Uterine Tissue Engineering

Translational approaches using animal models

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It’s kind of fun to do the impossible

- Walt Disney

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ABSTRACT

Recent advancements, such as uterus transplantation (UTx), have led to groundbreaking solutions for complete uterine dysfunction. Yet, there are inherent risks and limitations, including challenges of sourcing compatible donor organs and potential post-transplantation complications. These limitations underscore the need for innovative approaches that can improve infertility treatments while mitigating these risks.

This thesis describes the development of bioengineering techniques for patient-specific uterine tissue segments with regenerative potential. Our approach was centered on utilizing modified uterine tissue as a scaffold to facilitate the reconstruction of defective uterine tissue. While these techniques are still experimental and require optimization and safety evaluation, our work concentrated on translating well-established scaffold production protocols from small to large animal models, including the rat, sheep, cow, and the clinically relevant non-human primate (baboon). Results showed that our established sheep decellularization protocol was feasible in the larger cow and baboon tissue, with effective removal of cellular content without excessive damage to the extracellular matrix (ECM). Furthermore, we conducted *in vivo* experiments in both the rat and the sheep model to evaluate the feasibility of our biomaterial together with mesenchymal stem cells (MSCs). It is crucial to understand the regenerative processes after transplantation, particularly the involvement of the immune response, which plays a pivotal role in the success or failure of biomaterial integration. Results from the in vivo studies highlight the pro-regenerative effect of MSCs when combined with our biomaterial in the rat model. In addition, the same effect from MSCs was not observed in the sheep model, but instead, T-cell subpopulations seemed to play an important role in the transplantation outcomes.

In conclusion, this thesis demonstrates that our scaffold generation protocols are translational and applicable with minor adjustments to different large animal models, which facilitates clinical translations and scientific conclusions. Moreover, we evaluated the feasibility of transplanting larger grafts into both a small and large animal model, where we also gained essential and novel insights into the immune response following transplantation of tissue-engineered uterine grafts. The collective knowledge gained through these efforts not only contributes to the growing field of regenerative medicine but also to the development of clinically applicable products to treat uterus-related infertility.

Keywords: tissue engineering, female infertility, translational models, rat, sheep, cow, baboon, uterus

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SAMMANFATTNING PÅ SVENSKA

Livmoderstransplantation har lett till banbrytande lösningar för kvinnor som lider av livmodersrelaterad infertilitet. Trots lovande resultat så medför denna intervention risker och begränsningar, såsom svårigheter att hitta donatororgan samt risker för potentiella komplikationer efter transplantation. Dessa begränsningar understryker behovet av nya tillvägagångsätt som kan förbättra infertilitetsbehandlingar samtidigt som dessa risker minimeras.

Denna avhandling fokuserar på att utveckla metoder för att framställa patient-specifika biomaterial med regenerativ potential, där livmodersmaterial används för att rekonstruera en defekt livmoder. Trots att dessa tekniker fortfarande är experimentella och kräver säkerhetsutvärdering, så har vårt arbete fokuserat på att översätta väletablerade protokoll från mindre djurmodeller till större modeller vilket inkluderar råtta, får, ko och icke-mänskliga primater (babian). Resultaten visade att vårt etablerade protokoll för av för avcellularisering av får var genomförbart i ko och babianvävnad, med effektiv borttagning av cell komponenter, utan att skada den extracellulära matrisen. Parallellt utförde vi *in vivo* experiment i både råtta och får för att utvärdera implementering av vårt biomaterial tillsammans med stamceller. Dessutom ville vi förstå de regenerativa processerna efter transplantation, särskilt immunförsvarets påverkan som kan spela både en positiv och negativ roll i biomaterialets integration. Resultaten av *in vivo*-studierna påvisade en pro-regenerativ effekt av stamceller i kombination med vårt biomaterial i råttmodellen, medan effekten inte observerades får modellen, där T-cells subpopulationer i stället spelade en viktig roll för transplantationens utfall.

