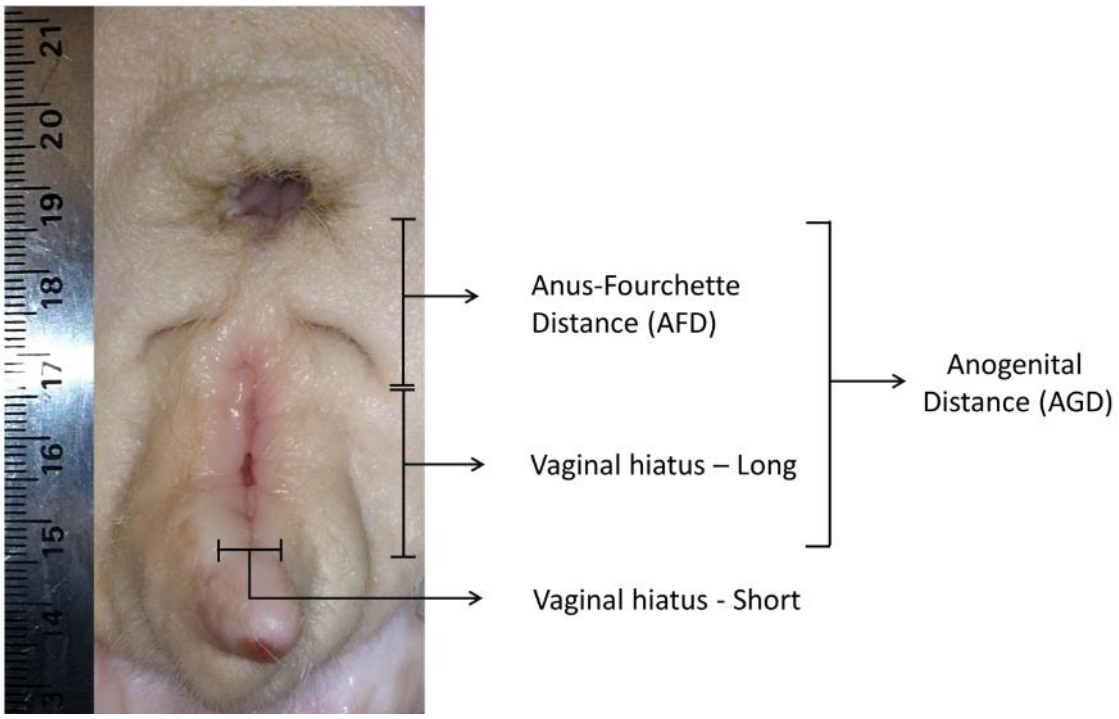

Figure 2.6 Illustration of anatomical parameters – VH Long, VH short, AFD and AGD.

2.5.2 Quantitative Anatomical measurement results

2.5.2.1 Vaginal tract measurements (VH long, VH short, TVL & V. dia)

Vaginal hiatus, used in this study, is similar to genital hiatus (GH) in humans. GH is known anatomical biomarker which quantifies changes in pelvic floor due to pregnancy and prolapse (Vakili, Zheng et al. 2005, Lewicky-Gaupp, Yousuf et al. 2010, Ghafar, Chesson et al. 2013). In our study, we applied the same concept to sheep samples. We hypothesize that due to the eversion of vaginal wall and edema of the external genitalia during vaginal prolapse in sheep, we will observe a change in vaginal orifice. We measured the length of the vaginal orifice (VH long) (i.e. the distance from base of the clitoris to the vagina), as well as the width of vaginal orifice (VH short) (Fig 2.6). TVL was measured from the tip of the vaginal opening to the base of the cervix as shown in Fig 2.7. Vaginal diameter was calculated at the interface of top and middle third regions of the sheep vaginal tract (Fig. 2.7), similar to mid sagittal diameter measurement in humans (Hsu, Chen et al. 2005).

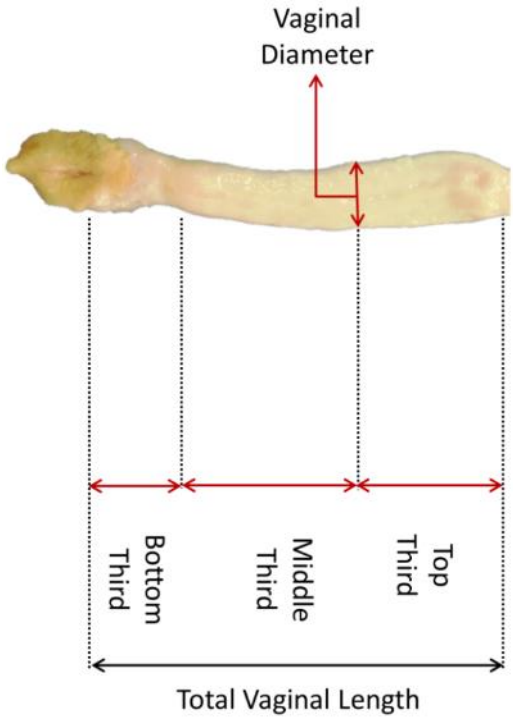

Figure 2.7 Anatomical measurements of explanted sheep reproductive tract – TVL and V. Dia.

Different parts and organs of the sheep reproductive tract are shown here. The tract is divided as bottom third, middle third and top third.

Anatomical parameters related to vaginal tract (VH long, VH short, TVL and V. dia) were recorded for all the sheep breeds (Fig 2.8). VH long, VH short and V. dia was found to be significantly different across the breeds. Data was also significantly different in pairwise comparison amongst the breeds for VH long and VH short. TVL was not significantly different across the breeds.

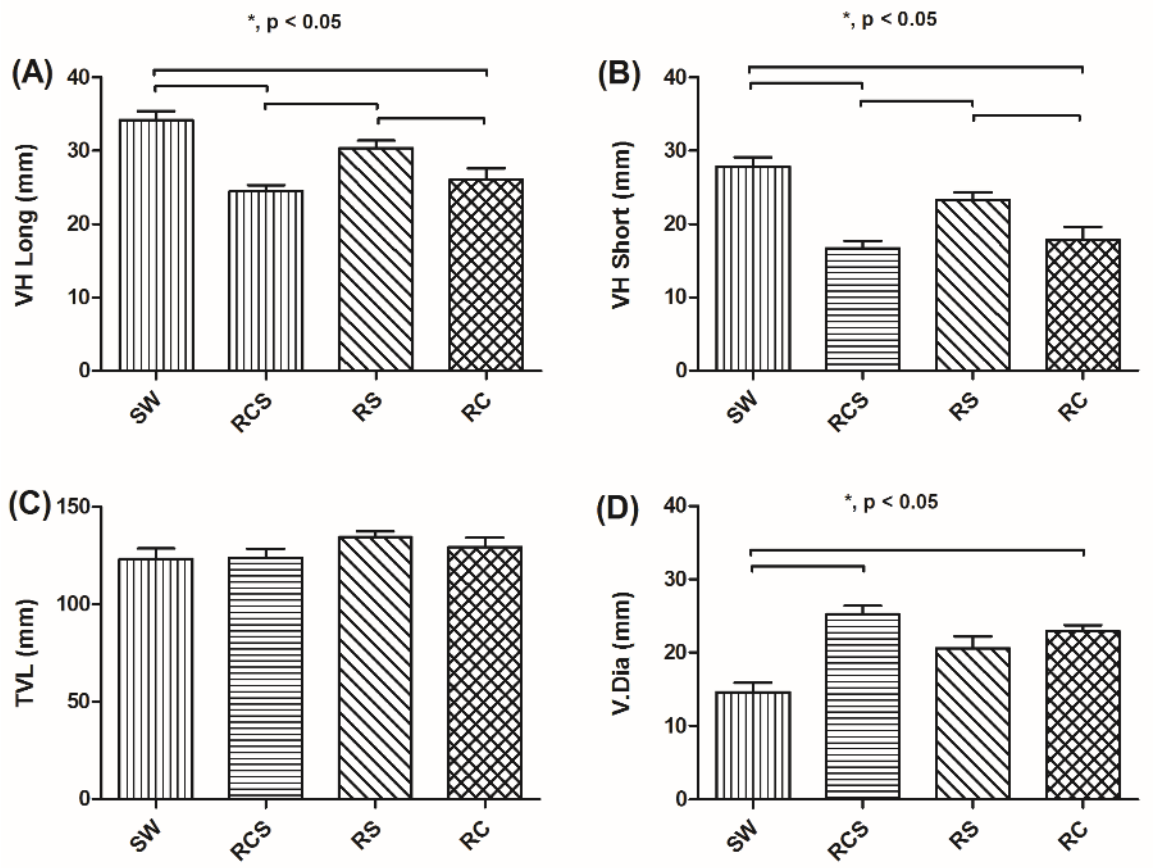

Figure 2.8 Anatomical measurement data of the vaginal tract for each sheep breed. Comparative measurements (A) VH long, (B) VH short, (C) TVL and (D) V. Dia. of four sheep breeds (SW, RCS, RS, RC) are shown here.

VH long and VH short were highest in magnitude for SW breed (34.19 ± 2.96 mm, and 27.85 ± 3.082 mm, respectively), whereas V.dia was highest in RCS breed (25.25 ± 4.061 mm). For VH long, after SW the magnitude of RS was highest, followed by RC and lastly RCS (30.34 ± 4.210 mm, 26.05 ± 4.924 mm, and 24.50 ± 2.969 mm, respectively). Similarly for VH short, after SW the magnitude of RS was highest, followed by RC and lastly RCS (23.28 ±4.187, 17.81 ± 5.701 mm, and 16.67 ± 2.736 mm, respectively). For V.dia, after RCS the magnitude of RC was the highest, followed

by RS and lastly SW (25.25 ± 4.061mm, 22.94 ± 2.691mm, and 14.64 ± 3.23mm, respectively).

2.5.2.2 Anogenital distance (AGD) and Anus-Fourchette distance (AFD)

Anus-fourchette distance (AFD) is the distance from the center of the anus to fourchette, while anogenital distance (AGD) is the distance from base of the clitoris to the anus (Fig 2.6). The relationship of human anogenital distance (AGD) with in utero developments and environmental exposure to phthalates is well characterized and established (Salazar-Martinez, Romano-Riquer et al. 2004, Barrett, Parlett et al. 2014). Additionally, AGD serves as a sexually dimorphic marker measurement in newborns (Salazar-Martinez, Romano-Riquer et al. 2004, Sathyanarayana, Beard et al. 2010), as an anthropometric marker in term infants (Kim, Lee et al. 2014), as a marker for female reproductive development (Barrett, Parlett et al. 2014), as a marker for gestational age and birth weight (Papadopoulou, Vafeiadi et al. 2013), and as a determinant of prenatal exposure to stressful life events (Barrett, Parlett et al. 2013). In this study, we demonstrate the feasibility of using AGD and AFD (anus-fourchette distance) as possible anatomical markers for vaginal prolapse.

Table 2.3 Anogenital distances of female population from previous studies.

Study population	Parameter	Avg. Age (yrs)	Average AGD (mm)	Err		Source
Female Human - 1 year old (USA; phthalate exposure)	Anogenital Distance (mm)	1	48.3	7.1	53	(Swan 2008)
Female Human - approx. 0.2 months (UK)	Anogenital Distance (mm)	0.02	9.1	2.5	279	(Thankamony, Ong et al. 2009)
Female Human - approx 1 year (UK)	Anogenital Distance (mm)	1.5	14.5	3.3	223	(Thankamony, Ong et al. 2009)
Female Human - approx. 1.5 years (UK)	Anogenital Distance (mm)	1.25	15.8	3.5	168	(Thankamony, Ong et al. 2009)
Female Human - approx. 16 months (USA)	Anoclitoral/ Anogenital Distance (mm)	1.25	47.2	20	273	(Barrett, Parlett et al. 2013)
Female Human - approx. 16 months (USA)	Anogenital Distance (mm)	1.25	20.3	11	273	(Barrett, Parlett et al. 2013)
Female Human - approx. 2 years (Crete)	Anogenital Distance (mm)	2	21.7	3.9	732	(Papadopoulou, Vafeiadi et al. 2013)
Female Human - approx. 2 years (UK)	Anogenital Distance (mm)	2	15.3	3	37	(Thankamony, Ong et al. 2009)
Female Human - approx. 3 months (UK)	Anogenital Distance (mm)	0.25	12.2	2.9	220	(Thankamony, Ong et al. 2009)

Table 2.3 (Continued)

Female Human - Birth (Greece)	Anogenital Distance (mm)	0.4	14.4	3	165	(Papadopoulou, Vafeiadi et al. 2013)
Female Human - Birth (Spain)	Anogenital Distance (mm)	0.4	13.8	2.5	187	(Papadopoulou, Vafeiadi et al. 2013)
Female Human - New Born (Barcelona)	Anoclitoral Distance (mm)	0.4	35	3.3	118	(Vafeiadi , Agramunt et al. 2013)
Female Human - New Born (Barcelona)	Anogenital Distance (mm)	0.4	14.3	3	118	(Vafeiadi , Agramunt et al. 2013)
Female Human - New Born (Mexico)	Anogenital Distance (mm)	0.4	11	0.27	42	(Salazar-Martinez, Romano-Riquer et al. 2004)
Female Human - New Born (Nigeria)	Anogenital Distance (mm)	0.4	13.89	0.26	100	(Avidime , Avidime et al. 2011)
Female Human - New Born (Nigeria)	Anogenital Distance (mm)	0.4	25.8	1.1	59	(Orish and Didia 2009)
Female Human - New Born (Turkey)	Anogenital Distance (mm)	0.4	13.4	3.2	300	(Özkan, Konak et al. 2011)
Female Human - New Born (Turkey)	Anogenital Distance (mm)	0.4	10.3	0.2	115	(Özkan, Konak et al. 2011)
Female Human - New Born (Washington, USA)	Anoclitoral Distance (mm)	0.4	37.2	3.7	87	(Sathyanarayana, Beard et al. 2010)

Table 2.3 (Continued)

Female Human - New Born (Washington, USA)	Anogenital Distance (mm)	0.4	15.1	2.9	87	(Sathyanarayana, Beard et al. 2010)
Female Human - Nulliparous	Anogenital Distance (mm)	18-22	37.7	6.3	99	(Mira-Escolano, Mendiola et al. 2014)
Female Human - Young Girls (Barcelona)	Anoclitoral Distance (mm)	18-22	49.1	0.6	223	(Vafeiadi , Agramunt et al. 2013)
Female Human - Young Girls (Barcelona)	Anus-fourchette distance (mm)	18-22	21.7	3.9	223	(Vafeiadi , Agramunt et al. 2013)

Anogenital distance (AGD) and anus-fourchette distance (AFD) were also recorded for the four sheep breeds (Fig. 2.9). AGD data were found to be statistically significant across the breeds, while AFD data was not statistically significant. In pairwise comparisons, AGD data were also significant different between RCS and SW, and RCS and RS groups, respectively. For AGD, the highest magnitude was observed in the SW breed (62.82 ± 3.969 mm), followed by RS (56.89 ± 4.626 mm), RC (54.36 ± 5.679 mm) and lastly RCS (51.07 ± 4.626 mm).

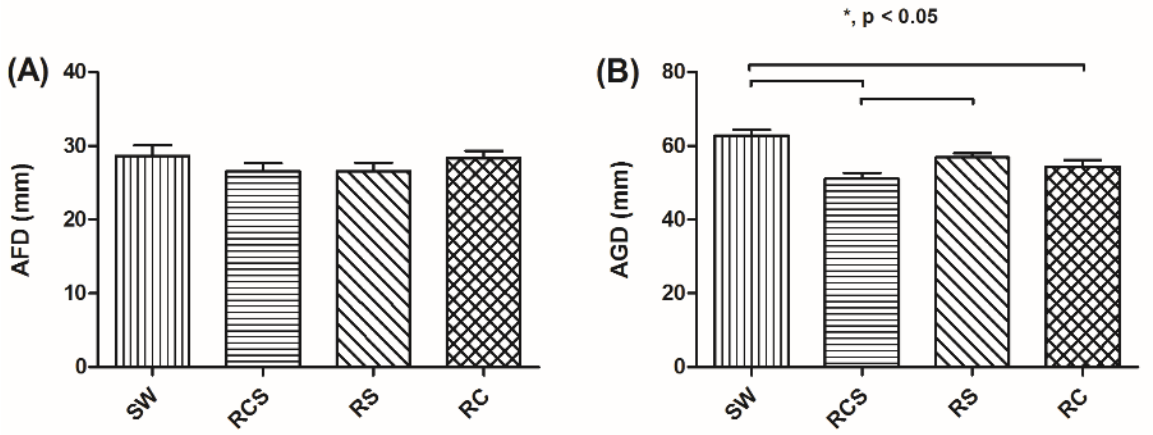

Figure 2.9 Anogenital distance (AGD) and anus-fourchette distance (AFD) measurements for the four sheep breeds.

(A) AFD data was not statistically significant, while (B) AGD data was significantly different across the breeds.

Stepwise multiple regression analyses were performed and predictive equations were generated using measurements for live weight, age, VH Long, AFD, AGD, TVL, and V.dia to estimate the expected vaginal anatomical parameters (Table 2.4). After stepwise regression analyzes, least useful parameters were eliminated, and VH Long, AFD, and V.dia were selected for the regression model.

Table 2.4 Predictive equations for vaginal tract anatomical parameters

Anatomical Parameters	**Equation**
VH Long	2.628 + (2.593 X age)
AFD	55.926 – (2.909 X age)
V. Dia	51.379 – (3.010 X age)

Regression coefficients of the predictive equation for vaginal tract as a function of age are listed in the multiple regression Table 2.5. VH long showed positive correlation

with age, while AFD and V.dia showed negative correlation with age. Distribution of VH Long, AFD and V. dia data is shown here in Fig. 2.10.

Table 2.5 Regression coefficients for vaginal tract measurements parameters as a function of age.

Independent Variables	**Regression Coefficients (mm)**	**p-value**
VH Long		
Invariable	2.628	
Age	2.593	0.046
AFD		
Invariable	55.926	
Age	-2.909	0.003
V. Dia		
Invariable	51.379	
Age	-3.01	0.04

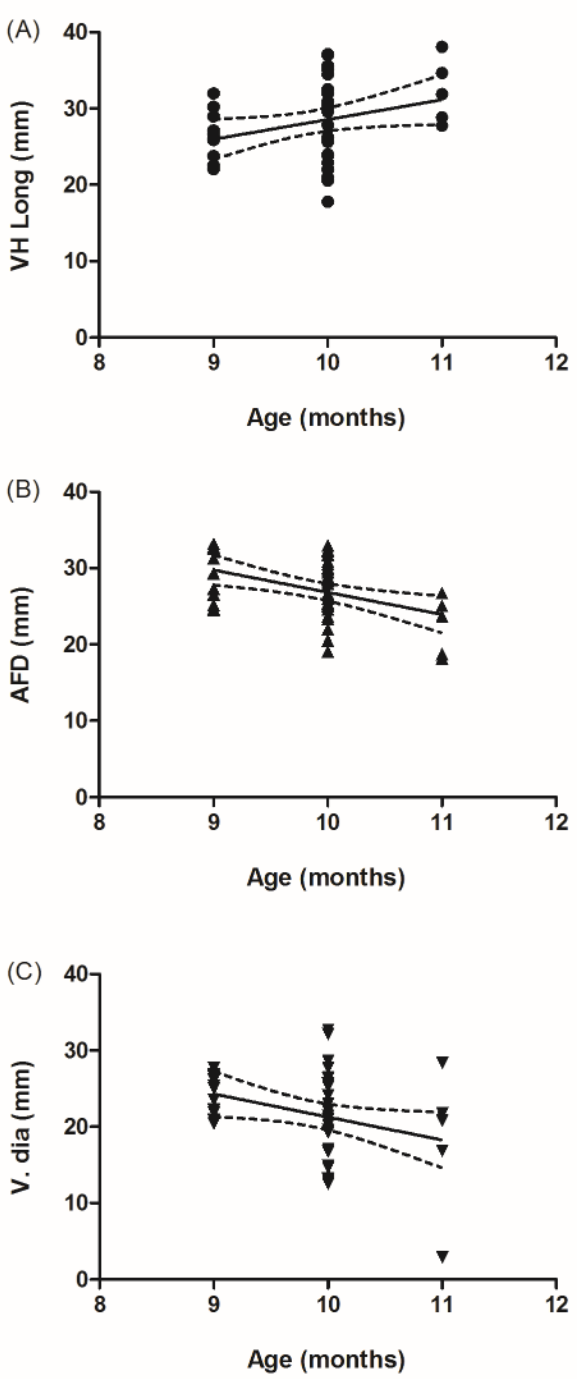

Figure 2.10 Distribution of vaginal tract anatomical parameters by age.