Denna avhandling visar att våra protokoll är användbara med mindre modifikationer i större djurmodeller, vilket främjar kliniska tillämpningar och vetenskapliga slutsatser. Vi utvärderade möjligheten att transplantera större biomaterial i både små och stora djurmodeller, där vi även fick nya och viktiga insikter i immunförsvarets roll efter transplantation av livmodersmaterial. Denna avhandling bidrar inte bara till en samlad kunskap inom ämnet av regenerativ medicin, utan även till utvecklingen av kliniskt användbara produkter för behandling av livmoders-relaterad infertilitet.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Sehic E, Thorén E, Gudmundsdottir I, Oltean M, Brännström M, Hellström M. **Mesenchymal stem cells establish a pro-regenerative immune milieu after decellularized rat uterus tissue transplantation.** *Journal of Tissue Engineering*. 2022;13. doi:10.1177/20417314221118858

II. Sehic E, Miguel-Gómez L, Rabe H, Thorén E, Gudmundsdottir I, Oltean M, Brännström M, Hellström M. **Transplantation of a bioengineered tissue patch promotes uterine repair in the sheep.** *Submitted to Acta Biomaterialia*

III. Sehic E, Miguel-Gómez L, Thorén E, Sameus J, Bäckdahl H, Oltean M, Brännström M, Hellström M. **Decellularization and enzymatic preconditioning of bovine uterus for improved recellularization.** *Submitted to Translational Medicine Communications*

IV. Miguel-Gómez L, Sehic E, Rabe H, Thorén E, Ahlström J, Oltean M, Brännström M, Hellström M. **Towards uterus tissue engineering: a standardized cross-species decellularization protocol exemplified with the baboon uterus.** *In manuscript*

CONTENT

ABBREVIATIONS

3D	three-dimensional
AB	Alcian blue
ART	assisted reproductive technology
AUFI	absolute uterine factor infertility
BCA	bicinchoninic acid assay
CAM	choriollantonic membrane
DAMPs	damage association patterns
DAPI	4′,6-diamidino-2-phenylindole
ddPCR	digital droplet polymerase chain reaction
dECM	decellularized extracellular matrix
dH20	deionized water
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
ERα	estrogen receptor alpha
FACS	fluorescence-activated cell sorting
FGF2	fibroblast growth factor 2
GAGs	glycosaminoglycans
H&E	hematoxylin and eosin
HOXA10	homeobox 10A
IL	interleukin

INFγ	interferon-gamma
iPSC	induced pluripotent stem cell
IVF	in vitro fertilization
MMPs	matrix metalloproteinase
MRKH	Mayer Rokitansky-Küster-Hauser
MSCs	mesenchymal stem cells
MT	Masson trichrome
NHP	non-human primate
PBS	phosphate-buffered saline
PCOS	polycystic ovarian syndrome
PGR	Progesterone receptor
PPIH	peptidyl-prolyl cis-trans isomerase
SDC	sodium deoxycholate
SDS	sodium dodecyl sulfate
SEM	scanning electron microscope.
TNFα	tumor necrosis alpha
UTx	uterus transplantation
VEGF	vascular endothelial growth factor
VVG	Verhoef van Geison
vWF	von Willebrand factor

1 INTRODUCTION

Female infertility is a complex condition affecting many women of reproductive age and stems from factors such as hormonal imbalances and anatomical and reproductive tract complications [1]. Assisted reproductive technology (ART), together with medications and surgeries, has made significant advancements to help couples overcome infertility challenges. Nonetheless, infertility often results in a negative impact on an individual's psychological and emotional well-being, leading to stress, depression, and feelings of hopelessness and guilt [2].

Recent advancements, such as uterus transplantation (UTx), have revolutionized the field by providing a potential cure for infertile women with uterine dysfunction or for women who lack a uterus for congenital or acquired reasons [3]. Additional novel female infertility treatments, such as ovarian tissue transplantation and tissue cryopreservation, are successful methods to treat and preserve fertility, mainly in women treated for cancer [4]. Further, platelet-rich plasma [5-7] and stem cells [8-10] have been tested as a treatment for endometrial regeneration, although these treatments have not been scientifically evaluated in larger studies yet. While various promising fertility treatments continue to evolve, a promising field known as tissue engineering offers significant clinical potential for female infertility treatment. Tissue engineering seeks to create functional organs and tissues through the development of biomaterials, usually in combination with various cell types [11].