(A) VH long, (B) AFD, and (D) V.dia, were plotted with respect to the age of the animal. The solid line represents the predictive trend and dashed line represents the confidence interval.

2.5.3 Organ weights

Body and organ weights are usually determined in animals for their reproductive potential and commercial success. Jalali et al. investigated the effect of noise stress on reproductive organ development (weight) in rats (Jalali, Saki et al. 2012). For intact organ weight measurements in humans are not possible; and hence, the volume or dimensions of the organ are determined using radiological techniques in which the organ weight is then extrapolated using allometric equations with respect to body weight. A linear regression model was then used to determine relation between the live weight and the organ weights.

From the statistical analyses of genital organ weight data, bladder weights and vagina weights were significantly different across the breeds (Fig 2.11). Uterus and cervix weights were not significantly different. For bladder weight, RCS (14.06 ± 1.205 g) was highest in magnitude, followed by RC (11.45 ± 2.514 g), then SW (10.7 ± 0.8075 g), and lastly, RS (10.09 ± 2.492 g). Conversely, for vaginal weight, SW (21.3 ± 4.656 g) was highest in magnitude, followed by RS (15.73 ± 7.378 g), then RC (14.58 ± 3.306 g) and finally, RCS (14.35 ± 3.765 g).

Similar to vaginal anatomical parameters, genital organ weights were also subjected to stepwise multiple regression analyzes and predictive equations were also formulated. After elimination of least useful variables, only vaginal weight was used for the regression model. Predictive equation of the vaginal weight was then formulated as a function of age and live weight. The regression coefficients of the equations are listed in Table 2.6.

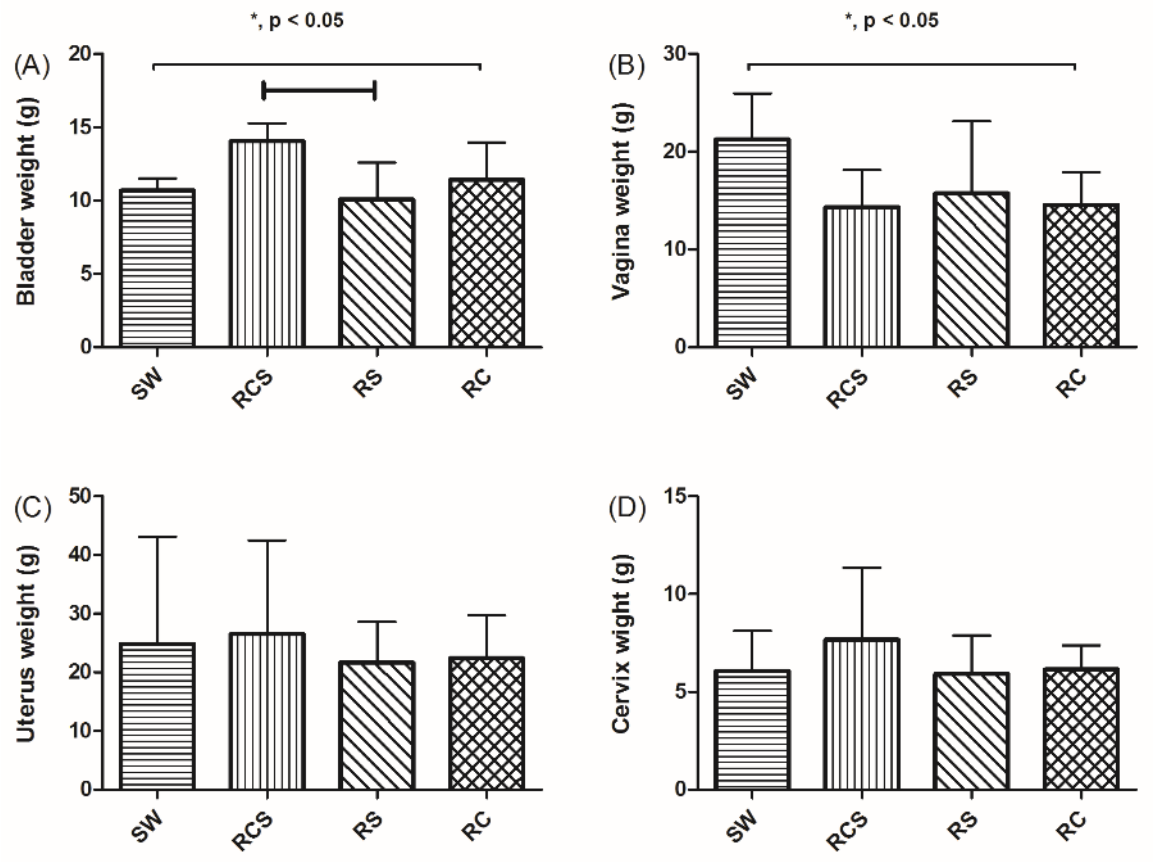

Figure 2.11 Comparative account of gential weight (bladder, vagina, uterus and cervix) measurements from four sheep breeds.

Bladder weights (A) and vagina weights (B) were significantly different across the breeds. On the other hand, uterus (C) and cervical weights (D) were not statistically different.

Table 2.6 Predictive equation for vagina weight as a function of age and live weight.

Genital Organs	**Equation**
Vagina weight	20.698 + (0.00033 X Live weight) - (2.891 X Age)

From the predictive equation, vaginal weight showed positive correlation with live weight, whereas it showed negative correlation with age (Table 2.6). Distribution of vaginal weight as per age and live weight is shown in Figure 2.12.

Table 2.7 Regression coefficients for vaginal weight as a function of age and live weight.

Independent Variables	**Regression Coefficients (g)**	**p-value**
Vagina weight		
Invariable	20.698	
Live weight	0.00033	0.047
Age	-2.891	0.04

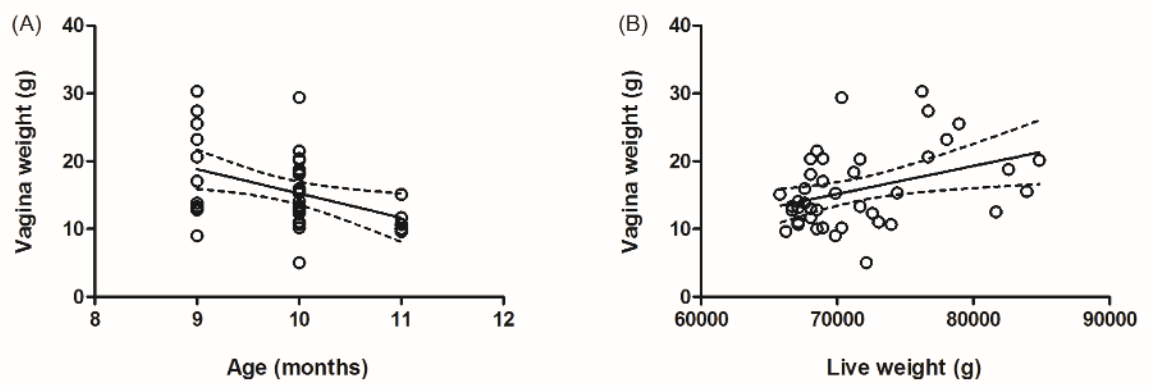

Figure 2.12 Distribution of vaginal weight as per age (A) and live weight (B).

Predictive trend (dark solid line) of the dependent variable (here it is vagina weight) is shown here as a function of age and live weight. The dark dashes lines are the confidence intervals of the predicted trend.

2.5.4 Tissue density (vagina)

Density of reproductive tissues have been previously estimated via Archimedes principle (Swierstra 1968). To our knowledge, this is the first report of vaginal tissue density measurement by Archimedes principle of volume displacement. Tissue density values will be obtained from three different regions of the vagina – top third, middle third and bottom third. The obtained data will be further correlated with anatomical and biomechanical parameters. Vaginal tissue density was not significantly different across

the group (Fig 2.13). This shows that the tissue density was conserved across throughout the sheep vaginal tract.

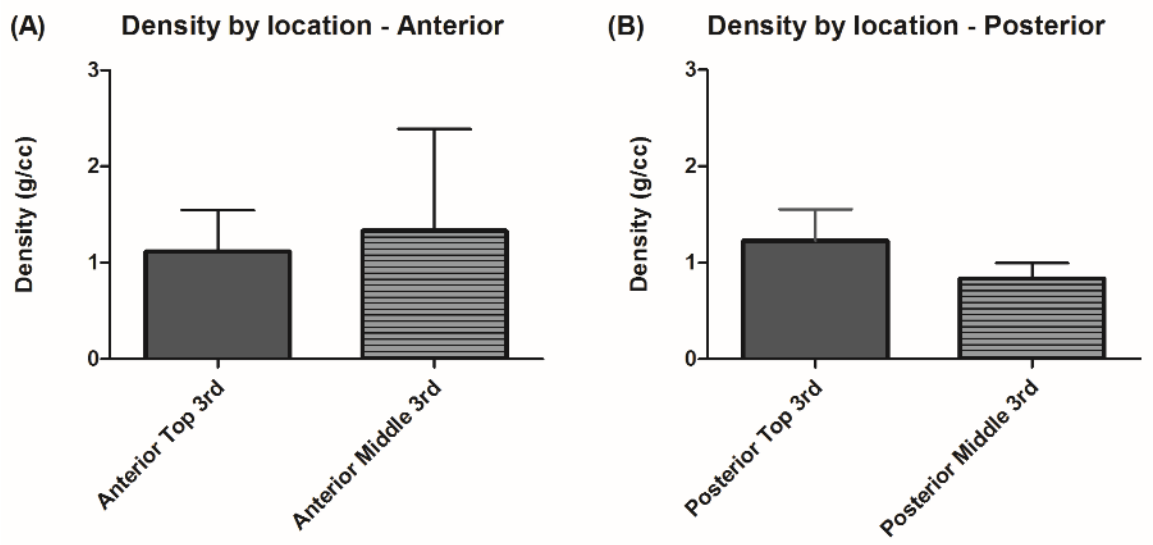

Figure 2.13 Density of sheep vagina wall tissue.

Density of the sheep vaginal wall tissue was measured for top third and bottom third regions. Additionally, samples were further divide as per anterior and posterior locations. No significant differences were observed across the locations.

2.5.5 Tortuosity

Tortuosity values of sheep vaginal wall tissues were obtained from multiple location of the sheep vaginal tract (Fig. 2.14). Additionally, samples were obtained either along the longitudinal axis of the vaginal canal (along fibers) or along the circumferential direction (cross fiber). Tortuosity data was found to be significantly different for the two groups (along fiber vs. across fiber).

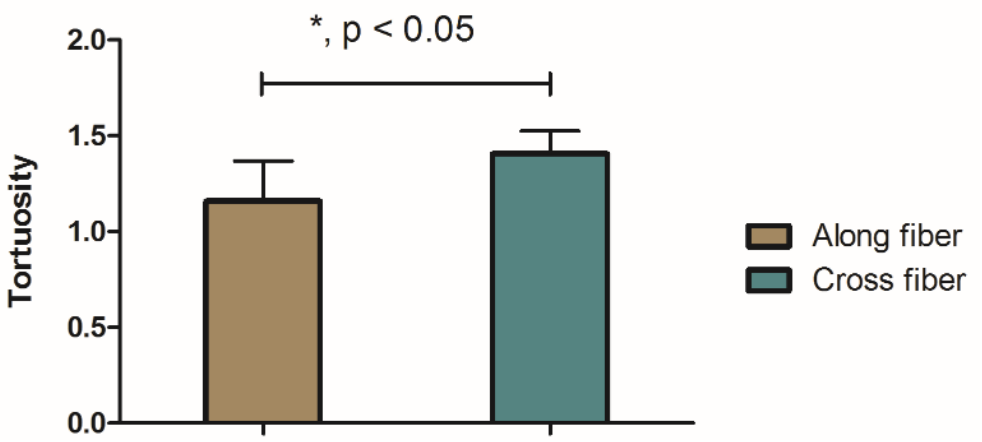

Figure 2.14 Histological tortuosity of sheep vaginal tissue.

Tortuosity data of sheep vaginal tissue were significantly different in the longitudinal direction and circumferential direction.

2.5.6 DT-MRI

Sheep vaginal wall tissue is mostly composed of smooth muscle cells, squamous epithelial cells and extracellular matrices. The 3D reconstruction of fiber pathways showed that the smooth muscle orientation has location dependence along the tract (Fig. 2.15). The FA values for the top third and middle third regions were found to be 0.1100 ± 0.0308 and 0.1060 ± 0.0346, respectively. FA values were significantly difference across the groups but were found to be almost similar in magnitude. Differences in anisotropy in these regions shown here can be correlated with the tissue structure-function relationship.

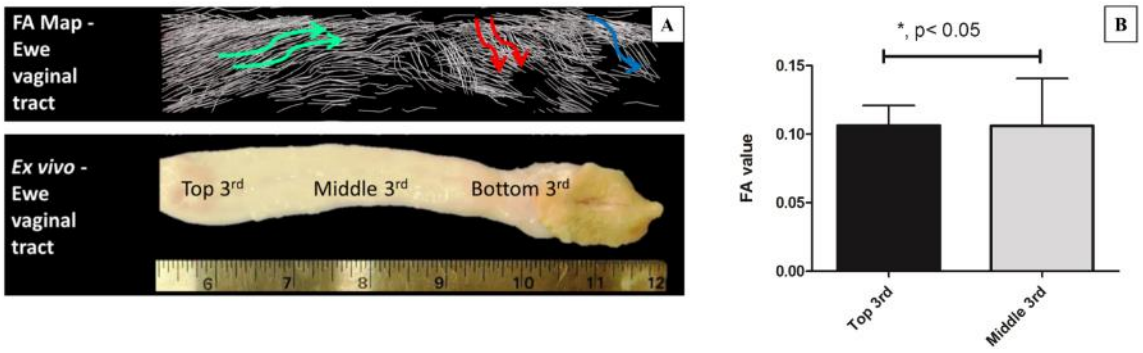

Figure 2.15 Fiber tractography of explanted sheep vaginal wall tissue.

(A) Fractional anisotropy map of underlying smooth muscle fiber architecture is shown here fiber architecture is shown here – the "green" arrows represent fibers along the long axis of the vaginal tract, "red" arrows are having cross-sectional orientation and "blue" arrows denote fiber with mixed orientations. (B) FA values of two regions – top third & middle (n = 10 per group) are shown here.

2.6 Discussion

Gross anatomical descriptions alone are not a complete answer for comparing animal models for POP research. A quantified anatomical or morphological parameter is a better approach for develop and compare the anatomical structures of humans with animal models of prolapse. We have formulated a set of baseline measurements of nulliparous adult sheep pelvic floor. Regardless of pregnancy, any increase in live weight in female sheep leads to increase in intra-abdominal pressure (McLean and Claxton 1960); which in turn is the primary risk factor for onset of prolapse. Further, vaginal prolapse is commonly observed in older and multi-gravid female sheep (McLean 1956, Zacharin 1969). Hence, multivariate analyzes were used to elucidate the relationship of anatomical parameters with live weight and age. For the length measures, we found that only VH long was positive correlated with age, while AFD and V.dia were negatively correlated with age (Fig 2.10). No association between anatomical measures (lengths)

and live weight were observed. Conversely, vagina weight was positively correlated with both age and live weight (Fig. 2.12). The obtained anatomical measurements can be used to compare the anatomical features of a normal female sheep with a prolapsed one (or possibly for comparison to humans).

2.6.1 Anatomical Measurements

The following anatomical measurements were recorded as a part of this study- the vaginal hiatus (VH long and VH short) (VH long is same as GH in humans), total vaginal length (TVL), anogenital distance (AGD) (same as PB measurements in humans), anus-fourchette distance (AFD) and vaginal diameter (V. dia). PB, GH and TVL are also a part of the routine Pelvic Organ Prolapse – Quantification (POP-Q) system in humans (Bump, Mattiasson et al. 1996, Mouritsen 2005, Persu, Chapple et al. 2011). However, there is no standard POP-Q system for sheep. Bosse et al. devised a grading system for sheep prolapse but it was not as detailed as POP-Q system (Bosse, Grimand et al. 1989, Noakes, Parkinson et al. 2001). Additionally, AFD and V.dia measurements have not been applied to human POP studies.

Trauma and damage to the pelvic floor tissues from childbirth leads to increase in genital hiatus (Vakili, Zheng et al. 2005, Shek and Dietz 2009, Ghafar, Chesson et al. 2013, Siafarikas, Stær-Jensen et al. 2015), which leads ultimately leads to POP (Mouritsen 2005, Kim, Jeon et al. 2007, Ghafar, Chesson et al. 2013). Similarly, adaptation of pelvic organs/tissues during pregnancy (Bassett and Phillips 1955) combined with anatomical variations in the animals, could possibly explain the incidence of vaginal prolapse only in a particular fraction of sheep in a herd (Bassett and Phillips 1955, Jackson, Hilson et al. 2014).

For prolapsed patients, mid-sagittal diameter, vaginal perimeter and cross-sectional area of the vaginal tract were found to be higher in prolapse patients than nulliparous ones (Hsu, Chen et al. 2005). They observed that vaginal perimeter for prolapse patients (~ 60 years) were about 111.0 ± 2.4 mm compared with controls (~ 60 years) which were 99.6 ± 2.2 mm. We did not calculate vaginal perimeter in this study, but based on Basset and Philips method of "vaginal perimeter" measurement (Bassett and Phillips 1955), the published values for sheep were same as our vaginal hiatus measurements (VH long). Bassett and Phillips measured either the length of the vaginal orifice (similar to VH long), then doubled these values, and termed them as "vaginal perimeter". Although there is no prolapsed sheep data for comparison, vaginal perimeters of nulliparous sheep were about 102.0 ± 5.4 cm in dry oestrous, about 90.0 ± 5.6 mm in the first month of pregnancy, and about 203.0 ± 5.5 mm 6 hours post-partum (Bassett and Phillips 1955). For sake of comparison to our nulliparous sheep data, VH long from their study will be about 50 mm vs. our data, which will be in the range of 25-35 mm (Fig. 2.9). However, it is difficult to compare these values to our data directly as they do not mention the age of their animals (Bassett and Phillips 1955). If we consider the correlation of VH long with age (Fig. 2.10), then with increasing age of the female sheep we can possibly observe an increased vaginal hiatus.

Total vaginal length (TVL), which is a primary anatomical biomarker for human prolapse (Barnhart, Izquierdo et al. 2006, Lewicky-Gaupp, Yousuf et al. 2010), was not statistically different across the sheep breeds (range 120 mm – 130 mm) (Fig. 2.9) and does not show any association with age. Similar to "vaginal perimeter" trend, Basset and Phillips found that the length of the vaginal tract is lowest measurement during first

month of pregnancy (166 ± 8.5 mm) and highest measurements during full term pregnancy stage (238 ± 11.4 mm) (Bassett and Phillips 1955). Additionally, post-partum (1-3 months) TVL of sheep were lesser in magnitude (170 – 180 mm) than full term measurements, and closer to oestrous stage measurements (169 -179 mm) (Bassett and Phillips 1955). As mentioned earlier, there is not associated weight information of these animals to compare the relationship of any anatomical parameter with age. Conversely, Khatun et al. did not find any difference in vaginal length from 2 weeks to 3 months of gestation (Khatun, Wani et al. 2010). Thus, TVL changes may be more pronounced with pregnancy, in a diseased state or perhaps with presence of multiple fetuses, and may be more conserved with age. However, we can hypothesize that TVL of prolapse sheep will be different in comparison to control ones.