One proposed tissue engineering solution is the development of a personalized uterus graft that could address UTx limitations, including organ shortages, and eliminate the risks associated with post-transplantation immunosuppression. In this context, the focus of the presented work in this thesis was to develop a bioengineering technique to develop a uterine tissue segment/patch with regenerative capacities. This approach could potentially treat infertility caused by substantial scaring or other uterine defects caused by interventions e.g., removal of extensive myomas, placental tumors, or repeated cesarean sections [12-16]. However, many of these techniques are in the experimental stage and require extensive optimization, safety evaluation, and validation before progressing to clinical trials. As tissue engineering continues to advance, it offers hope for a future where all types of infertility caused by uterine defects can effectively be managed and treated.

1.1 INFERTILITY

Infertility is defined as the failure to achieve pregnancy after 12 months of regular unprotected sexual intercourse [1] and affects around 10-15% of couples of reproductive ages. The leading cause of infertility is usually divided into primary and secondary circumstances. The absence of a successful conception is classified as a primary infertility disorder, while secondary infertility relates to couples that have had at least one prior pregnancy achieved. This classification may then be divided further into male (30%), female (35%) combined (20%), or unexplained and other factors (15%) [17].

Male infertility is characterized by a range of factors, including sperm abnormalities and genetic factors. However, previous infections and lifestyle factors, e.g., smoking, intake of androgens, and obesity, also impact male fertility [18] (Figure 1). Different types of anatomical abnormalities on the ovaries, uterus, or Fallopian tubes may cause female infertility. Such abnormalities may include e.g., tubal blocks, uterine septum, endometriosis, or uterine fibroids (Figure 1-2). Additionally, hormonal imbalances may cause ovulatory dysfunction that also influences fertility. Polycystic ovary syndrome (PCOS) affects about 5-10% of women and is the most prevalent endocrine disorder, leading to anovulation [1, 19]. Age-related decline in ovarian function is also a significant factor in female infertility. With age, the pool of primordial follicles and the oocyte quality decline, leading to a reduced ability to become pregnant, but also to an increased risk of chromosomal abnormalities and miscarriage [20]

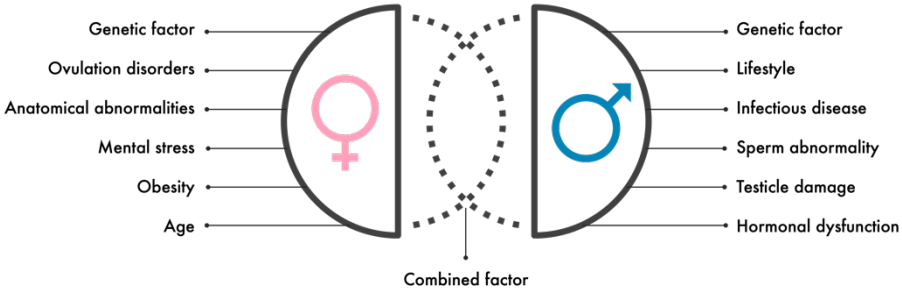

Figure 1. Common factors for female and male infertility.

1.1.1 ABSOLUTE UTERINE FACTOR INFERTILITY

Absolute uterine factor infertility (AUFI) is another form of infertility that affects about 1 in 500 women of fertile age [21]. AUFI is defined as a complete dysfunction or absence of the uterus. It may be congenital, such as in women with intrauterine malformations, or in the Mayer Rokitansky-Küster-Hauser (MRKH) syndrome, where a woman is born without a uterus. It can also be acquired following hysterectomy, adhesions, scarring from irradiation cancer therapy, or from severe myoma resections or repeated uterine incisions (Figure 2) [22]. Although many cases of female infertility can be treated with ART, surgery or hormone treatment, women without a functional uterus face a major limitation in becoming pregnant. Due to economic, legal, ethical, and religious reasons, surrogacy and adoption are not an option for many AUFI patients [23]. However, there is a glimmer of hope for these patients due to the recently developed procedure of UTx. However, this curative treatment is still considered as experimental procedure since it has not yet been fully established at centers around the world [24].