Vaginal diameter (V.dia) in sheep were measured similar to mid-sagittal diameter in prolapse patients (Hsu, Chen et al. 2005). Most of the patients (prolapse and control) in their study were about 60 years of age and the magnitude of V.dia was about 130mm for both groups. Comparatively, the V. dia for nulliparous sheep used in our study were almost ten times less as compared to humans (15- 25 mm) (Fig. 2.8). We did not find any data on sheep vaginal diameter with respect to nulliparous, pregnant or prolapsed sheep. The V. dia measurements were negatively correlated with age and can be predicted using the following equation:

$$\text{V. dia} = 51.379 - (3.01 \times \text{age}) \qquad \text{(Eq. 2.2)}$$

In the case of adults, longer AGD (or perineal body length (PBL)) was a characteristic of irregular menstrual cycles in their mother before pregnancy (Mira-

Escolano, Mendiola et al. 2014). Moreover, women with longer anus-fourchette (AFD) distance and anus-clitoris distance (AGD) are more likely to have more than six ovarian follicles (Mendiola, Roca et al. 2012). In animal models, AGD is inversely linked to onset of vagina estrus in rats (Zehr, Gans et al. 2001), and exposure to environmental chemicals (Honma, Suzuki et al. 2002). Currently, AGD measurements have not been applied to POP research. This is the first report of AGD and AFD measurements in nulliparous sheep. Interestingly, AGD was significantly different across the breeds, whereas AFD was selected in the multivariate analyses. Multivariate analyzes found a negative correlation of AFD with age and prediction equation is:

$$\text{AFD} = 55.926 - (2.909 \text{ X age}) \quad \text{(Eq. 2.3)}$$

2.6.2 Organ weight

From the genital organ weight data, only bladder and vagina weights were significantly different across the four sheep breeds. Further, multivariate analyzes revealed that vagina weight was positively correlated with live weight and negatively with age (Fig. 2.12). From our obtained results, sheep vaginal weight can be predicted by using the following equation:

$$\text{Vagina weight} = 20.698 + (0.00033 \text{ X Live weight}) - (2.891 \text{ X Age}) \quad \text{(Eq. 2.4)}$$

Not only does estrogen influence the microstructure of the vagina, its influence is also observed in the anatomy and physiology of other genital organs (ovary, oviduct, uterus, cervix, and mammary glands) as well (Adams 1977, Adams 1986, Burton and Wells 2002). Alteration in the weight of vagina and uterus were witnessed with *in utero*

exposure of bisphenolA (BPA) (an environmental estrogen) in mice models (Schönfelder, Flick et al. 2002), and with exposure of Chlorpyrifos-methyl (CPM) (an organophosphate insecticide) in rat models as well (Kang, Jeong et al. 2004). Similar observations were reported in a rabbit animal model, where the increase in concentration of neem leaf meal (0 to 15%) led to the progressive reduction of uterus, cervix and vagina weights (Princewil 2008). At the peak of the estrous cycle in sheep, blood flow to the reproductive tract is maximized, and hence, weights of uterus and vagina were found to be higher than normal (Moor and Bruce 1976). Under the influence of certain phytoestrogens, female sheep exhibit a marked pathophysiological abnormality and variation is the weight of genital organs in sheep (Adams 1977, Nwannenna, Lundh et al. 1995, Cantero, Sancha et al. 1996, Burton and Wells 2002). Furthermore, excess of phytoestrogen in female sheep diet may lead to development of vaginal or uterine prolapse as well (Sobiraj 1990, Burton and Wells 2002). Thus, we hypothesize that genital organ weights will be different in sheep with vaginal prolapse than normal ones. The data presented here will be a baseline for comparing organ weights of prolapse sheep and a possible anatomical biomarker for comparing sheep models in prolapse research.

2.6.3 Density

This is the first report of density measurement of sheep vaginal tissue. The average density was found to be between 1.2-1.5 g/cc (Fig. 2.13). However, we did not observe any differences between the locations. This clearly indicates that the tissue density of the vaginal tract does not change with location. However, due to the swelling and edema presentations in prolapsed sheep tissues, we may find a difference in density of prolapse vaginal tissue as compared to the native one. Density measurements of the

vaginal tissues and other soft tissues can be taken as inputs for computational modelling applications.

2.6.4 Tortuosity

The tortuosity measurement of the superficial layer (epithelium) of the sheep vaginal wall tissue was significantly different along the longitudinal axis of vagina vs. along the circumferential direction (Fig. 2.14). Accommodation of pregnancy, support to pelvic organs, and stretching during delivery are some of the primary functions of the vaginal wall. Due to these dynamic functions of the vagina, the stretching of the vaginal wall in circumferential directions will be different than the longitudinal directions. In order to accommodate these vital functions, microstructural arrangement of the vaginal tissue in general will differ in either axes of the tract. Hence, these differences in tissue tortuosity observations are primarily a function of the innate microstructural arrangement.

Similar to humans (Patton, Thwin et al. 2000), sheep vaginal epithelium layer undergoes cyclic changes (from thick stratified squamous type to low-cuboidal type) (Nalbandov and Cook 1976) as well as variation in thickness along the entire length of the tract. This alteration is the epithelial layer of sheep vaginal tract is primarily under the influence of estrogen. Change in thickness of the epithelial layer will be witnessed by a variation in the overall tortuosity measurement of the vaginal tissue. Although, our study was not controlled for cyclic changes, the histological examination of the tissues revealed a consistency in the epithelial morphology and overall cellular microstructure. As prolapsed sheep have shown an alteration in estrogen levels, we can further hypothesize that tortuosity characteristic of prolapse vaginal tissues will be different from non-prolapsed ones. Moreover, prolapsed human vaginal tissues have shown an altercation in

epithelial and sub-epithelial layer thickness in comparison to control patients (Inal, Kaplan et al. 2010, Kaplan, Usta et al. 2011). Hence, we can enlist tortuosity measurements as a quantitative anatomical parameter for comparing prolapsed vs. non-prolapse tissues for both humans and sheep.

2.6.5 DT-MRI

The strength of this study is the three dimensional visualization and quantification of the smooth muscle fibers in the sheep reproductive tract. DT-MRI has been previously utilized to study the fiber tractography of the female pelvic floor muscles and organs (Zijta, Froeling et al. 2011, Rousset, Delmas et al. 2012, Zijta, Froeling et al. 2013); however, this is the first report of DT-MRI application in sheep vaginal wall tissue. Fractional anisotropy (FA) and fiber tractography from DT-MRI provides quantitative description of the microstructural properties and a vivid 3D visualization of the anatomical features, which are not easily observed in conventional MRI scans. Of the various studies reported on DT-MRI of pelvic floor tissues, FA value of pelvic muscles in general range from 0.26 to 0.28 (Zijta, Froeling et al. 2011). No FA values were found for sheep or human vaginal tract, and hence, we could not directly compare our results with other published literature. The magnitude of fractional anisotropy (FA) was significantly different as per locations, but the magnitudes of both locations (top 3rd vs. mid 3rd) were almost similar (0.1063 vs. 0.0147 vs. 0.1060 ± 0.03459, respectively).

To the best of our knowledge, this is the first report of a successful report of sheep vaginal wall diffusion tensor imaging. We found that the local smooth muscle fiber orientation changes from the top third to the bottom third region. The difference in smooth muscle fiber orientation in the sheep vaginal tract is clearly evident from the local

fiber distribution profile obtained from the DT-MRI scans (Fig 2.15). Similar to humans, histological analysis of sheep vaginal wall revealed that the smooth muscle orientation is both whirled along the circumferential direction, and parallel to the longitudinal axis of the vaginal canal. Similar to POP patients, we can expect an altered fiber orientation and anisotropic characteristics in the DT-MRI scans of a prolapsed sheep pelvic floor. This DT –MRI protocol will be translated into clinical studies to determine the localized defect in the female pelvic floor tissues of POP patients (Zijta, Froeling et al. 2011, Rousset, Delmas et al. 2012, Zijta, Froeling et al. 2013). The ongoing effort is to correlate the smooth muscle fiber orientation and FA with local tissue mechanical anisotropy. The ultimate goal is to use this protocol for *in vivo* sheep DT-MRI study, and build a structure-based 3D computational model of the female sheep reproductive tract.

2.7 Similarities between human and sheep prolapse

As expected, the sheep and human pelvic floor anatomy were found to be similar and the larger size of the model is advantageous in urognecology research. Detailed comparison of human and sheep pelvic floor tissue anatomy, and prolapse characteristics are provided in Table 2.8. It is interesting to note the close resemblance of pathophysiology of vaginal prolapse in sheep and humans. With the progress of pregnancy in sheep, the pelvic floor tissues become weaker, and there is an increase in fibroblasts and smooth muscle cells content (Aughey, Calder et al. 1983). Conversely, there is also a reduction in collagen and fibrocytes (Aughey, Calder et al. 1983), which may lead to reduction in the biomechanical properties of the supporting tissue structures and ultimately lead to vaginal prolapse. This effect is further compounded by the presence of over-sized and multiple fetuses (McLean 1956, Grimard, Bosse et al. 1990,

Sobiraj 1994, Noakes, Parkinson et al. 2001, Hosie 2008, Jackson, Hilson et al. 2014). Ennen et al. found that in prolapsed sheep vaginal tissues exhibited a reduction in α-2 chain of collagen I, estrogen reception – α (ER- α), and tissue inhibitor of matrix metalloprotease -1 (TIMP1); and an increased expression of matrix metalloprotease -1 (MMP1), as compared to late term pregnant sheep (Ennen, Kloss et al. 2011). Likewise, alternation in expressions of ER- α receptor, TIMP1 and collagen I, were observed in pelvic floor tissues of POP patients (Albertazzi and Sharma 2005, Chen, Chung et al. 2008, Strinic, Vulic et al. 2009, Liang, Huang et al. 2010, Strinic, Vulic et al. 2010, Knuuti, Kauppila et al. 2011, Vulic, Strinic et al. 2011, Ferrari, Rossi et al. 2012). Comparable to humans, the exact role of estrogen in the development of vaginal prolapse is not completely understood (Noakes, Parkinson et al. 2001, Kerkhof, Hendriks et al. 2009, Ennen, Kloss et al. 2011). The quantity of collagen, elastin and smooth muscle cells varies with each pelvic tissue in humans (Moalli, Shand et al. 2005). Except for arcus tendineus fasciae pelvis, most of these human pelvic floor tissues have abundance of smooth muscle cells (Goh 2003, Moalli, Talarico et al. 2004, Kerkhof, Hendriks et al. 2009). Previous studies have shown that prolapse patients (both premenopausal and postmenopausal) exhibits a reduction in smooth muscle content and irregular distribution pattern in their vaginal wall (Boreham, Wai et al. 2002, Badiou, Granier et al. 2008, Takacs, Gualtieri et al. 2008, Kerkhof, Ruiz-Zapata et al. 2014). Likewise, a reduction in smooth muscle content is also observed in pelvic ligaments and other supporting structures of patients with pelvic organ prolapse (Han, Wang et al. 2014). Interestingly, in pregnant rats, vaginal wall smooth muscle cells switch their phenotypic expression from a contractile to a synthetic one, but this behavior is again reverted back from synthetic to

contractile during postpartum period (Daucher, Clark et al. 2007). Northington et al. found that in *in vitro* cell culture studies of cells (anterior vaginal muscular) obtained from POP patients, the vaginal smooth muscle cells showed a reduced contractility as well as lowered α (1A) adrenergic receptor expression (Northington, Basha et al. 2011). Similar observations were also reported by Poncet et al. and Meyers et al. (Poncet, Meyer et al. 2005, Meyer, Achtari et al. 2008). However, there is not clear evidence on the quantity or contractile properties of vaginal smooth muscle cells in prolapsed sheep. Data obtained from these studies will be used to establish a high-fidelity computational model with the information of the vaginal wall smooth muscle orientation and local tissue biomechanics.

Table 2.8 Similarities between sheep and human vaginal tissue.

Characteristic	**Humans**	**Sheep (*Ovis aries*)**	**Source**
Reproductive Cycle			
Gestation length (days)	270	140-160	(Frandson 1974, Hafez 2000)
Estrous cycle (days)	21-28	16-17	(Vincent, Bourne et al. 2009)
Puberty (age)	13-15 years	4-14 months	(Vincent, Bourne et al. 2009)
Hormonal			
Dominant endocrine system during parturition	Maternal	Fetal	(Liggins 1994, Hafez 2000)
Pelvic ligament relaxation	Prominent	Prominent	(Bassett and Phillips 1954, Richardson, Lyon et al. 1976, Beecham 1980, Hafez 2000)
Anatomical Comparison			
Uterine tube			
Oviduct length	8-15cm	15-19 cm	(Frandson 1974, Dixson 2009, Vincent, Bourne et al. 2009, Weed, Borazjani et al. 2012)

Anatomical measurements of an adult non-pregnant sheep are provided here.

Table 2.8 (Continued)

Uterus			
Type	Simplex	Bipartite	(Frandson 1974)
Length of horn	N/A	10-12 cm	(Sisson and Grossman 1941, Frandson 1974, Liggins 1994, Hafez 2000)
Length of body	6.25- 7 cm	1-2 cm	(Gray 1918, Bassett and Phillips 1954, Frandson 1974, Richardson, Lyon et al. 1976, Beecham 1980, Hafez 2000)
Uterine Cavity	Similar	Same as humans	(Fuchs and Fuchs 1984, Liggins 1994, Hafez 2000, Elovitz and Mrinalini 2004, Vincent, Bourne et al. 2009)
Cervix			
Cervix length	2.5- 6 cm	4-10 cm	(Frandson 1974, Zemlyn 1981)
Cervical Diameter	2.5 to 3 cm	1-1.5 cm	(Frandson 1974, Vincent, Bourne et al. 2009)
Vagina			
Total Vagina length	8-12 cm	8-13 cm	(Sisson and Grossman 1941, Mishell 1997, Elovitz and Mrinalini 2004, Vincent, Bourne et al. 2009)

Table 2.8 (Continued)

Vagina (cont'd)			
Vaginal epithelial layer • Thickness • cell layers	•~ 175-284 µm •~ 22-29	• 86-114µm • 8-13	(Frandson 1974, Patton, Thwin et al. 2000, Vincent, Bourne et al. 2009)
Fornix vagina	Yes	Yes	(Frandson 1974, Hafez 2000, Barnhart, Izquierdo et al. 2006)
Origin of vagina	Mesoderm	Mesoderm/Endoderm	(Frandson 1974, Ulfelder and Robboy 1976, Patnaik, Brazile et al. 2015)
Widest vaginal portion	Proximal vagina	Proximal/Cranial vagina	(Barnhart, Izquierdo et al. 2006)
Narrowest portion	Vaginal orifice	Vaginal orifice	(Barnhart, Izquierdo et al. 2006)
Vaginal Prolapse Characteristics			
Spontaneous	Yes	Yes	(Sisson and Grossman 1941, Mishell 1997, Abramowitch, Feola et al. 2009, Vincent, Bourne et al. 2009, Couri, Lenis et al. 2012)
Pelvic organ mobility	Increased	Increased	(Dietz, Eldridge et al. 2004, Brækken, Majida et al. 2009)

Table 2.8 (Continued)

Vaginal Prolapse Characteristics (cont'd)			
Mineral imbalance	No	Maybe	(Litherland, Knight et al. 2007)
Connective tissue disorder (or metabolism issue) • Collagen • Elastin Proteoglycan	Yes Yes Possibly	Yes Possibly N/A	(Frandson 1974, Jackson, Avery et al. 1996, Hafez 2000, Chen, Wen et al. 2005, Alperin and Moalli 2006, Barnhart, Izquierdo et al. 2006, Kerkhof, Hendriks et al. 2009, Kerkhof, Hendriks et al. 2009, Ennen, Kloss et al. 2011, Moon, Choi et al. 2011, Lammers, Lince et al. 2012)
Reduction in smooth muscle cells	Yes	Yes	(Hosie 2008, Kerkhof, Hendriks et al. 2009)
Multiple fetus as a risk factor	Yes	Yes	(Frandson 1974, Hendrix, Clark et al. 2002)
Birth related injury or trauma , eventually progressing to prolapse	Yes	Maybe	(Frandson 1974, Ewies, Al-Azzawi et al. 2003, Ashton-Miller and DeLancey 2009, Shek and Dietz 2009)

Table 2.8 (Continued)

Offspring weight	Similar	Closest to humans	(Frandson 1974, Carter 2007, Barry and Anthony 2008)
Anatomical disproportion as risk factor	Yes	Yes	(Noakes, Parkinson et al. 2001)

With the limited range of age (9-11 months) and live weight (148-180 lbs.) of our samples, we were able to able to establish some juxtaposition between the anatomical parameters and the age or weight of the animal. Future studies with either prolapsed sheep and/or varied age groups will provide us more robust anatomical data. Like POP-Q system, these anatomical parameters could be possibly used as a standard measurement system for staging or grading of vaginal prolapse in sheep. Also like the POP-Q and other prolapse grading systems, these measurements in a sheep flocks will be time intensive and its clinical application will depend on the economic feasibility. The obtained values and measurement methods will help formulate a baseline for future studies, which will compare the anatomical features of prolapsed female sheep with the non-prolapsed ones.

CHAPTER III

LOCATION DEPENDENT VARIATIONS IN MECHANICAL AND STRUCTURAL PROPERTIES OF SHEEP VAGINAL WALL TISSUES

3.1 Introduction

3.1.1 Pelvic organ prolapse: from a biomechanical perspective

The stretching of the vaginal tissue indicates an intrinsic biomechanical issue, which could be a result of a pathological condition, injury, altered connective tissue, or biochemical alteration in the patients' body (Hendrix, Clark et al. 2002, Nygaard, Bradley et al. 2004, Fritel, Varnoux et al. 2009, Miedel, Tegerstedt et al. 2009, Tinelli, Malvasi et al. 2010, Skorupski, Jankiewicz et al. 2013, Durnea, Khashan et al. 2014). From a mechanical perspective, Richardson et al. (Richardson, Lyon et al. 1976) believed that vaginal prolapse occurred due to the tears in the endopelvic connective tissue. Similarly, previous theories suggested a possible thinning or stretching of the anterior vaginal wall to be the root cause of pelvic organ prolapse (White 1912, Hegde 2015). However, with the advent of modern imaging technology and advanced research, experts now believe that pelvic organ prolapse is a result of the failure of the pelvic floor support and specifically, Delancey's three levels of support (DeLancey 1994). The soft tissue structures comprising the female pelvis, which supports the pelvic organs, have complex responsibilities including neurological support and mechanical support of the region (DeLancey 2005). It is highly likely that patients with mild prolapse patients (cranial to

hymen) will not exhibit any typical symptoms or issues (Mouritsen 2005). Although, patients with severe anterior vaginal prolapse may show dragging of the vagina and other urinary issues; on the other hand, patients with posterior vaginal prolapse may exhibit defecatory issues (Chaliha and Khullar 2006). About a century ago, Stevens pointed out that the anterior compartment defect was the most common presentation of pelvic organ prolapse (Stevens 1928); and till date, it is still highly prevalent followed by posterior and apical defects (Ellerkmann, Cundiff et al. 2001).

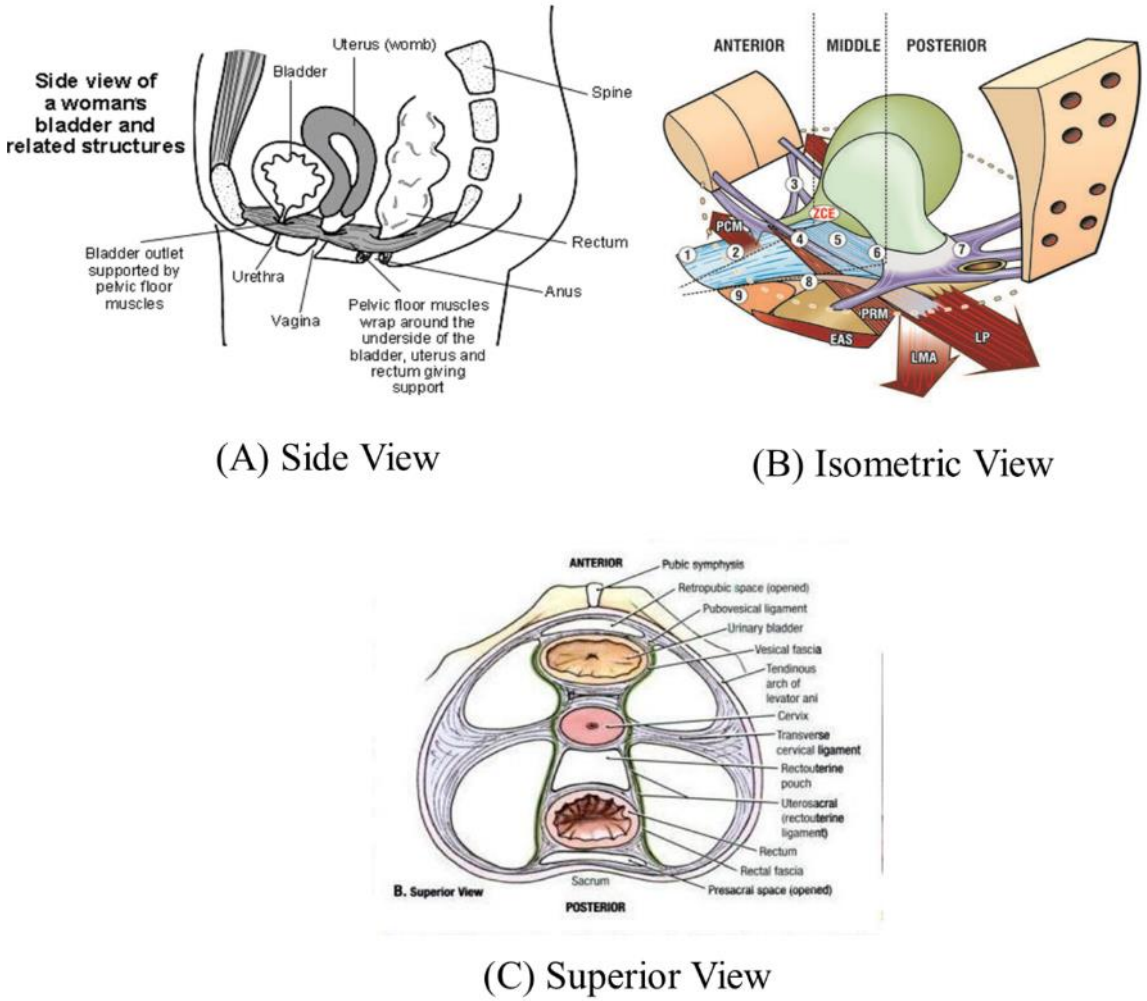

Figure 3.1 Tissues and supporting structures of the pelvic floor[3].

(A) Side view, (B) Isometric View and (C) Superior view. Details of the isometric view (B) PCM = pubococcygeus muscle force; LP = levator plate muscleforce; LMA = longitudinal muscle of the anus force; ZCE = Zone of Critical Elasticity. Anterior zone (1 = external urethral ligament (EUL) ;2 = pubourethral ligament (PUL); 3 = suburethral vagina (hammock)), Middle zone (4 = arcus tendineus fascia pelvis (ATFP), 5 = pubocervical fascia (PCF) ,6 = anterior cervical ring/cardinal ligament (CL) ZCE = excess tightness, usually scar tissue below bladder neck ('tethered vagina'), Posterior

[3] Source : Oxford Gynecology http://www.oxfordgynaecology.com/Investigations/Pelvic-Floor-Exercises.aspx; Pelvic Perineology - http://www.pelviperineology.org/ano_rectal_function/tables/study11_ligamentous_repair_using_tissue_fixation_fig.html and Kegel8 Pelvic Health - http://www.kegel8.co.uk/glossary/term/ligament/

zone (7 = uterosacral ligament (USL); 8 = rectovaginal fascia (RVF); 9 = perineal body (PB)).

Patients with posterior vaginal wall prolapse presentations are associated with longer posterior vaginal wall and the pelvic structures are more caudal relative to non-prolapse individuals (Lewicky-Gaupp, Yousuf et al. 2010). Furthermore, in surgical terms, the risk of mesh exposure is higher for the anterior wall prolapse repairs than posterior wall prolapse repair (Frankman, Alperin et al. 2013). Even though the prolapse frequency may differ as per each respective location, the severity of the prolapse symptoms are not associated with the location of the defect (Ellerkmann, Cundiff et al. 2001). This anatomy or location dependent variation exhibited by prolapse patients shows the level of complexity exhibited in human pelvic floor tissue. The biomechanical strength and structural integrity of these supportive tissues are dependent on the mixture of hormonal effects, and a dynamic interplay of several genes; POP is hypothesized to be a resultant product of one of these defects. However, the exact cause or etiology still remains elusive.

3.1.2 Pregnancy and POP

Studies of pregnant and non-pregnant women have shown that the anatomy and structural support of the pelvic organs changes during pregnancy. Pregnancy in general and vaginal delivery in particular, leads to a significant amount of pelvic floor damage and dwindling of its biomechanical properties (Manabe and Yoshida 1986, Sze, Sherard III et al. 2002, O'Boyle, O'Boyle et al. 2003, Dietz, Eldridge et al. 2004, Lowder, Debes et al. 2007, Rahn, Ruff et al. 2008, Feola, Moalli et al. 2011, Ulrich, Edwards et al. 2014). The leading risk factor for POP is vaginal childbirth, with each additional delivery (up to

5 births) increasing the risk of prolapse by 10-20% (Hendrix, Clark et al. 2002, Jelovsek, Maher et al. 2007). Women having given birth to 2 or more children are 8.4 times more likely than their nulliparous counterparts to be diagnosed with POP and require hospitalization (Hendrix, Clark et al. 2002, Mant, Painter et al. 2005).

Dietz et al. found that bladder and urethral mobility begins to increase as early as the 6th week of gestation and is significantly increased by late gestation (Dietz, Eldridge et al. 2004). Explicit tissue damage during vaginal delivery may, in part, be attributable, but there is evidence that shows that Cesarean-section (c-section) delivery is only partially protective in the development of FPFDs (Sze, Sherard III et al. 2002, O'Boyle, O'Boyle et al. 2003, Rortveit, Daltveit et al. 2003, Gustilo-Ashby, Lee et al. 2010). In contrast, some investigators have shown that, compared with c-section, spontaneous vaginal delivery significantly increases the odds for both SUI and POP (Handa, Blomquist et al. 2011). In the same way, Yeniel et al. reported a strong association of POP with multiparous women with vaginal deliveries, including a higher risk with multiple births and no association with C-section deliveries (Yeniel, Ergenoglu et al. 2013). An animal study utilizing the LOXL1-KO mouse model of POP showed that c-section only delayed the development of POP (Gustilo-Ashby, Lee et al. 2010). Similarly, others have suggested that the protective effects of c-section observed in some studies may disappear with increasing age (Phillips and Monga 2005). Although it may be the case that c-section reduces the risk or delays the onset of POP, it is clear that it does not eliminate the risk all together. Pregnancy, itself, has been documented to cause significant changes in pelvic support in both humans and animals. It can be inferred from

these studies that tissue remodeling that accompanies pregnancy may indeed play a role in the pathophysiology of POP.

Although this risk is likely in part attributable to the biomechanical trauma of childbirth that may lead to neural, muscular, and connective tissue injury, scientific evidence suggests that hormones and molecular regulation of pelvic tissue modeling play a significant role in the development of POP (Lang, Zhu et al. 2003, Albertazzi and Sharma 2005, Jelovsek, Barber et al. 2005, Chung and Bai 2006, Daucher, Clark et al. 2007, Jelovsek, Maher et al. 2007, Chen, Chung et al. 2008, Chen, Wan et al. 2008, Kerkhof, Hendriks et al. 2009, Gustilo-Ashby, Lee et al. 2010). Studies investigating the effects of pregnancy on the histological and biomechanical changes in pelvic tissues are valuable in understanding the results of studies performed contrasting vaginal and caesarean deliveries. Daucher et al, found that collagen fiber area decreases during pregnancy and returns to normal levels in the late postpartum period (3 weeks after delivery) (Daucher, Clark et al. 2007). Interestingly, they also found that smooth muscle cells shift from a contractile to a synthetic phenotype during pregnancy and shift back to a contractile phenotype in the late postpartum period (Daucher, Clark et al. 2007). The reports of collagen density from this study are similar to previous publications by Manabe and Yoshida who studied partial-thickness vaginal tissue biopsies from women in three different groups: nonpregnant, pregnant, and immediately following vaginal delivery (Manabe and Yoshida 1986). They found that during pregnancy and immediately after delivery collagen fibers are less dense than in the non-pregnant state (Manabe and Yoshida 1986). Such results likely have functional outcomes as depicted by

changes in biomechanical properties of vaginal tissues during the perinatal periods (Lowder, Debes et al. 2007).

Lowder et al, found that the biomechanical behavior of the vagina and its supportive tissues are dynamic during the perinatal period helping confirm the studies by Daucher et al (Daucher, Clark et al. 2007, Lowder, Debes et al. 2007). Pregnancy resulted in decreased stiffness and decreased failure load in tissues, and the route of delivery (C-section or vaginal) was not associated with the observed changes in biomechanical properties. They also found that after delivery (C-section or vaginal) the tissues are more distensible than during the virgin or pregnant state. Most importantly, it was found that the biomechanical properties are recovered to the non-pregnant state by 4-weeks postpartum (Lowder, Debes et al. 2007). These studies collectively describe an adaptation in rats which allow for non-traumatic delivery of fetuses in a manner that accommodates favorable neonatal and maternal outcomes (Daucher, Clark et al. 2007, Lowder, Debes et al. 2007). This was a novel study, illustrating the adaptation of the biomechanics of the pelvic tissues that occur in a species that does not develop POP and that does not have difficult or traumatic births. Perhaps these adaptations have been partially lost in human evolution where an emphasis has been put on producing only a few quality offspring (i.e. it is more important for a quality offspring be produced at least one time, than for the mother being able to deliver numerous times in her life). In rats, alternatively, evolution has emphasized a reproduction modality that requires the mother to delivery in a manner that favors quantity as appose to quality of offspring. As cited by Lowder et al, it may be the case that in humans adaptations of the pelvic tissues to pregnancy may often be deficient, incomplete, or exceeded (Lowder, Debes et al. 2007).

This may account for both the high incidence of obstetric trauma and development of POP in parous women.

3.1.3 Previous biomechanical studies with prolapse and native vaginal tissues

3.1.3.1 In-vivo and In-vitro studies

Recently, several *in vivo* biomechanical devices have been developed for measuring pressure profiles and degree of elasticity of the patient's vaginal wall (Coutty, Lambaudie et al. 2007, Epstein, Graham et al. 2007, Egorov, van Raalte et al. 2010, Egorov, van Raalte et al. 2012, Hollenstein, Bajka et al. 2012, Lopez, Dissertations et al. 2013). However, basic science research in pelvic organ prolapse has been primarily focused on *ex vivo* biomechanical techniques such as uniaxial tensile mechanical testing, biaxial puncture testing, etc. Biomechanical techniques are widely used in human, as well as rat and mouse prolapse models, to gauge the strength of tissue explants as it relates to collagen and elastin content (Daucher, Clark et al. 2007, Lowder, Debes et al. 2007, Rahn, Acevedo et al. 2008, Abramowitch, Feola et al. 2009, Ho, Heydarkhan et al. 2009, Couri, Lenis et al. 2012, Rubod, Brieu et al. 2012). In comparison to bladder and rectum, human vaginal wall tissue exhibits a lower extensibility and a general non-linear behavior, including regional variations (Rubod, Brieu et al. 2012). Detailed list of previously published biomechanical studies of vaginal tissues are listed in Table 3.1.

Table 3.1 Previous biomechanical studies with human and sheep tissues

Tissue Source	**Test Type**	**Groups**	**Strain rate**	**Mechanical Property (Units)**	**Source**
Human vaginal wall	Tensile	• Post-operative failure • Recovered patients	N/A	Modulus (MPa) • 3.8-7.6 • N/A	(Gilchrist, Gupta et al. 2010)
Human – anterior vaginal wall	Tensile	• Post-operative anatomical failure • Post-operative anatomical success	0.05 mm/sec	Modulus (no units) • 0.0196-0.0321 • 0.0172-0.0369	(Khaja, Winlove et al. 2014)
Anterior and posterior middle third	Tensile	• Non- POP cadavers - Anterior • POP patients – Anterior • Non- POP cadavers - Posterior • POP patients – Posterior		Stiffness (E) (MPa) & Max stress (S) (MPa) • E (13.1 ± 0.8 vs. 9.5 ± 0.7) & S (5.3 ± 0.5 vs. 3.2 ± 0.9) • E (6.9 ± 1.1 vs. 10.5 ± 1.0) & S (2.6 ± 0.4 vs. 3.5 ± 0.4)	(Martins, Lopes Silva-Filho et al. 2013)
Post-menopausal multiparous sheep	Tensile	• Top third • Middle Third • Bottom third	10 mm/min	Modulus (MPa) • ~12 • ~15 • ~30	(Ulrich, Edwards et al. 2014)

Table 3.1 (Continued)

In vivo					
Human	Tactile imaging	• Normal Anterior • Normal Posterior • Stage III prolapse – anterior • Stage III prolapse – posterior	N/A	Modulus (kPa) • 7.4 ± 4.3 • 6.2 ±3.1 • 1.8 ± 0.7 • 1.8 ± 0.5	(Egorov, van Raalte et al. 2012)
Human	Cutometer MPA 580	• Normal • POP patients	N/A	• initial stiffness index 7.3 (POP) vs 10.9 kpa (Normal) • final stiffness index 5.9 (POP) vs 10.7 kpa (Normal)	(Epstein, Graham et al. 2007)

In addition to humans and sheep, vaginal tissues from primates, rat, mouse and rabbit have also been studies for their biomechanical characteristics (Couri, Lenis et al. 2012). The further development of animal models will help us understand the etiology behind female pelvic floor disorders (Abramowitch, Feola et al. 2009, Couri, Lenis et al. 2012). Also, sheep have been used for studying performance and biomechanical behavior of surgical grafts used in pelvic reconstructive surgeries (De Tayrac, Alves et al. 2007,

Rezapour, Novara et al. 2007, Krause and Goh 2009, Manodoro, Endo et al. 2013, Alcalay, Livneh et al. 2014, Feola, Endo et al. 2014). However, thorough biomechanical and microstructural characterization is still warranted.

3.1.4 Knowledge gap and our objective

We hypothesize that region variation behavior in humans will also be exhibited by the sheep vaginal tissues. In this study, sheep vaginal wall tissues were studied with the aim of validating this tissue as a robust biomechanical animal model for POP. Uniaxial and biaxial testing can be used to understand many key tissue parameters, and to explore complex anisotropic mechanical responses present in many biological tissues, including the vagina. As an example, biaxial testing can evaluate tissue behavior of vaginal wall under physiological loading conditions (Sacks 2000, Sacks and Sun 2003, Nagatomi, Gloeckner et al. 2004). Uniaxial mechanical testing will be carried out to assess failure properties and viscoelastic properties of tissues. These biomechanical characterizations will help us provide a description of the differences or similarities amongst the three different regions of the sheep vaginal wall. Vaginal wall tissues will be grouped by either anterior or posterior groups and further by either top, middle or bottom third regions, to elucidate any important regional variations. Biaxial and uniaxial tension specimens will be further subdivided into longitudinal or circumferential based on their fiber distribution.

3.2 Materials and Methods

3.2.1 Sample Collections

For the present study, vaginal wall samples from ten female sheep (Avg. wt.: 131.67 ± 14.36 lbs) were obtained from a commercial abattoir. Excess fat or connective

tissues were removed from the samples. Samples were first categorized into anterior or posterior groups and further divided as per their locations as top third and middle third, based on a previous study (thirds are based on one-third of the total vaginal length) (Baggish and Karram 2006, Ulrich, Edwards et al. 2014) (Fig. 3.2). Hence, the four groups were formulated: anterior top third, anterior middle third posterior top third, and posterior middle third. Specimens were further processed for biomechanical (n= 8 for each group) and histological characterizations (n = 2 for each group).

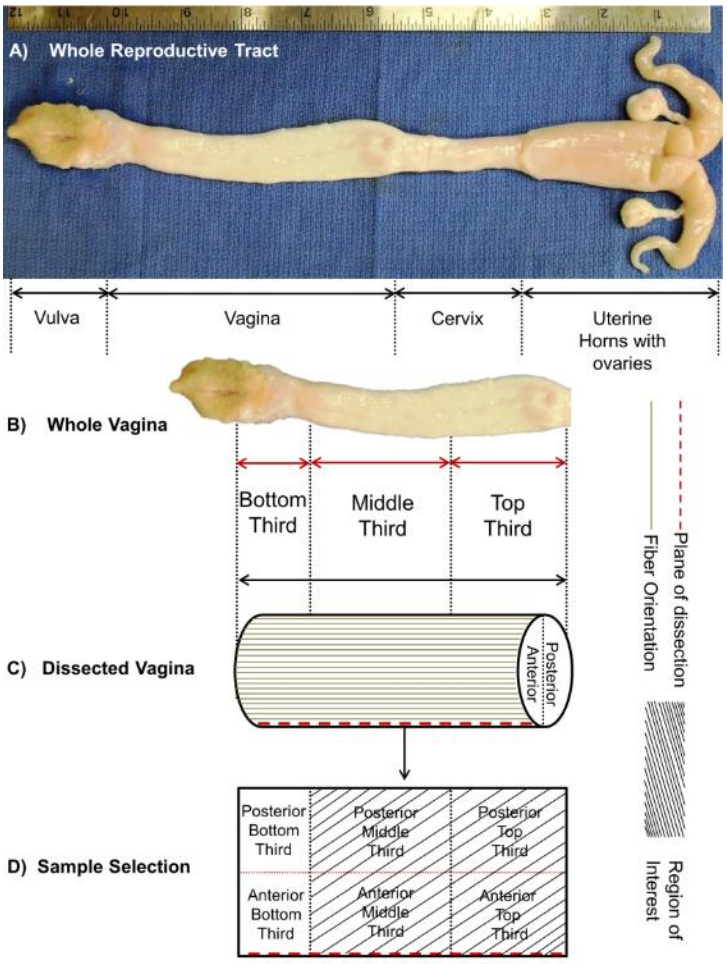

Figure 3.2 Sample processing for location dependent study.

(A) Intact sheep reproductive tract is shown here with intact vulva, vagina, cervix, uterus and ovaries. (B) Vagina was trimmed out of the entire tract and further divided into anterior and posterior portions. (C) Similar to human vagina, these tissues were further divided into one thirds i.e. one third of total vaginal length. The obtained groups were posterior top third, posterior middle third, anterior top third and anterior middle third.

3.2.2 Biomechanical Testing: Biaxial

Biaxial mechanical testing was carried out as per previously established protocols (Wang et al. 2012, Chow et al. 2014). From each group, the excised sheep vaginal wall tissue was dissected into ~ 20 mm x 20 mm square samples, with one edge of the sample aligned along longitudinal direction and the other edge aligned along circumferential

direction. Four black markers were placed on the tissue specimens for tracking local deformation. Tissue specimens were securely connected to each actuator arms of the biaxial device using custom designed hooks, which were looped with 000 non-absorbent sutures (4 hooks per edge). Specimens were preloaded up to 2 grams, preconditioned ten times in physiological range (0.05N) and subjected to an equibiaxial tension of 60N/m (each half cycle = 15 seconds). Maximum stretch of the samples, in either direction, due to the applied load was recorded. All the tests were performed in a Phosphate Buffered Saline (PBS) at 37˚C.

3.2.3 Biomechanical Testing: Uniaxial Tensile Test

The uniaxial tensile testing for each group (n =6 for each group) was characterized by a micromechanical testing machine (Mach-1, Biosyntech, MN) in the longitudinal and circumferential directions as per previously published protocols (Borazjani, Weed et al. 2011, Patnaik, Weed et al. 2012, Weed, Patnaik et al. 2012). Dog bone shaped samples (~12 mm x ~4 mm) were dissected from each location (anterior top third, anterior middle third, posterior top third, or posterior middle third), and thickness was measured using digital calipers in triplicate to ensure accuracy (Rubod, Boukerrou et al. 2007, Ulrich, Edwards et al. 2014). Samples were loaded onto the Mach 1 testing machine by custom designed tensile grips. Samples were preloaded to 2 grams, preconditioned 10 times, and pulled to failure at a strain rate of 10% per second. The stress strain curves were then recorded. The stress was calculated by normalizing the force to the initial cross-sectional area, and the strain was calculated by dividing the displacement with the initial grip-to-grip distance. All the tests were performed in a Phosphate Buffered Saline (PBS) bath at 37˚C.

3.2.4 Microstructural Characterization – Histology & Scanning Electron Microscopy

Routine Movat's pentachrome staining of vaginal tissue samples were done to show full thickness microstructural details. Samples were fixed in 10% phosphate buffered saline for at least 48 hours and embedded in paraffin wax. These embedded tissues were further sectioned in to 5 μm sections and transferred onto a glass slide. Movat's pentachrome staining was used for revealing the microstructural arrangement of the sheep vaginal wall tissues. Samples from each group were processed in triplicates.

For SEM, specimens were fixed using a solution of half-strength Karnovsky's in PBS (2.5% glutaraldehyde, 2% paraformaldehyde, 0.1M PBS) for a minimum of 48 hours. Specimens were then rinsed with distilled water, fixed by osmium tetroxide, and subjected to a series of increasing concentration alcohol solution treatment and overnight chemical drying using Hexamethyldisilazane (HMDS) solution. Samples were loaded onto aluminum stubs using carbon paste and sputter coated with platinum (30μm). Samples were then viewed using JEOL JSM-6500F Field Emission Scanning Electron Microscope (SEM) with secondary electron detectors (SEI), and the voltage was set to 5 keV. Micrographs were obtained at multiple magnifications to understand the details of sheep vaginal tissue ultrastructure.

3.2.5 Statistical Analysis

The obtained data was imported and analyzed using SigmaPlot ver.10 (©Sysat Software 2006). ANOVA was used to check if the data was significantly different. Pairwise comparisons were carried out using Tukey's tests. Data was considered significant at $p < 0.05$.

3.3 Results

3.3.1 Biomechanical Testing: Biaxial

Tension-Stretch curves were plotted for all groups to observe the anisotropic characteristic of the sheep vaginal tissue. Anisotropy index, areal train, longitudinal extensibility, circumferential extensibility and longitudinal modulus values were calculated from the obtained curves (Table 3.1). Data was not significant across the groups.

Table 3.2 Measured biaxial parameters for each group.