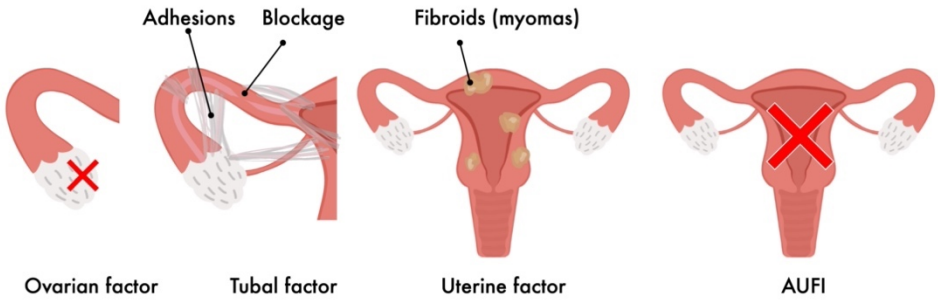

Figure 2. Illustration of common factors for female infertility

1.2 INFERTILITY TREATMENTS

Treatment options for infertility vary depending on the underlying cause and the individual circumstances. In men with issues related to sperm quality, intracytoplasmic sperm injection is a common solution [25]. For women, it can be more complex due to a range of factors. Disorders affecting ovulation, like PCOS, can often be treated with ovulation-regulated medications. In cases where ovarian stimulation alone is not enough, mature eggs can be retrieved for *in vitro*

fertilization (IVF) [1]. The fertilized egg develops into an embryo which is then transferred into the uterine cavity usually after five days [26].

Surgery is an option for women with structural abnormalities, including intrauterine adhesions, polyps, or uterine fibroids [27, 28]. Procedures like myomectomy or laparoscopic surgery can be employed to restore uterine anatomy and improve fertility outcomes [29].

In cases when a woman cannot produce any viable eggs or when the sperm quality of a man cannot be restored, donor oocytes or sperm may be considered. Fertilized donor eggs, or a woman´s own oocytes, fertilized by donor sperms, are then used in an IVF procedure with embryo transfer into the recipient's uterus [30]. While clinical interventions exist, lifestyle modifications can also positively impact fertility. Managing stress and weight, quitting smoking, and reducing alcohol intake can complement clinical treatments [31].

1.2.1 UTERUS TRANSPLANTATION

Uterus transplantation (UTx) has emerged as a viable cure to AUFI. This groundbreaking solution emerged from advancements made in organ transplantation and ART [32]. However, it is important to recognize that UTx is a complex and ethically sensitive procedure that involves significant medical and ethical considerations. The potential risks encompass a spectrum of challenges, including surgical complications for both the live donor and the recipient, immunosuppression-related issues, and the long-term health impacts for the donor, the recipient and the child(ren) born. All these aspects must be thoroughly evaluated and managed [33]. As UTx remains a relatively recent addition to fertility interventions, ongoing research and progressive refinements of the procedure will contribute to advancing its efficacy and overall outcomes.

1.2.1.1 TRANSLATIONAL PROGRESS OF UTx

The first scientific publication on UTx date back to the 1960s with combined uterine-oviduct transplantation in dogs [34]. However, early outcomes were unfavorable, mainly due to the low effectiveness of available immunosuppressive drugs at that time. Subsequent studies, including the first clinical attempt in Saudi Arabia in 2000 faced technical hurdles, as the patient developed acute vascular thrombosis about three months after transplantation resulting in a hysterectomy [35]. That was a live donor case. A deceased donor case was reported a decade later in Turkey, where the patient had a menstrual cycle after transplantation and repeated embryo transfers [36]. After vascular re-surgery, to increase venous out-

flow of blood flow, the patient finally got pregnant and delivered a baby 9 years after the procedure [37]. Although that procedure was the second UTx in the world, more than 40 live births after UTx had occurred previously, during the period from 2014 to 2020 [32]. Both the Saudi and the Turkish UTx procedures were signs of ingenuity, however, they were conducted without any significant preparation or preclinical research which might explain outcomes.