Parameters	Anterior Top 3rd	Anterior Mid 3rd	Posterior Top 3rd	Posterior Mid 3rd
Anisotropy Index	0.085 ± 0.065	0.202 ± 0.102	0.136 ± 0.105	0.136 ± 0.060
Areal Strain (mm/mm)	0.183 ± 0.009	0.261 ± 0.091	0.231 ± 0.103	0.208 ± 0.104
Longitudinal extensibility (mm/mm)	1.135 ± 0.034	1.245 ± 0.107	1.190 ± 0.112	1.123 ± 0.138
Circumferential extensibility (mm/mm)	1.043 ± 0.037	1.014 ± 0.022	1.035 ± 0.030	1.079 ± 0.055
Longitudinal modulus (kPa)	0.538 ± 0.193	0.439 ± 0.158	0.515 ± 0.238	0.929 ± 0.697

Biaxial data showed that circumferential direction was found to be stiffer than longitudinal direction for all groups (Fig 3.3). Anterior middle third group had the highest anisotropy index (0.202 ± 0.102) than other groups (almost twice), while anterior top third had the least difference between the longitudinal and circumferential curves (Table 3.1). Circumferential direction of all groups, showed similar trend and their extensibility values were also similar. On the other hand, extensibility in the longitudinal direction was

highest for anterior mid third (1.245 ± 0.107 mm/mm), followed by posterior top third (1.190 ± 0.112 mm/mm), then posterior mid 3rd (1.123 ± 0.138 mm/mm) and lastly, anterior top third (1.135 ± 0.034 mm/mm). Conversely, longitudinal modulus was highest for posterior mid-third (0.929 ± 0.697 kPa), followed by anterior top third (0.538 ± 0.193 kPa), and posterior top third (0.515 ± 0.238 kPa), and lastly, anterior mid third (0.439 ± 0.158 kPa). No location based trend was observed, and anisotropy was not observed in posterior middle third region.

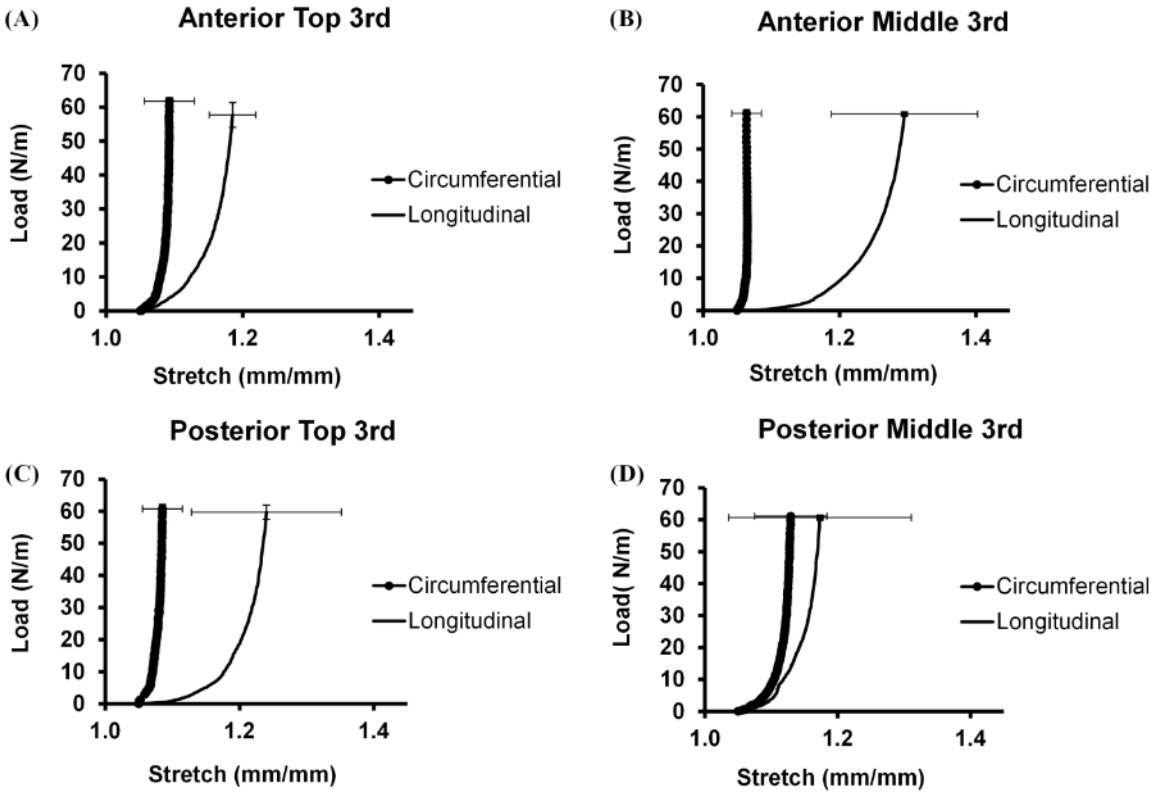

Figure 3.3 Biaxial mechanical data of sheep vaginal wall tissue.

(A) Anterior Top 3rd, (B) Anterior Middle 3rd, (C) Posterior Top 3rd, and (D) Posterior Middle 3rd. Circumferential was stiffer than longitudinal direction. Biaxial curves of anterior middle third group had the highest difference in anisotropy index and posterior middle third were almost coincident.

3.3.2 Biomechanical Testing: Uniaxial

Uniaxial mechanical testing of sheep vaginal tissue was performed for all groups in transverse and longitudinal directions. For each group, tensile modulus, ultimate tensile strength and strain to failure, were calculated (Table 3.2, 3.3, 3.4, & 3.5). Tensile modulus measurements were significantly different for anterior top third (longitudinal vs. circumferential) group, and posterior middle third (longitudinal vs. circumferential) group. In addition, data for ultimate tensile strength (longitudinal vs. circumferential) for anterior top third group, and strain to failure (longitudinal vs. circumferential) for posterior mid third group, were also significantly different. Stress-strain curves of circumferential direction of anterior middle third and posterior middle third, were stiffer than longitudinal direction. No observable trend was recorded for the top third regions.

3.3.2.1 Anterior region

In the anterior top third group, anisotropic behavior was observed, and circumferential direction was found to be stiffer than longitudinal direction (Fig. 3.4 (A)). On the other hand, in the anterior middle third region, longitudinal direction was stiffer than the circumferential directions (Fig. 3.4 (B)), but the degree of anisotropy was less as compared to anterior top third. The strain to failure for both regions was similar (Table. 3.2 and 3.3). Furthermore, the ultimate tensile strength (0.592 ± 0.119 MPa) and tensile modulus (1.833 ± 0.335 MPa) was highest for circumferential direction of anterior top third as compared to other groups.

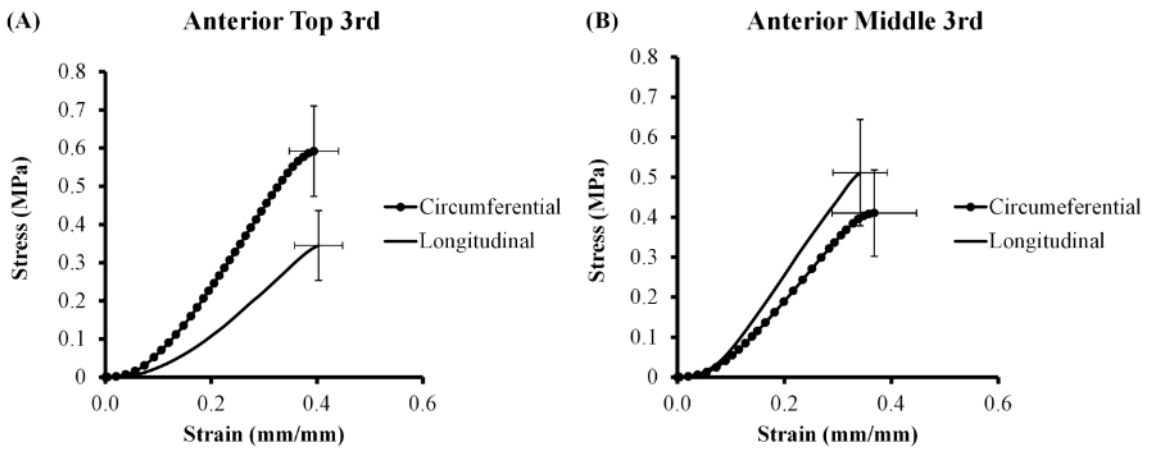

Figure 3.4 Uniaxial tensile testing data for sheep vaginal tissue - anterior.

(A) Anterior top third and (B) Anterior middle third regions. Comparison of stress-strain curves obtained from circumferential and longitudinal directions are shown here.

Similar to biaxial data, circumferential direction of the anterior top third group exhibited a stiffer stress-strain curve as compared to the longitudinal one. Ultimate tensile strength and tensile modulus data was also found to significantly different for anterior top third group (circumferential vs. longitudinal).

Table 3.3 Biomechanical parameters obtained from mechanical testing data of the anterior top third region of the sheep vaginal tract.

Anterior Top 3rd	**Tensile Modulus (MPa)***	**Ultimate Tensile Strength (MPa)***	**Strain to failure (mm/mm)**
Circumferential Direction	1.833 ± 0.335	0.592 ± 0.119	0.394 ± 0.047
Longitudinal Direction	1.034 ± 0.582	0.345 ± 0.092	0.403 ± 0.045
*denotes significant difference			

Table 3.4 Biomechanical parameters obtained from mechanical testing data of the anterior middle third region of the sheep vaginal tract.

Anterior Middle 3rd	**Tensile Modulus (MPa)**	**Ultimate Tensile Strength (MPa)**	**Strain to failure (mm/mm)**
Circumferential Direction	1.402 ± 0.273	0.410 ± 0.108	0.368 ± 0.079
Longitudinal Direction	1.849 ± 0.470	0.511 ± 0.132	0.342 ± 0.051

3.3.2.2 Posterior region

Biomechanical properties of the posterior vaginal wall tissues were slightly different as compared to the anterior region. Both uniaxial testing curves for posterior region showed an inverse trend as compared to their corresponding biaxial data. Moreover, anisotropy was only observed in posterior middle third region; circumferential and longitudinal curves of posterior top third region were almost co-incident (Fig. 3.5). The magnitude of the both transverse and longitudinal curves in either posterior group was lower in comparison to the anterior ones (Table 3.4 and 3.5). As mentioned previously, the tensile modulus and strain to failure data was significantly different for posterior middle third regions (Table 3.4 and 3.5). However, the magnitude of tensile modulus of the anterior region along longitudinal directions were higher in comparison to the corresponding posterior regions (1.849 ± 0.470 MPa vs. 1.523 ± 0.642 MPa, respectively); whereas the tensile modulus of their corresponding circumferential directions were almost similar in magnitude (1.402 ± 0.273 MPa vs. 1.445 ± 0.419 MPa, respectively).

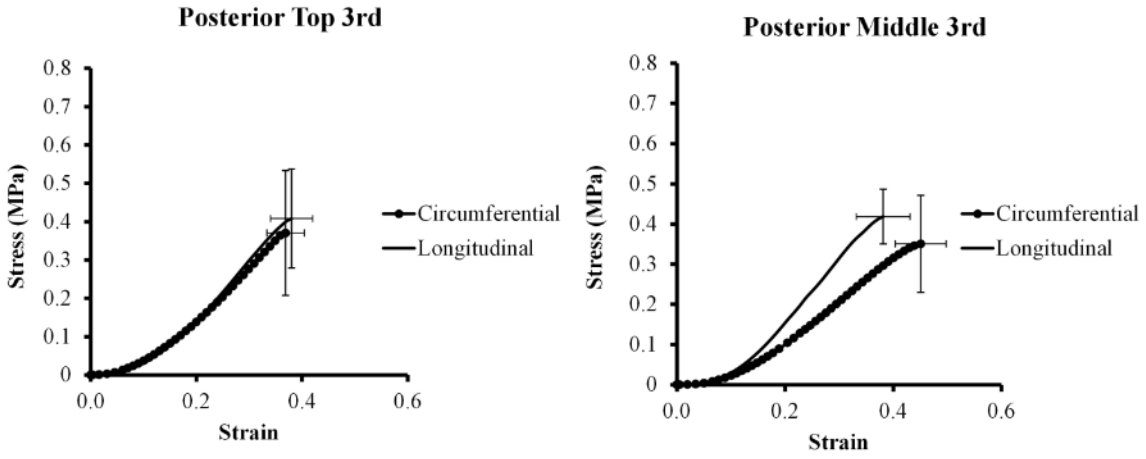

Figure 3.5 Uniaxial tensile testing data for sheep vaginal tissue – posterior.

(A) Posterior top third and (B) Posterior middle third regions. Comparison of stress-strain curves obtained from circumferential and longitudinal directions are shown here.

Some degree of anisotropy is exhibited by the posterior middle third region, whereas no difference was observed between the circumferential and longitudinal curves of posterior top third group. Similar to biaxial data, posterior middle third regions exhibited similar trend; circumferential direction was stiffer than longitudinal direction. Strain to failure and tensile modulus data was also found to significantly different for this group (posterior top third – circumferential vs. longitudinal).

Table 3.5 Biomechanical parameters obtained from mechanical testing data of the posterior top third region of the sheep vaginal tract.

Posterior Top 3rd	**Tensile Modulus (MPa)**	**Ultimate Tensile Strength (MPa)**	**Strain to failure (mm/mm)**
Circumferential Direction	1.445 ± 0.419	0.371 ± 0.162	0.369 ± 0.035
Longitudinal Direction	1.523 ± 0.642	0.408 ± 0.129	0.380 ±0.040

Table 3.6 Biomechanical parameters obtained from mechanical testing data of the Posterior middle third region of the sheep vaginal tract.

Posterior Middle 3rd	**Tensile Modulus (MPa)***	**Ultimate Tensile Strength (MPa)**	**Strain to failure (mm/mm)***
Circumferential Direction	0.975 ± 0.354	0.351 ± 0.121	0.451 ± 0.047
Longitudinal Direction	1.473 ± 0.281	0.419 ± 0.068	0.381 ± 0.050
*denotes significant difference			

3.3.3 Microstructural Characterization – Histology & Scanning Electron Microscopy (SEM)

Histological sections of sheep vaginal wall tissue along the longitudinal axis were taken and stained with Movat's pentachrome (Figure 3.6 & 3.7). Movat's pentachrome staining of the histological sections, collagen fiber orientation (yellow), smooth muscle orientation (pink bundles), and other microstructural features are clearly observed. The tissue was mostly composed of smooth muscle cells, squamous epithelial cells and extracellular matrices. Four distinct layers characteristic of vaginal tissue – epithelium, lamina propria, adventitia, and muscularis - were observed in the Movat's pentachrome stained sections (Fig 3.6 & 3.7). Sheep vaginal tissue is highly vascularized, as observed in Figure 3.6 and 3.7 (pink and black fibers surrounding the voids).

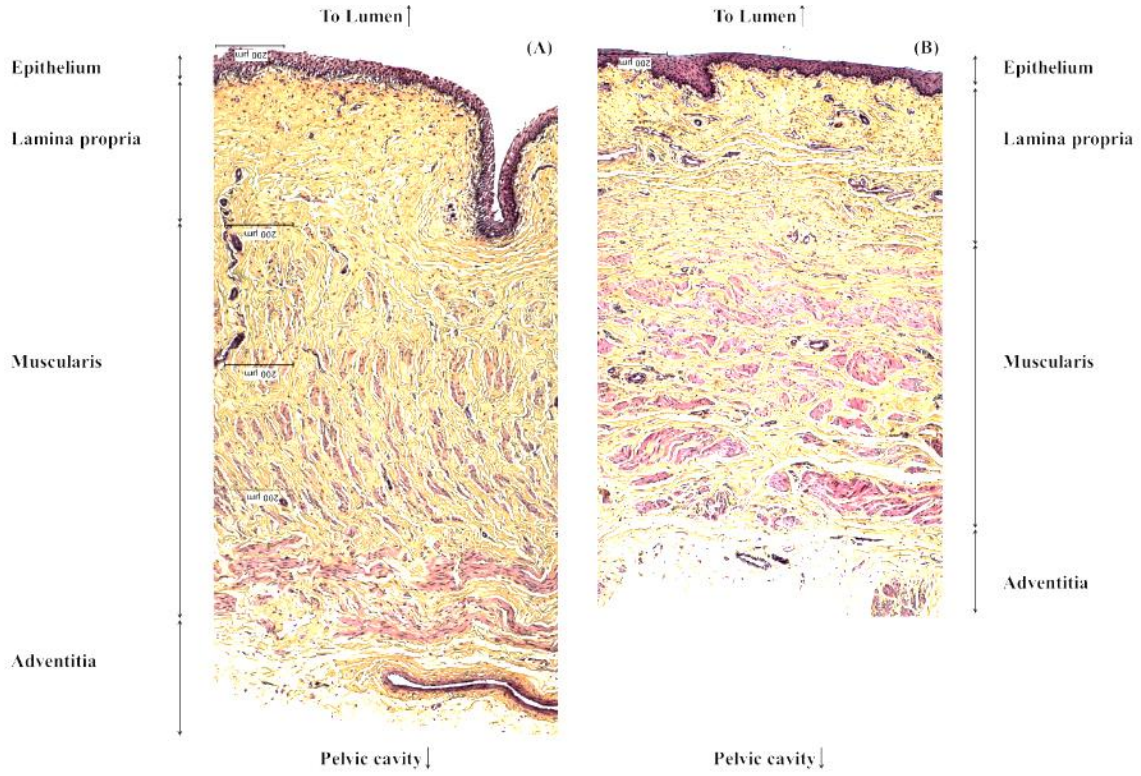

Figure 3.6 Full thickness histological sections of anterior vaginal wall in sheep (along fibers).

(A) Top third and (B) Middle third. Movat's pentachrome staining of the histological sections, collagen fiber orientation (yellow), smooth muscle orientation (pink bundles), and other microstructural features are clearly observed. Moving from the lumen towards pelvic cavity, vaginal layer is composed of four layers – epithelium, lamina propria, muscularis, and adventitia. In addition to the vaginal tissue thickness, the arrangement and distribution of smooth muscle fibers in the muscularis layer is different in top third and bottom third layers of anterior vaginal wall in sheep.

Smooth muscle fibers distribution varied greatly as per location. For anterior top third region, smooth muscle fibers of the muscularis layer were oriented circumferentially, whereas the smooth muscle bundles located closed to adventitia layer were oriented parallel to the epithelial layer (Fig. 3.6 A). For anterior middle third region, thickness or diameter of the smooth muscle fibers bundles were greater as compared to anterior top third region (Fig. 3.6 B). More fibers were oriented circumferentially in the

muscularis layer and only a few fibers were oriented along the longitudinal axis. Additionally, distance of the muscularis layer from the epithelium was more in anterior top third as compared to anterior middle third (Fig. 3.6). Blood vessels were less prominent in the anterior top third region as compared to anterior mid third region. Epithelial layer invaginations were more gradual in the anterior top third region, whereas the invaginations were more irregular in the anterior middle third region. Further, collagen fibers in the muscularis layer of anterior top third region are oriented perpendicular to the epithelial layer, whereas the collagen fiber orientation is more parallel to the epithelial layer.

Similar to anterior region, microstructural arrangements of posterior top third and posterior middle third regions were varied (Fig. 3.7). Like the anterior region, the densities of muscle fiber bundle were more prominent in the middle third regions than top third region of the posterior vaginal wall. Conversely, there was noticeable presence of smooth muscle fibers oriented parallel to the epithelial layer; in the bottom half of the muscularis layer and some parts of the adventitia layer (Fig. 3.7 A-B). In addition, distance of the muscularis layer from the epithelium was similar in both posterior groups. In the posterior top third region, collagen fibers in the muscularis layer were more diagonally oriented, while in the rest of the tissues these fibers were oriented parallel to the epithelial layer. Similarly, collagen fiber bundles were also oriented parallel to the epithelial layer in the posterior middle third region. Blood vessels were sparsely located in either group. As observed previously in anterior region, epithelial invaginations were more sporadic in posterior middle third region, while the invaginations were more graduation in the posterior top third regions.