In 1999, Prof. Mats Brännström initiated a comprehensive research project on UTx (Figure 3), starting with years of rodent studies that eventually demonstrated pregnancies and live births [38, 39]. Further rodent work in the mouse led to refined transplantation methods wherein the uterus was anastomosed to the aorta and vena cava using a heterotopic approach [40]. Subsequent rat studies marked the progression with successful pregnancy outcomes through natural mating and full-term pregnancy with normally developed offspring [41, 42].

The focus then shifted to translate these achievements using larger animal models. Sheep, in particular, played a pivotal role in refining the surgical techniques [43, 44] and ultimately achieving pregnancies and live births [45, 46]. This became a milestone towards the development of a clinical UTx protocol and additional UTx studies on the sheep model were performed in the leadup for human UTx attempts [47, 48]. The model has since become the main surgical training model for surgical teams preparing for human UTx.

Non-human primate (NHP) studies were also conducted [49] prior to the first registered clinical trial on UTx, but it was a Japanese group, using the smaller NHP model of cynomolgus macaques, who presented the first live birth after UTx in a primate species, and this was after autologous UTx, without the need for immunosuppression transplantation [50]. Follow-up studies on allogenic UTx settings in baboons initially showed mild signs of rejection, where optimization of the immunosuppression regimen was suggested [51].

The major breakthrough was achieved by Prof. Brännström's team at the Sahlgrenska University Hospital in Gothenburg, Sweden, marking the first successful human UTx that resulted in a live birth in 2014 [3]. In this live donor case, a close family friend of the recipient donated her uterus. Continued efforts by the same team yielded several follow-up transplantation studies with refined operating protocols [52]. Subsequent refinements have expanded the UTx possibilities, including deceased donor cases and robotic surgery, improving outcomes, and reducing bleeding and recovery times for both the donor and recipient [32]. While ongoing protocol optimization is crucial, UTx is a

groundbreaking fertility treatment that has to date resulted in a total of 71 reported cases worldwide with a 77% surgical success rate [32], with around 50 babies born around the world, as reported on PubMed and other public media [24]. This journey highlights the importance of maintaining a scientific and translational approach using several animal models prior to human studies to achieve clinical success.

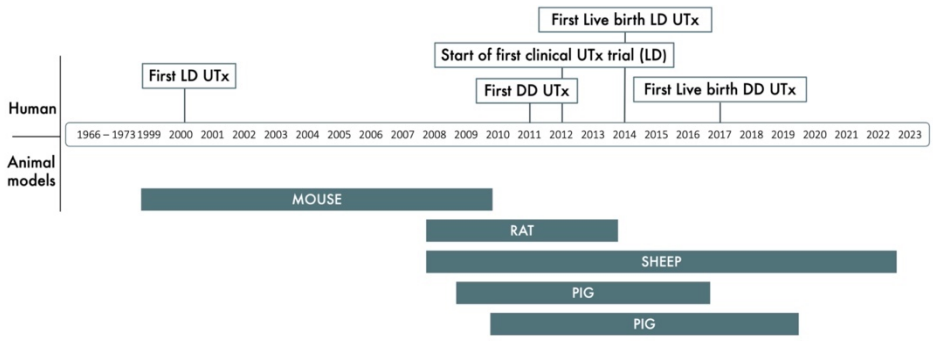

Figure 3. Figure modified and used with permission from the publisher and the authors [32]. A timeline of animal models used for UTx trials and human uterus transplantation accomplishments. LD; live donor, DD; deceased donor

1.2.1.2 FUTURE OF UTX AND TISSUE ENGINEERING

The future prospect of UTx presents exciting potential, but there are also some significant challenges to consider. Successful UTx requires a multidisciplinary team of experts, including gyne-oncology surgeons, transplant surgeons, reproductive specialists, immunologists, and psychologists, to ensure the safety and efficacy of the transplantation process and the ultimate health of the patient. Furthermore, ethical considerations related to donor selection, organ availability, and long-term implications continue to be subjects of active discussion and research [53]. Ongoing advancements in surgical techniques, immunosuppression strategies, and post-transplant care are essential for optimizing outcomes and expanding the availability of this life-changing procedure. However, UTx includes risky live-donor surgeries and immunosuppression-related negative side effects that raise several concerns [54] (Figure 4 A). Another issue with UTx is finding a matching donor organ for the numerous patients waiting for a UTx [53].