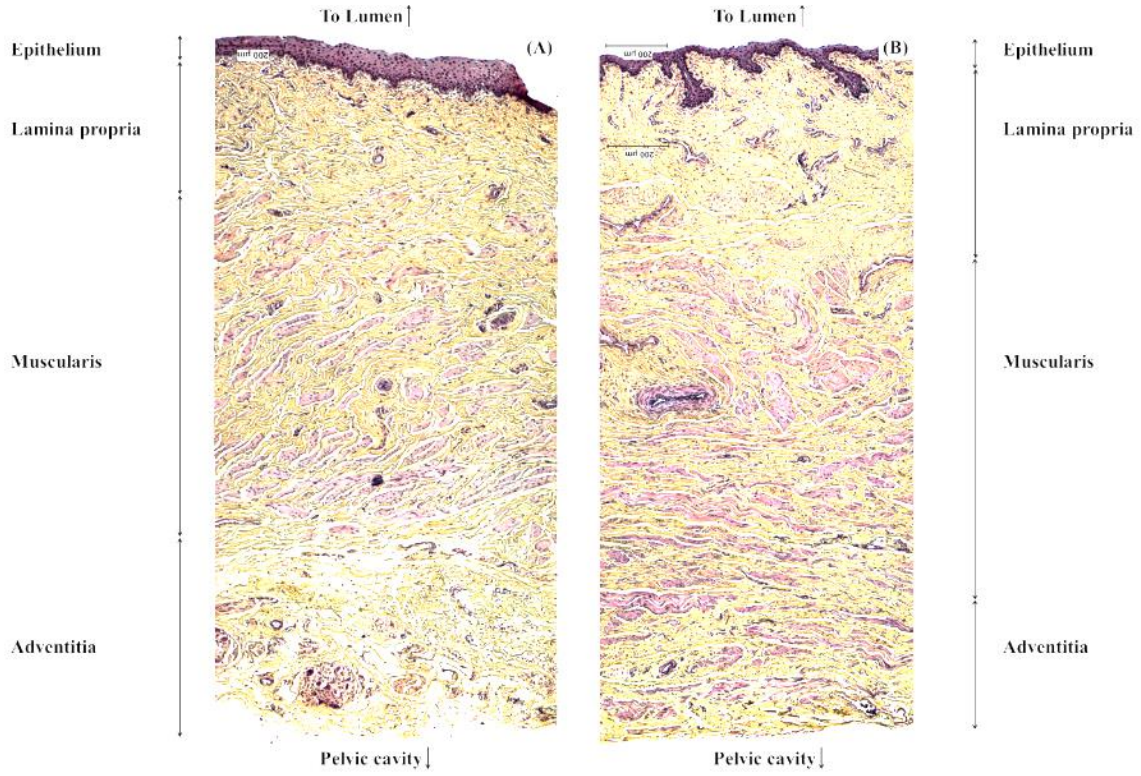

Figure 3.7 Full thickness histological section of posterior vaginal wall (along fibers) in sheep.

(A) Top third and (B) Middle third. Movat's pentachrome staining of the histological sections, collagen fiber orientation (yellow), smooth muscle orientation (pink bundles), and other microstructural features are clearly observed. Similar to anterior vaginal wall, the distribution and orientation of smooth muscle fibers vary in top third and middle third. Additionally, adventitia layer is less prominent in middle third than top third.

3.3.4 Scanning Electron Microscopy (SEM)

SEM images of the four groups of sheep vaginal tissues were viewed at 5000x magnification to study the surface characteristics. Surface characteristics of the samples obtained from each group did not show any marked difference. Image of anterior top third region is shown in Fig. 3.8. The surface was somewhat flat and mosaic in appearance. Vaginal tissue surface showed prominent presence of "club tipped"

microvilli, which was a common presentation of the diestrus period (Rubio 1976, Centola 1978).

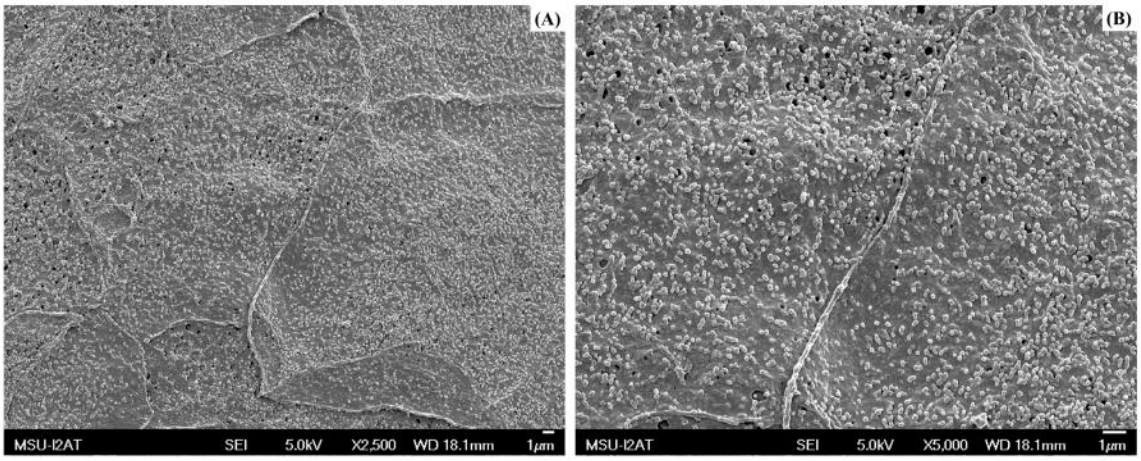

Figure 3.8 Scanning Electron Microscopy (SEM) of sheep vaginal wall surface.

(A) anterior top third surface and (B) zoomed in section of the same image. SEM image of the sheep vaginal wall surface revealed the presence of "club tipped" microvilli, which is a characteristic presentation in diestrus cycle period (sexual inactivity between cycles)(Rubio 1976, Centola 1978). No difference was found in surface characteristics across the groups.

3.4 Discussion

Due to issues with in vivo human testing and difficulties with obtaining healthy human reproductive tissue specimens, animal models are a valuable tool in understanding the pathophysiology and biomechanics behind the pelvic organ prolapse. Histomorphological studies of vaginal tissues obtained from women have shown that the smooth muscle, collagen, elastin and other connective tissue components are altered in patients with POP and hence would result in change in mechanical properties (Kerkhof, Hendriks et al. 2009). The sheep was selected as animal model due microstructural and anatomical similarity with humans.

We evaluated location based biomechanical and histological studies for sheep vaginal wall tissue in the anterior (top third and middle third) and posterior (top third and middle third) regions. Not only there was location based difference in biomechanics, there were differences in biaxial and uniaxial properties of the tissues obtained from the same group. Movat's pentachrome staining of histological samples revealed the prominent difference in size and distribution of smooth muscle cells, as well as overall thickness of the muscularis layer in the sheep vaginal tissue (Fig 3.6-3.7). Regional difference in smooth muscle contractility, distribution, and overall tissue composition could possibly explain the location based difference in biomechanical properties of sheep vaginal tissue (Ulrich, Edwards et al. 2014).

Rubod et al. reported that sheep vaginal tissue was isotropic and longitudinal direction of the tissue were stiffer than the circumferential direction (Rubod, Boukerrou et al. 2007). In contrast, we found that sheep vaginal wall tissue to be anisotropic from our biaxial and uniaxial data (with the exception of poster top third) (Fig 3.3-3.5). Conversely, the tensile modulus from their study is close to our results; we found the overall tensile modulus of sheep vaginal tissues to be in the range of 1.2 – 1.8 MPa (Table 3.2 – 3.5). As per Ulrich et al., tensile modulus of anterior region was higher than posterior region in nulliparous sheep (40-50 MPa vs. 13-15 MPa, respectively) (Ulrich, Edwards et al. 2014). Our study supports this finding (1.8 MPa vs. 1.2 MPa); however the magnitude to the modulus exhibit almost difference in magnitude (Table 3.2-3.5). Additionally, Rubod et al. did not find any difference in biomechanics of the samples obtained from anterior and posterior region of the sheep vaginal wall (Rubod, Boukerrou et al. 2007). There are no studies evaluating the biaxial data for sheep vaginal wall tissue

biomechanics; hence, the anisotropic behavior of the sheep vaginal wall tissue reported here is pretty novel. Anisotropy of the vaginal tissue is evident of the fact that the vaginal canal has to stretch circumferentially to allow the passage of the fetus. To accommodate this stretching and extension of the vaginal canal during childbirth, the smooth muscle fibers are arranged both circumferentially and longitudinally to the lumen of the sheep vagina – both anterior and posterior locations (Fig. 3.6 and 3.7, respectively).

The histological data obtained from this study shows marked similarity between human and sheep vaginal tissues (Martins 2010, Ulrich, Edwards et al. 2014). We found the mechanical behavior of the sheep vaginal tissue (Figure 3.4-3.5) to be similar to previous publications (Rubod, Boukerrou et al. 2007, Ulrich, Edwards et al. 2014, Ulrich, Edwards et al. 2014) and comparable to human tissue as well (Gabriel 2011). Data obtained from our studies will be used as input for finite element models of the vaginal wall tissue. Differences in biomechanical properties of sheep vaginal tissue have been reported with respect to menopause status, pregnancy and multiple births (Knight, Moalli et al. 2013, Ulrich, Edwards et al. 2014). As hormones, pregnancy and parity play a role in the biomechanics of the vaginal tissue; it is safe to consider than prolapsed vaginal tissues will also exhibit a difference biomechanical traits as compared to the normal ones. However, to date there are no reports of ex vivo biomechanical characterization of sheep vaginal prolapse tissues. Our study will provide a baseline measurement for future biomechanical studies with sheep as an animal model for vaginal prolapse. Biomechanical studies of the sheep vaginal tissues (obtained from healthy animal and sheep POP models) will not only provide us insight into the etiology of the POP, but also help the development of novel biomaterials which can be used for pelvic reconstructive

surgeries (Rubod, Boukerrou et al. 2007, Mangera 2011). Further mechanical studies of these tissues will better correlate the mechanical properties with clinical findings and help establish sheep as an animal model for human reproductive tract and pelvic floor disorders.

CHAPTER IV

BIOENGINEERING APPLICATIONS: DEVELOPMENT OF PATCH MATERIAL FROM DECELLULARIZED VAGINAL TISSUE

4.1 Introduction

4.1.1 Current issues with gynecological meshes

Pelvic floor disorders, including stress urinary incontinence and pelvic organ prolapse, results from the failure of DeLancey's three levels of structural support (DeLancey 1994). In order to restore this support to hold the organs in place, clinicians employed various surgical meshes (artificial, biological, and hybrid) and grafts in pelvic reconstructive surgeries (Le, Kon et al. 2007, Ridgeway, Chen et al. 2008, Peppas, Gkegkes et al. 2010). There is no unique or widely accepted "gold standard" surgical technique for correcting pelvic organ prolapse, and hence, several different types of gynecological meshes are utilized. With the high volume of polypropylene meshes used for pelvic reconstructive surgeries in the past decade, the resulting complications and mesh related issues are excessive (Jonsson Funk, Edenfield et al. 2013). In 2011, the FDA issued a safety warning regarding the use of polypropylene meshes for pelvic organ prolapse repair (FDA 2011). The problem with the polypropylene meshes were primarily due to the lack of studies with level I evidence, high rate of recurring prolapse surgeries, surgical complications, increasing volume of lawsuits against the manufacturers and lack of sufficient preclinical data (Silva and Karram 2005, Murphy, Holzberg et al. 2012,

Patel, Ostergard et al. 2012, Maher, Feiner et al. 2013, van Geelen and Dwyer 2013). Moreover, the outcome of a mesh implantation not only depends on the material type, but also on its pore size, pore shape, pore density, mesh weight and its biofilm formation (Falagas, Velakoulis et al. 2007, Patel, Ostergard et al. 2012).

After the issued FDA warning, there has been no overall change in the use of synthetic meshes or biological grafts (Reynolds, Gold et al. 2013), but the rate of mesh revision has been greater than before (Elterman, Chughtai et al. 2014), and there has also been an increase in native tissue repair rates and robotic assisted surgeries as well (Lee, Mottrie et al. 2014, Serati, Bogani et al. 2014, Skoczylas, Turner et al. 2014, Flack, Monn et al. 2015). Along with vaginal mesh complications, a number of problems in sacral colpopexy (attachment of vagina to the spine) and mid-urethral sling (attachment of sling to hold the organs in place) procedures have also been reported (Rice, Hu et al. 2013); however the number of repeat surgeries are higher with vaginal mesh placements as compared to sacral colpopexy (Nygaard, McCreery et al. 2004, Diwadkar, Barber et al. 2009). With the given problems with synthetic meshes, surgeons have resorted to biological grafts (allografts, autografts or xenografts), and even after the FDA warning the use of synthetic meshes in pelvic reconstructive surgeries have been unvarying (Chen, Ridgeway et al. 2007, Clemons, Weinstein et al. 2013, Skoczylas, Turner et al. 2014). On the other hand, use of xenograft (animal derives surgical biomaterials) has increased recently owing to their lower rates of rejection, erosion, infections, and revision; although the cost of xenograft materials are relatively higher compared to synthetic meshes (Jeon and Bai 2007, Le, Kon et al. 2007, Peppas, Gkegkes et al. 2010, Wong, Nguyen et al. 2013). These xenogenic grafts exhibit low levels of mesh exposure (0.9%), but are

frequently associated with high prolapse recurrent rates (> 20%) as compared to synthetic meshes (Meschia, Pifarotti et al. 2007, Jia, Glazener et al. 2008). However, as per the current Cochrane review, clinical trials comparing different types of meshes for anterior compartment repair have suggested that native tissue repairs were associated with a higher degree of recurrent prolapse than absorbable or xenogenic grafts (Maher, Feiner et al. 2013). The Cochrane review (along with other studies) indicate that findings from mesh related studies were relatively generalized, which may have resulted in unfortunate retraction of several commercial transvaginal mesh kits (Jia, Glazener et al. 2008, Maher, Feiner et al. 2013); Prolift® mesh was retracted from market in early 2013. With the given scenario, a long term solution is essential for confronting this issue, and experts believe that the answer lies with regenerative medicine and tissue engineering research, which has shown tremendous potential in recent years.

4.1.2 Tissue engineering as an alternative to mesh usage

Regenerative medicine and tissue engineering technologies have shown promising results in cardiovascular and orthopedic areas; however, their applications are still novel in the urogyencological field. Over the years, several tissue-engineering (TE) based repair and replacement strategies/techniques have been formulated for regeneration of urologic organs and pelvic floor tissues (Kim, Baez et al. 2000, Sievert, Amend et al. 2007, Atala 2008). However, a lot of ground work is required before we can move towards our ultimate goal i.e. a completely functional engineered organ. Tissue engineering utilizes principles of biology, medicine and engineering to regenerate artificial organs in a laboratory setting to obtain an engineered tissue (or product) which is structurally and functionally similar to the native one. This tissue engineered product

should ideally mimic the functions of the native organ/tissue, bind to the patient's body and work cohesively with the patient's organ systems. In urogyencological applications, two modes of tissue engineered products can be employed: an acellular approach and a cellular-based approach (Yamzon, Kokorowski et al. 2008). The acellular scaffolds rely on the host to incorporate new cells, extracellular matrix (ECM) and tissue growth; while the cellular-based approach incorporates donor cells into the scaffolds, which creates the required microenvironment for growth and nourishment of the tissue. The acellular approach includes both naturally sourced biomaterials (Chung, Tu et al. 2014) and commercially available collagen matrices (Friedman and Meltzer 1970, Chen, Yoo et al. 1999); many of which are currently being used for surgical purposes (Jeon and Bai 2007). For our study, we will focus on the development of female sheep vaginal tissue derived scaffolds by commonly used decellularization methods. Various structural and biomechanical studies, including studies conducted in our lab, have shown that the baseline dimensions of sheep vaginas are similar to humans (Abramowitch, Feola et al. 2009, Vincent, Bourne et al. 2009, Knight, Moalli et al. 2013, Ulrich, Edwards et al. 2014, Ulrich, Edwards et al. 2014). Additionally, sheep vagina wall tissues were found to be similar to humans in their microstructural characteristics and extracellular matrix distribution; similar findings were also reported by Abramowitch et al. and Couri et al. (Abramowitch, Feola et al. 2009, Couri, Lenis et al. 2012).

4.1.3 Decellularized Sheep Vaginal Scaffolds

A scaffold is the base material which allows the growth and development of the cells that are seeded onto it. A scaffold also sets the groundwork for new extracellular matrix (ECM) development, which is the basis of the mechanical support of any tissue.

Moreover, prolapsed tissues exhibit an increased matrix metalloproteinase (MMP-9) activity which evidently causes an altered collagen metabolism leading to loss of structural and mechanical support (Jackson, Avery et al. 1996, Kerkhof, Hendriks et al. 2009). Hence, any defect or damage to the ECM framework of the pelvic floor tissues (esp. the vaginal wall and pelvic ligaments) is directly reflected in the integrity of the pelvic floor organs and in the onset of pelvic organ prolapse (Kerkhof, Hendriks et al. 2009). New tissue and ECM growth around the implant or mesh will dictate the type of integration for the graft with the host and ultimately, its long term adaption and functional characteristics. In tissue engineering experiments, a number of polymeric, biological and hybrid (biological and polymeric) biomaterials are used (Patnaik, Wang et al. 2014); each type of scaffold has its own pros and cons, which is conditional on its applications and the ultimate goal. Ideally, a scaffold should provide adequate mechanical strength, provide the framework for new tissue/cellular growth and integrate cohesively with the host. In addition, the microstructure of the scaffold dictates the cellular growth and differentiation of the seeded cells, and thus, crucial for the eventual experimental outcome (Brehmer, Rohrmann et al. 2007). Hence, in order to fix the issues with the current gynecological meshes, we have to understand the structure-property relationship between the grafts and their surrounding tissues. For the purpose of this study, we will be using acellular xenogenic (animal derived) scaffolds. Decellularization removes any cellular and genetic components, and only the ECM makes up the bulk of the material (Patnaik, Wang et al. 2014). Most of the urological tissue engineering applications have utilized the use of commercially acellular matrices (e.g. Xenoform (Goldstein, Maccarone et al. 2010), etc.), xenogenic matrices (porcine sub-intestinal

submucosa (SIS) (Ho, Heydarkhan et al. 2009), bovine pericardium (Guerette, Peterson et al. 2009), etc.) or customized polymeric constructs (polyamide (Ulrich, Edwards et al. 2014), poly (L-lactic acid)(PLLA), etc.) (Kollhoff, Cheng et al. 2011). On the other hand, acellular and biological matrices are useful as tissue engineering experiments owing to their ability to retain ECM components like collagen, elastic, glycosaminoglycans, fibronectins, laminins and among others (Patnaik, Wang et al. 2014). This distinct advantage gives xenogenic and allogenic matrices an unparalleled edge over polymeric constructs.

4.1.4 Knowledge gap and our objectives

Considerable research is warranted to assess the superiority of xenografts over synthetic meshes for pelvic reconstructive surgeries (Le, Kon et al. 2007). Our study aims to move a step further towards utilization of xenogenic biomaterials for pelvic organ prolapse. In this study we have used three types of commonly utilized decellularization methods for developing decellularized female sheep vaginal scaffolds. Thorough microstructural characterization and biomechanical evaluation of these scaffolds were performed to equate their performance with currently used gynecological meshes.

4.2 Materials and Methods

4.2.1 Sample collection and preparation

Sheep vaginal wall tissues were obtained from a commercial abattoir. After removal of connective tissues and fat, wall specimens were cut into ~ 40 x 40 mm square samples (1.5 - 2 mm thick) and subjected to one of three different decellularization protocols. Only top third and middle third samples were chosen for this study.

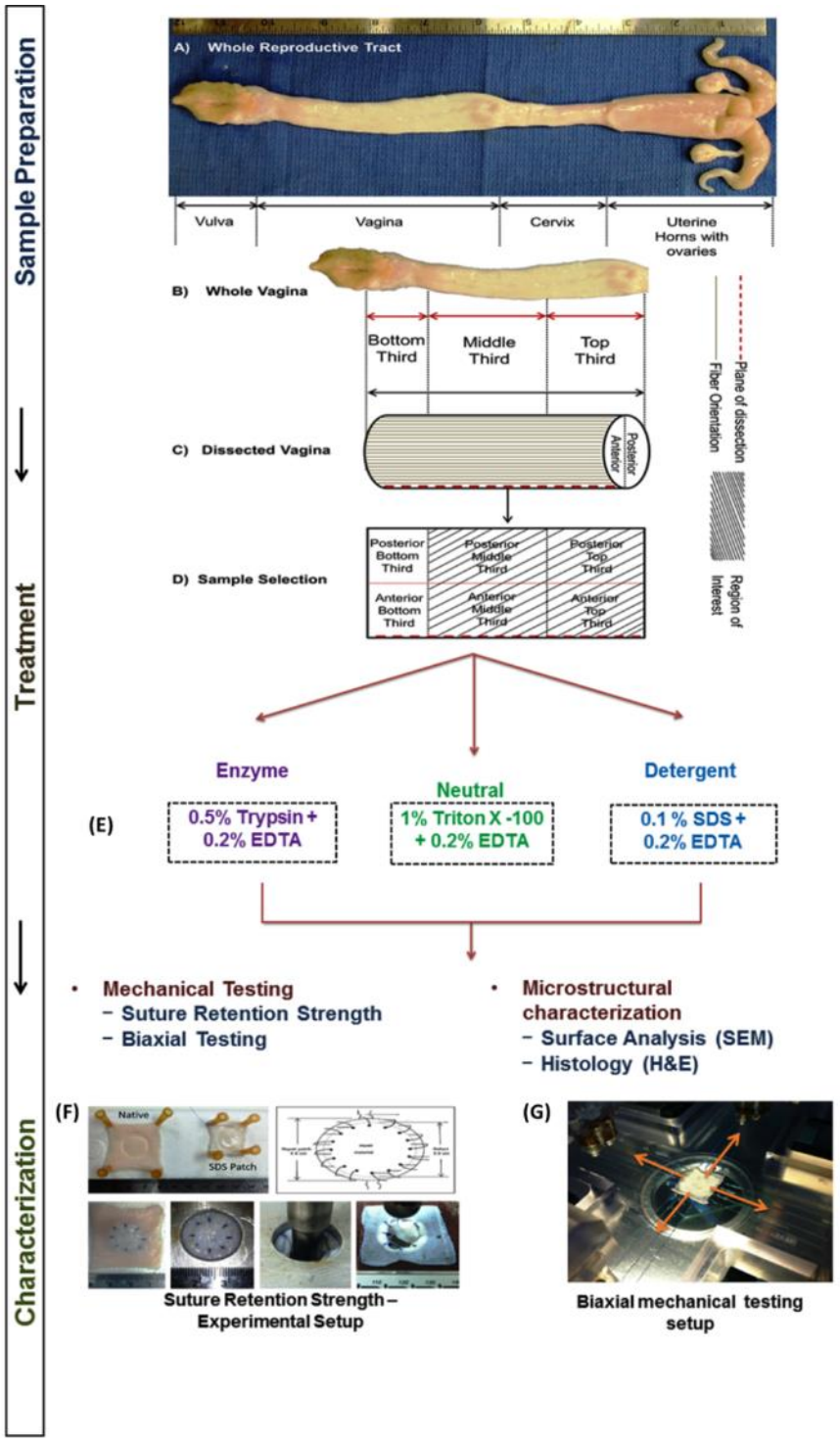

Figure 4.1 Overall layout of the experiment.

(A-E) Sample preparation and testing of scaffolds. (F) Suture retention testing setup. (G) Biaxial testing - Sheep vagina tissue loaded onto custom biaxial testing machine.

4.2.2 Decellularization Protocol

The decellularization solutions were each based on a stock solution (Wang, Tedder et al. 2012) which consists of 0.2% EDTA (Sigma) to promote solubility, 20 μg/ml RNase A (Sigma) and 0.2 mg/ml DNase (Sigma) to remove genetic material, and 1 mM phenylmethylsulfonylfluoride (PMSF, protease inhibitor) (Sigma) to inhibit extracellular matrix (ECM) degradation. The decellularization solutions then included one of the following decellularizing agents: (1) an ionic detergent: 0.1% sodium dodecyl sulfate (SDS) (Sigma), (2) an enzymatic decellularization agent: 0.5% Trypsin (VWR), or (3) a non-ionic detergent: 1.0% Triton X-100 (Sigma) (Liao, Joyce et al. 2008) (Fig. 4.1 (E)). These treatment agents have previously been evaluated in other tissues such as heart valves (Liao, Joyce et al. 2008), myocardium (Wang, Tedder et al. 2012), artery (Williams, Liao et al. 2009), etc. Specimens were treated with the decellularization agents for 48 hours under continuous gentle agitation using a commercially available shaker (Stovall Life Science, Greensboro, NC). Specimens were then thoroughly rinsed and stored in phosphate buffered saline (PBS) supplemented with 1% antibiotic-antimycotic for further analyses.

4.2.3 Patches for Suture Retention Testing

Decellularized specimens (i.e. Sodium Dodecyl Sulfate (SDS), Trypsin and, Triton-X 100), and native vaginal wall controls, were dissected to circular patches of 19.4mm (Obermiller, Hodde et al. 2004) (Fig 4.1 (F)). Each patch was then sutured, using eight interrupted stitches of polydioxanone (PDS) US Pharmacopeia (USP) size 2-0 suture with a taper needle (Ethicon, Johnson & Johnson Intl.), to a specimen of native female sheep vaginal wall tissue graft (~ 40 x 40 x 2 mm) in which a 14.9 mm circular

hole had been cut. All stitches were sutured by a Diplomat of the American College of Veterinary Surgeons. This patch-graft construct was then subjected to ball-burst tests to evaluate suture retention strength.

4.2.4 Suture Retention Testing (Ball Burst Testing)

Suture retention test or ball burst testing was carried out by a scaled American Society of Testing and Materials (ASTM) standard ball-burst apparatus using a stainless steel ball of 12.7 mm diameter and sample surface area of about ~1013.41 mm2 (ASTM Test Method D3787). Specimens (i.e. patch-graft constructs) were mounted between two metal plates with a 19.05mm diameter hole and ruptured at a rate of 10 mm per minute (Feola, Barone et al. 2013). The number of suture sites that failed after rupture was recorded for each specimen; tearing of the suture knot from the tissue constituted a "knot failure" event. Data from ball burst tests were processed to create load vs. displacement curves of the three decellularization treatments.

4.2.5 Biomechanical Testing: Biaxial

Biaxial mechanical testing was performed as per previously established protocols (Liao, Joyce et al. 2008, Zhang, Crow et al. 2010). From each group, the specimens were dissected into ~ 20 mm x 20 mm square samples, with one edge of the sample aligned along longitudinal direction and the other edge aligned along circumferential direction. Tissue specimens were securely connected to each actuator arms of the biaxial device using custom designed hooks, which were looped with 000 USP size non-absorbent sutures (4 hooks per edge) (Fig. 4.1 (G)). Specimens were preloaded up to 2 grams, preconditioned ten times in physiological range (0.05 N) and subjected to an equbiaxial

tension of 60N/m (each half cycle = 15 seconds). Maximum stretch of the samples, in either direction, due to the applied load was recorded. All the tests were performed at room temperature in Phosphate Buffered Saline (PBS).

4.2.6 Microstructural Characterization

4.2.6.1 Scanning Electron Microscopy

For SEM, specimens were fixed using a solution of half-strength Karnovsky's in PBS (2.5% glutaraldehyde, 2% paraformaldehyde, 0.1M PBS) for a minimum of 48 hours. Specimens were then rinsed with distilled water, fixed by osmium tetroxide, and subjected to a series of increasing concentration alcohol solution treatment and overnight chemical drying using Hexamethyldisilazane (HMDS) solution. Samples were loaded onto aluminum stubs using carbon paste and sputter coated with platinum (30μm). Samples were then viewed using JEOL JSM-6500F Field Emission Scanning Electron Microscope (SEM) with secondary electron detectors (SEI), and the voltage was set to 5 keV. Micrographs were obtained at multiple magnifications to understand the tissue ultrastructural alterations due to various decellularization treatments.

4.2.6.2 Histological analysis

Routine Hemotoxylin and Eosin (H&E) staining of vaginal tissue samples were done to show full thickness microstructural details. Samples from each region were obtained in triplicates.

4.2.7 Statistical analyses

The obtained data sets were stored and analyzed using SPSS ®. ANOVA was used to check if the data was significantly different. Bonferroni-Holm test was used for

comparison of native data with decellularized groups. Data was considered significant at $p < 0.05$.

4.3 Results

4.3.1 Suture retention Strength

Suture retention strength provides information regarding the implant performance under physiological loads. Details of the suture retention strength data for the three types of scaffolds are shown here in Fig. 4.2. In addition to the mechanical testing data, we also evaluated the failure of suture knots for each samples. The plot of the suture knots failure (tissue tear) with respect to the load is shown in Figure 4.3.

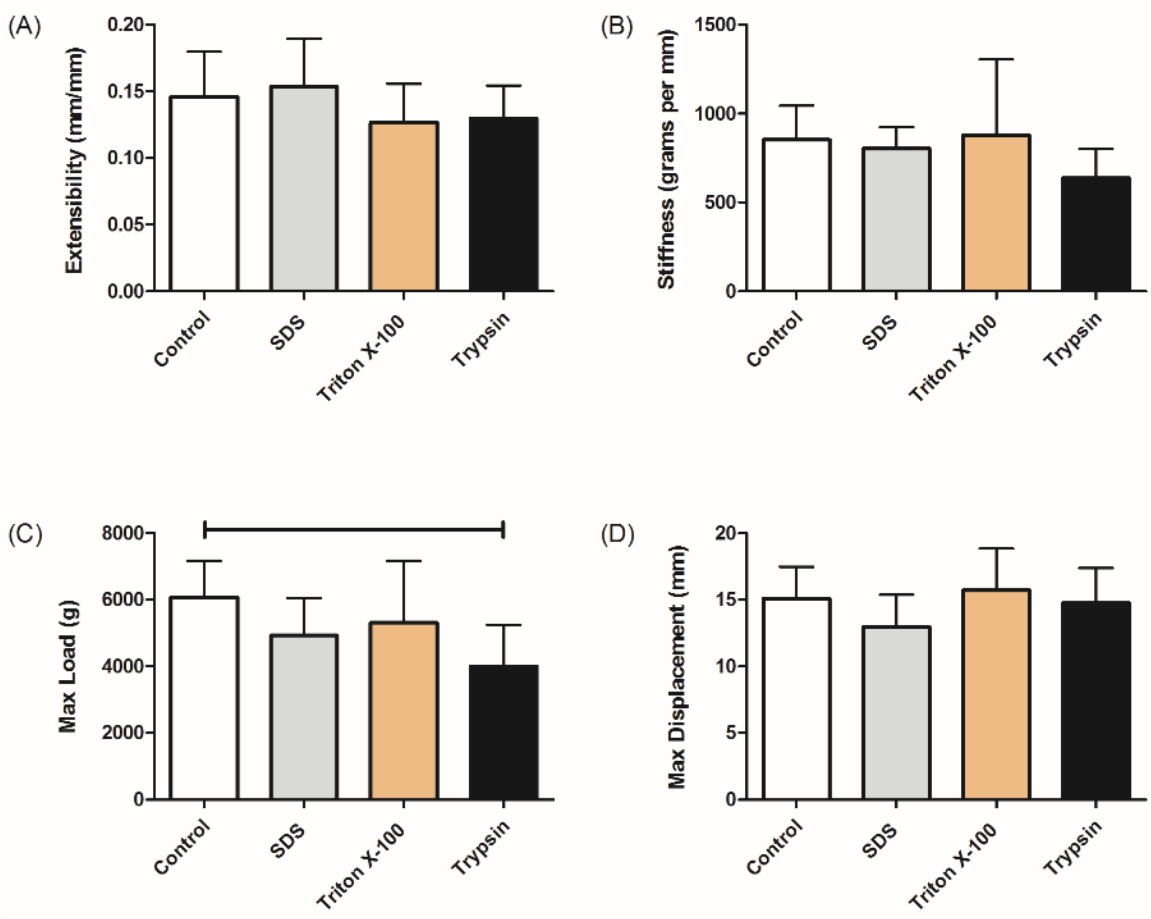

Figure 4.2 Biomechanical parameters from suture retention testing of vaginal scaffolds.

(A) Extensibility, (B) Stiffness, (C) Max load and (D) Max. Displacement.

There was no statistical significance across the groups; only max load was significantly different for trypsin vs. control (Fig 4.2 (A)). As expected, control had the maximum load bearing capacity, while SDS was most extensible Fig 4.2 (A and C)). Suture knot failure (tissue tear) was not statistically significant across the groups. Trypsin has the most failed sutures, followed by Trypsin and SDS (Fig 4.3).

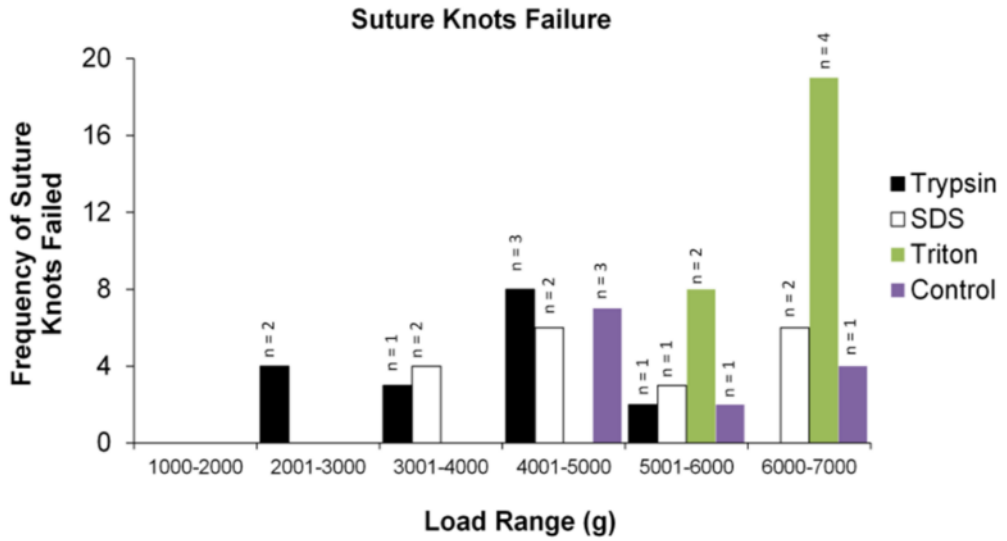

Figure 4.3 Suture knots failure of each patch is provided herewith.

Failure of knots (tissue tear) are in the order of Trypsin > Triton> SDS.

4.3.2 Biaxial Testing

Biaxial mechanical results show major changes in mechanical response following decellularization, including shift in anisotropic mechanical behaviors (Fig 4.4). Biaxial testing data for native vagina tissue, SDS treated, Triton-X treated and trypsin treated scaffolds are shown here in Fig 4.4. Biaxial mechanical testing showed tissue anisotropy amongst all the groups. Tissues become stiffer when cells are removed (as expected) and this behavior was exhibited by the tissue in circumferential direction. However, not much change was seen circumferential direction. Biaxial behaviors of native vaginal tissues were found to be stiffer in circumferential direction than longitudinal direction. However, for all other groups the trend is reversed. For SDS, Triton-X and trypsin groups, the biaxial data showed that the longitudinal curves were stiffer than the circumferential curves. However, anisotropic behavior of the scaffolds was preserved, and curves were

stiffer than the native tissue. This behavior is typically observed when cells are removed from the tissue.

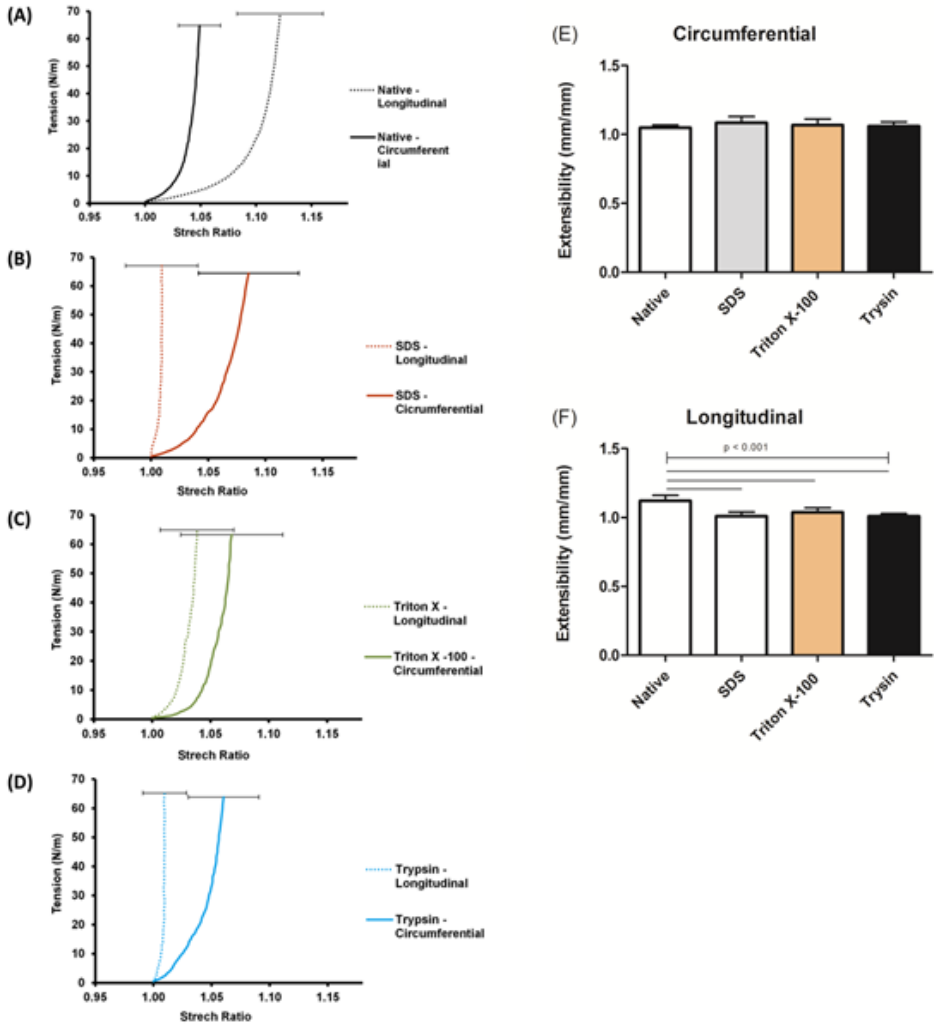

Figure 4.4 Biaxial mechanical data of native, SDS, Triton X-100 and Trypsin samples are shown here in A-D.

Extensibility of each group in circumferential and longitudinal direction are shown here E-F. Longitudinal extensibility was significantly different across the groups and between the groups as well.

4.3.3 Microstructural Characterization – SEM and Histology

SEM images of the native and decellularized vaginal scaffolds are shown here in Fig. 4.5 (A-D). Surface of the native tissue showed a "club" type villi presence, which was a characteristic feature of diestrus or "dry" period of the animal. Chemical disruptions of the tissues were more pronounced in trypsin, where the surface morphology is almost appears damaged.

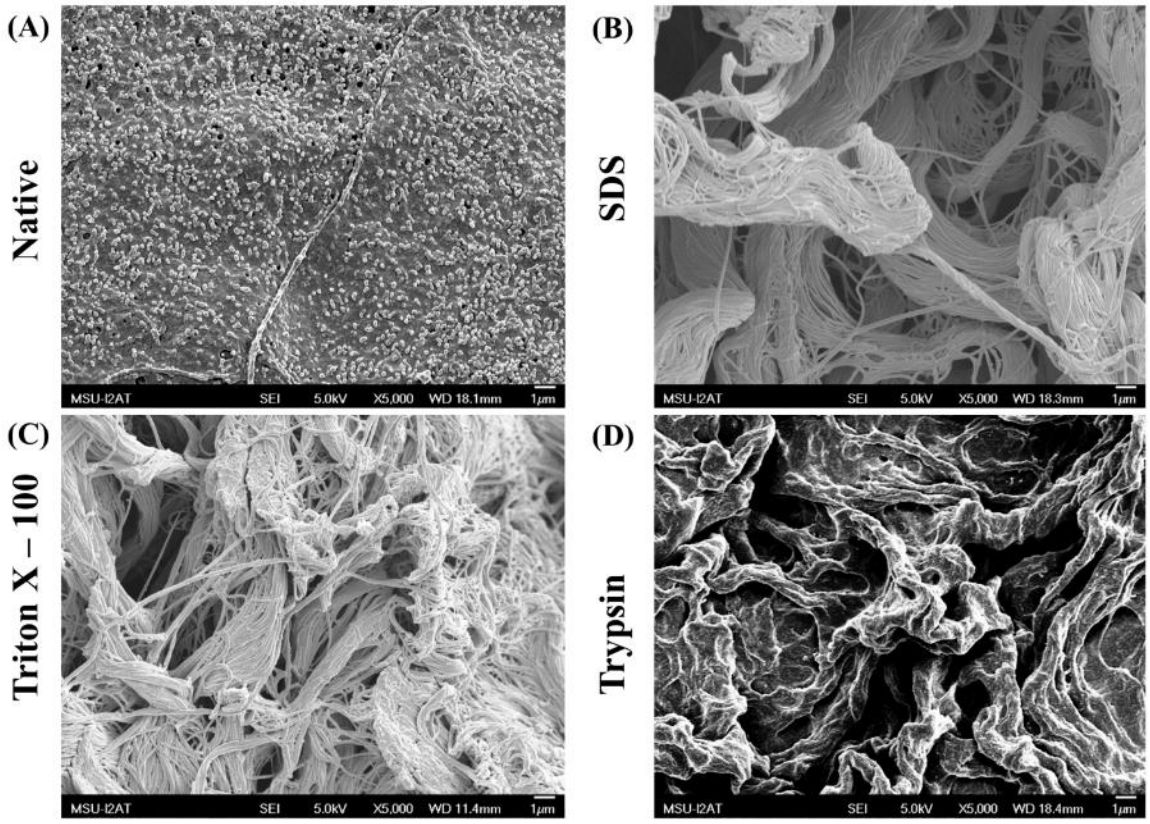

Figure 4.5 Surface characteristics of the decellularized vaginal scaffolds using SEM. Trypsin treatments were found to be the most corrosive agent on sheep vagina tissue. The degree of disruption can be stated as Trypsin>SDS>Triton X-100.

Pore formation was ideal in SDS treated samples, whereas a Triton x -100 did not achieve proper void formation or cell removal. This observation was again confirmed by

histological study of these samples. Histology of these scaffolds is shown in Fig. 4.6. Vaginal epithelium and smooth muscle cells are present prominently throughout the cross-section of the native vaginal tissue. Upon treatment with SDS, cell surface is ruptured which releases the cells and only the ECM is left. As observed in SEM imaging, trypsin caused damage to the extracellular matrix, whereas Triton X-100 was unsuccessful in removal of cellular debris from the vagina wall tissue.

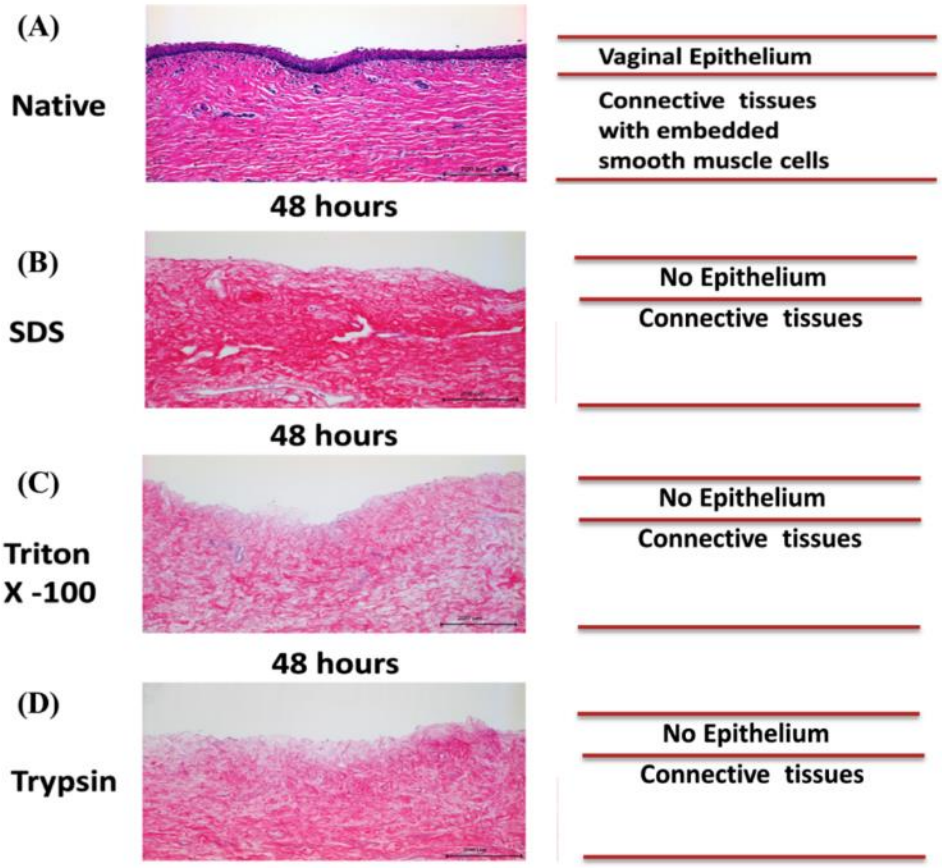

Figure 4.6 Histology of native sheep vaginal tissue and various decellularized tissues – SDS, Triton X-100 & Trypsin groups are shown here.

Removal of cells and preservation of ECM were best achieved by SDS. Triton X-100 and trypsin were not successful in complete removal of cells; moreover, trypsin caused more damage to the surface.

4.4 Discussion

Our objective was to develop a biocompatible, non-carcinogenic, cohesive, structurally and mechanically compliant scaffold that can be utilized as an alternative to the current gynecological meshes. To our knowledge this is the first report of the use of female sheep vaginal tissue as acellular or decellularized scaffolds; sheep forestomach is the only other sheep derived tissue studied as a tissue-engineered repair material for POP (Mangera, Bullock et al. 2013). We have compared the microstructural, biomechanical and suture retention strength of vaginal scaffold, which can be potentially utilized in gynecological surgeries.

The risk and severity of a complication arising from a prolapse surgery could be due to the choice of surgical technique applied and due to the patient characteristics (Alvarez, Cvach et al. 2013). Currently, the debate is not only between the mode of surgery or technique used in the surgery, but also for type of mesh material for a pelvic reconstructive surgery. Along with the issues with prolapse surgery related complications, suture/mesh erosion is one of the major issues faced by the surgeons' today (Brubaker, Maher et al. 2010). In addition, the type of suture material (Polydioxanone (PDS®); Polypropylene (Prolene®); Coated Polyester Polyester (Ethibond®), etc.) used and its physical characteristics (multifilament (braided/twisted) or monofilament type) in a prolapse surgery determines the overall success of the operation, and its long term outcomes (Goldstein, Vakili et al. 2007). With proper surgical management practices, mesh/suture erosion in abdominal sacrocolpopexy operations can be avoided by controlling risk factors such as smoking and concurrent hysterectomy (Cundiff, Varner et al. 2008). Moreover, the use of a delayed absorbable

monofilament suture, rather than a permanent one, could also reduce the possibility of suture/mesh erosion event (Tan-Kim, Menefee et al. 2014).

Usually the selection of suture used in a surgery is based on the surgeon's discretion or choice. Owing to their knot security and ease of handling, braided sutures are the primary choice for reconstructive surgeries. However, with the given microbial environment of the vagina, the braided sutures may harbor bacteria (and other microorganism) (Katz, Izhar et al. 1981, Fowler, Perkins et al. 2013) which may ultimately lead to post-operative complications such as infections, erosion and granulations (Chu and Williams 1984, Goldstein, Vakili et al. 2007, Henry-Stanley, Hess et al. 2010). Similar to gynecological meshes (de Tayrac and Letouzey 2011, Ostergard 2011, Azadi, Jasinski et al. 2014), improper handling and bacterial colonization could lead to degradation of the sutures, and further lead to post-operative complications. Correspondingly, multifilament sutures utilized in vaginal prolapse surgeries are more prone to post-operative morbidity, infections and vaginal discharge than single filament ones (Varner, Holley et al. 1998, Patil and Duckett 2012, Mizon and Duckett 2015).

In our study, PDSII USP 2-0 monofilament sutures were used for the suture retention testing of three types of decellularized female sheep vaginal tissue scaffolds. PDSII is an absorbable type suture material with minimal rate of degradation in the first 90 days, and it is further degraded by hydrolysis in 180-210 days (Hochberg, Meyer et al. 2009). PDSII sutures are more preferred by some surgeons' for prolapse surgeries owing to their lower post-operative complication rate, reduced affinity to microorganisms, lower infection rates, and minimal granulation characteristics (Katz, Izhar et al. 1981, Chu and Williams 1984, Goldstein, Vakili et al. 2007, Henry-Stanley, Hess et al. 2010,

Fowler, Perkins et al. 2013). Allahdin et al. compared the use of polydioxanone (PDS) or polyglactin (Vicryl) in a short term randomized controlled trial of patients undergoing prolapse surgeries, and did not find any difference in quality of life (QoL) scores and urinary incontinence scores (Allahdin, Glazener et al. 2008). Although, in their follow up study, patients undergoing prolapse repair with Vicryl suture showed better prolapse-related quality of life scores and urinary incontinence scores, as compared to PDS ones (Madhuvrata, Glazener et al. 2011). Moreover, PDS suture material exhibits less creep behavior (Nout, Lange et al. 2007) which is ideal for closing wounds under high loads (e.g. abdominal wall).

Along with the physical characteristics, biomechanical properties of the suture materials also play a vital role on the integrity of host-graft interface and ultimately the union and performance of the implant in a patients' body. Hence, in addition to the mechanical characterization of the scaffold material (via biaxial and uniaxial tensile testing), we have also evaluated the integrity of the scaffold-tissue interface by suture retention strength testing (Fig 4.2-4.3). By using the testing method devised by Obermiller et al. (Obermiller, Hodde et al. 2004), we have combined the (i) traditional suture test and (ii) ball burst tests. For traditional suture tests, the suture under investigation would be attached to the actuator (or moving arm) of the mechanical testing machine, and the other end will be knotted to a tissue (fixed). The basis of the test was to evaluate the magnitude of force necessary to uproot or tear the suture knot from the tissue. On the other hand, ball burst tests (or biaxial puncture tests) were conducted in accordance with ASTM standards D3787. Owing to biaxial nature of load exerted on the sample (similar to *in vivo* conditions), the ball burst test is now commonly utilized

biomechanical technique for preclinical evaluation of gynecological meshes (Freytes, Rundell et al. 2005, Feola, Barone et al. 2013, Endo, Feola et al. 2014, Feola, Endo et al. 2014, Feola, Pal et al. 2014).

The type of stich used to secure the scaffolds or implants in the host is key to the strength of the suture/mesh interface. Maguire et al. showed that women who underwent prolapse surgery experience more pain (as per visual analogue scale (VAS) scores) with interrupted suture knots compared to that of continuous suture knots (Maguire, Mayne et al. 2014). Similar findings were also reported by Kettle et al. in their Cochrane Reviews (Kettle and Johanson 2000, Kettle, Hills et al. 2007). Conversely, the amount of suture material used (in packs) for an interrupted suture knot is less than continuous type (Kettle, Dowswell et al. 2012). Further, long term effects of these knots are not completely understood. Future studies will determine the durability and long term effects of different type of suture knots in pelvic reconstructive surgeries. Similar to previously published studies from literature (Lazarou, Scotti et al. 2004, Margulies, Lewicky-Gaupp et al. 2008, Young 2009, Moreno Sierra, Ortiz Oshiro et al. 2011), we used interrupted suture to create the scaffold-tissue constructs for suture retention testing. Suture knots were applied evenly through our the scaffold-tissue interface and a bite size of 10 mm (nearest to 0.001 mm) was provided to each scaffold-tissue constructs to ensure proper overlap (Obermiller, Hodde et al. 2004); this also minimizes any uneven stress distribution that could potentially lead to errors in suture strength estimates. In our study, suture retention strength data indicated that Trypsin was substantially weaker in all respects when compared with SDS and Triton (Fig. 4.2-4.4). SDS and Triton had similar values for stiffness and load at failure, but Triton treatment appeared to create a patch that

could accommodate greater stretch. This is important as damaging stretch events such as high abdominal pressure or high strenuous activities.

Ultrastructural and microscopic analysis shows the effect of each treatment on the tissue matrix. The degree of disruption can be stated as Trypsin>SDS>Triton X-100. Tissue anisotropy or mechanical properties were reversed after decellularization process. This shift is mostly due to removal of native and muscle cells from native tissue. Future work will include the evaluation of the biocompatibility and cell support capability of this acellular wall scaffolds.

Biomechanical aspects of the surgical materials for pelvic organ prolapse are now considered very seriously. Hence, it is vital for any replacement tissue or patch (or mesh) to incorporate appropriate flexibility and elasticity that will allow the pelvic organs to stretch over time. Given the complex mechanical requirements of the vagina, the accommodation of a wider variety of deleterious scenarios may be advantageous. Additional investigations are needed before asserting complete superiority of one patch preparation method. With the given mixed results from synthetic and biological meshes, the current investigations are focused on the possibility of reducing the increased magnitude of foreign body responses, which is triggered by the introduction of the mesh in the host by introducing various biomaterial coatings on the meshes (such as titanium (Junge, Rosch et al. 2005), hydroxyapatite, collagen, plasma (Gerullis, Georgas et al. 2013), etc.). We believe that newer avenues of research, along with tissue engineering initiatives, will one day disentangle the current gynecological mesh issues and eventually increase the quality of life of female patients globally.

CHAPTER V

SUMMARY

Sheep are becoming a popular animal model for human pelvic organ prolapse because they have relatively similar size and anatomy such as a three level support system in the pelvic floor. Sheep are known to prolapse frequently in production settings and typically prolapse usually occurs during the final stages of pregnancy. These advantages are important because there is not an established large animal model for pelvic organ prolapse. Rodent models have the advantage of being easy to manage and prolapsing quickly, but their small size hampers certain studies, especially those related to biomechanics and surgical implants. Non-human primates have the most similar anatomy and physiology to humans, but carry many difficult logistical and ethical issues. Most importantly, though, the non-human primates take many years to prolapse, and do not prolapse at very high rates. This prevents many studies and experiments from being completed within a reasonable timeline. Sheep has promise to overcome many of these issues due to their size, anatomy, and relatively quick prolapse timeline.

Sheep prolapse has been documented to occur between 1% and 29%, with the higher values occurring in specific flocks where management concerns led to high rates of prolapse. Prolapse and dystocia are widely correlated in sheep, and these two pathologies account for a significant amount of loss in sheep production, due to lamb loss and culling. Unfortunately, many of the risk factors for these pathologies are symptoms

of management efforts to increase productivity, especially in meat operations where large animals are more valuable. Risk factors associated with high levels of prolapse in sheep include parity, higher body score, larger lambs and/or smaller pelvic inlet (high ratio of lamb size to pelvic inlet size), hillside grazing, history of dystocia, previous prolapse, pedigree history of prolapse, and the presence of phytoestrogens or other chemical influences in their feedstock. It is important to note that many of these risk factors have close analogues in humans, including high fetal weight, obesity, dystocia, parity, and family history of prolapse. These commonalities are important because research into sheep prolapse may be directly translated into human medicine, and vice versa. This makes sheep the ideal vehicle for advancing our knowledge of prolapse and prolapse related disorders across multiple species.

In this study, we have examined sheep vaginal wall tissue with the aim of validating this tissue as a robust biomechanical animal model for POP. We found that anatomical parameters are a good measure or biomarker for estimating structural and anatomical changes in the body of the animal. As anatomical measurements are applied to human vaginal prolapse, we can apply the same principles in sheep and further explore the feasibility of using sheep as an animal model for prolapse.

We found that the fractional anisotropy of the middle region was different from the top regions. The orientation of the smooth muscles in the vaginal tissue changes from location to location. The surface tortuosity of the vagina tissue is different between the circumferential direction and the longitudinal direction. Vaginal tissue density was also accessed via Archimedes principle. We found that tissue density is conserved across different locations of the sheep vaginal tract. We hypothesize that these tissue parameters

or anatomical measurements of the prolapsed sheep will be different from the normal ones, owing to their increase in muscularis layer thickness and other hormone related changes in the mucosa.

We have characterized the structure-property relationship of sheep vaginal wall tissue in the top third and middle third regions; bottom third regions were not included in this study. We found that in contrast to currently published research, sheep vaginal tissues are in fact anisotropic in nature. This anisotropic characteristic of the sheep vaginal wall tissue is a direct function of the microstructural arrangement of collagen, elastin, smooth muscle and other components. Muscle and ECM orientation (esp. collagen fibers) varies with location and depth as well. Epithelial invaginations are more gradual in the top third regions whereas these invaginations are more irregular in the middle third. One can speculate that, under the influence of abnormal cellular and tissue metabolism, structural defects are developed, which in turn leads to reduction in the mechanical strength of the supporting tissues and ultimately leading to prolapse.

We developed three different types of vaginal tissue scaffolds using SDS, Triton X-100 and trypsin for reconstructive surgery applications. These scaffolds were developed from sheep vaginal tissues by the use of the mentioned chemicals. During the decellularization, all of the cellular components are removed, which leaves the acellular ECM behind. Our clinical goal is to allow this decellularized scaffold to integrate with the host body and provide base for new tissue growth. Additionally, these scaffolds should also provide the required mechanical and anatomical support during the tissue regeneration process. Hence, we analyzed the biomechanical and microstructural properties of these scaffolds, and found that the SDS treated scaffold is better in all

aspects of the preclinical evaluation. Future studies will aim at applying these anatomical and biomechanical techniques to prolapsed sheep tissues.

CHAPTER VI

CURRENT LIMITATIONS AND FUTURE STUDIES

Sheep reach sexual maturity between 4-14 months depending of the breed and other environmental factors (Frandson 1974, Hafez 2000). Like most mammals, sheep also exhibit characteristic changes in their vulva as per their estrus cycle. The vulva is small and pale in seasonal breeders (sheep) in their anestrus (non-heat) period, while prominent swelling and protrusion is observed as they approach the estrus cycle (Kunz, Wemmer et al. 1996). The samples used in our study were obtained from 9-11 months old female lambs. Upon gross anatomical examination, no marked swelling of the vulva was observed and SEM images showed the presence of diestrus "club tipped" microvilli. Hence, we can rule out the estrus cycle effect (i.e. the morphological variations) on external genitalia in our sheep samples. This further minimizes the possibility of errors in PB, GH and AGD measurements due to estrus cycle variation. As compared to Archimedes principle, automated density calculation using pycnometer can be used to determine density of soft tissues with minimal error in the future.

We have utilized explanted reproductive tracts of nulliparous sheep for our study. Most of the anatomical parameters were measured using electronic calipers in accordance with previously published literature (Jelovsek, Sokol et al. 2005, Sathyanarayana, Beard et al. 2010, Alperin, Tuttle et al. 2014). However, the ideal morphometric study will utilize a combination of standardized camera setup and image analysis software (such as

NIH ImageJ, etc.). These images could also be further digitized using morphometric softwares (such a Morphologika, etc.) and used for developing detailed structural and statistical models. Furthermore, there will be some evident difference in anatomical measurements performed on explanted sheep tracts vs. *in vivo* measurements with live animals.

The intact reproductive tracts used for the DT-MRI were encased in a gelatin medium. The gel was radio-opaque and ideal for MRI scanning, but some degree of sample deformation may have occurred during the gel hardening process in the freezer. However, with given tensile strength of the female sheep vaginal wall tissue (Rubod, Boukerrou et al. 2007, Ulrich, Edwards et al. 2014), this deformation will have minimal effect on the overall smooth muscle pattern or fiber distribution visualized with DT-MRI. *In vivo* scanning of sheep pelvic floor will further provide a better understanding of the 3D smooth muscle structure of the supporting tissues and a high fidelity computational model for finite element simulations.

Further studies are necessary to visualize and understand the changes occurring in a prolapsed sheep and its similarities with the pelvic organ prolapse. Similar to MOP-Q for mice (Wieslander, Rahn et al. 2009), development of a POP-Q like system for sheep prolapse is necessary to classify and grade the severity of vaginal prolapse (as per Cox et al. 1982 (Cox 1982)). The female sheep is perhaps the ideal large animal model for studying POP due to the many similarities between human and sheep reproductive anatomy, as well as similar vaginal prolapse pathophysiology (Bassett 1956, McLean 1956, McLean and Claxton 1959, Zacharin 1969, Hay 1991, Mitchell 1993, Litherland, Lambert et al. 2000, Noakes 2001, Hosie 2008, Scott 2008, Abramowitch, Feola et al.

2009, Ennen, Kloss et al. 2011, Couri, Lenis et al. 2012). Knowledge of the biomechanics of healthy and prolapsed sheep vaginal tissues will help better design the biomechanically compatible meshes, which will reduce the incidence of complications.

We belive that the vaginal scaffolds developed from the decellularization methods have a great potential as biomaterials for POP surgical intervention. Furthermore, the acellular vaginal scaffolds can be utilized for quantification of cellular components as well as ECM components. In the future, these scaffolds will be used to evaluate the cell surface attachments and biocompatibility characteristics. A comparison of suture retention strength of different types of suture materials, multifilament vs. monofilament analysis, and a combination of different surgical knots will be performed. Last, *in vivo* sheep studies will be performed to assess the clinical potential of the acellular vaginal scaffolds.

In short, sheep have been previously used as a model for uterine transplantation, preterm birth, selected pregnancy, and placentation studies. Additionally, sheep have been used for studying biocompatibility of grafts used for pelvic reconstructive surgery (De Tayrac, Alves et al. 2007, Rezapour, Novara et al. 2007, Krause and Goh 2009, Manodoro, Endo et al. 2013, Alcalay, Livneh et al. 2014, Feola, Endo et al. 2014). The sheep animal model offers several advantageous similarities with humans including similar offspring weights, gestations, vaginal length, vaginal epithelial layers, cervical diameters, etc. But there are still many knowledge gaps in sheep perlvic floor tissues and their complicated biology and biomehcanics. One major gap is the changes that occur in the pelvic floor of sheep with age are not properly documented and warrant future work. Incidences and location dependent characteristics of sheep prolapse are also not reported

yet. There is a startling opportunity for a synergistic overlap between human and sheep prolapse research, and we have initiated the basic biomechanical standard for future prolapse studies.

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