Therefore, the development of an alternative organ donor source would greatly benefit the field. A proposed solution lies in the creation of a patient-specific bioengineered uterus [12] (Figure 4 B). Such organ construct would eliminate UTx transplantation limitations, including donor organ scarcity, rejection risks, and immunosuppression-related negative side-effects, a major concern following organ transplantation procedures [55] [56].

While early in development, the construction of a bioengineered uterus could revolutionize treatments for uterine factor infertility. Initial rodent studies have provided proof-of-concept with restored fertility following the transplantation of small uterine tissue segments [13-15, 57, 58]. However, further studies addressing the functionality, physiological similarity, and long-term safety of such bioengineered constructs remain crucial. These research goals are important and provide the basis for the construction of larger and more relevant-sized grafts that may be used clinically for partial uterus repair, ultimately leading up to the construction of a whole bioengineered uterus suitable to replace a donor in a UTx setting. However, the uterus is structurally a complex organ that requires a composition of many cell types, a functional vascular network, and hormonal regulation. Due to the complexity of constructing a whole bioengineered uterus [56], which is outside the scope of this thesis, the preclinical work described herein is focused on generating bioengineered uterus segments for both small and large animal models that can be used to repair a damaged or dysfunctional uterine tissue (Figure 4 C).

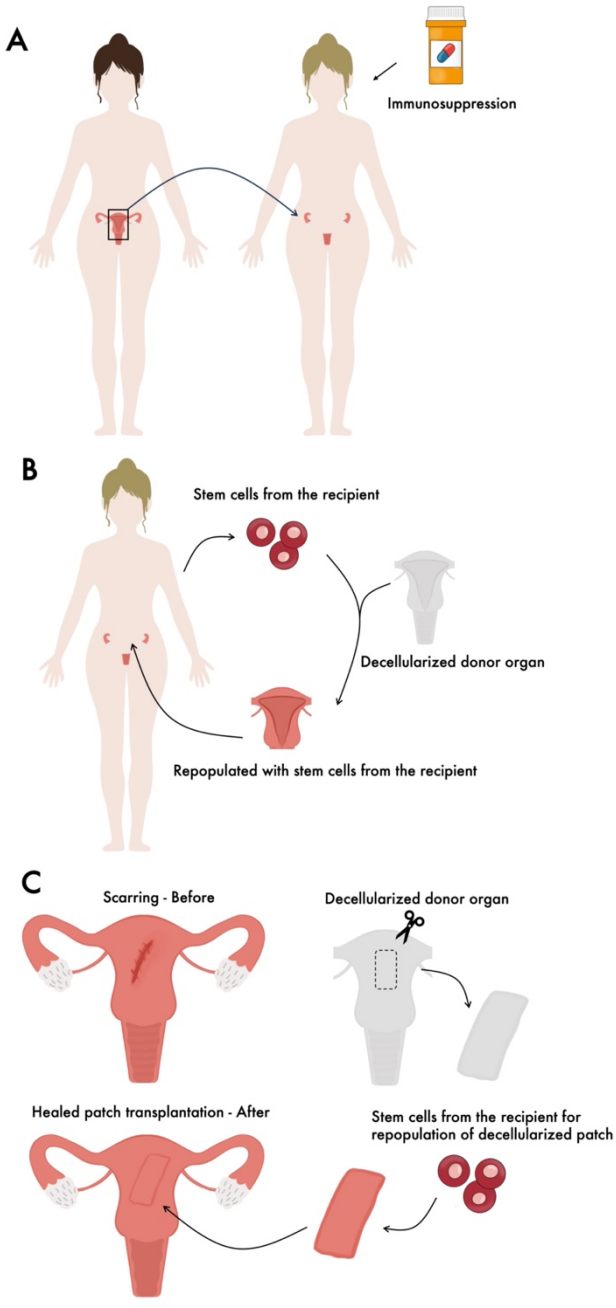

Figure 4. Schematic illustration of (A) UTx, (B) whole organ decellularization and recellularization followed by UTx and (C) partial patch repair of uterine defects

1.3 UTERINE ANATOMY AND PHYSIOLOGY

A comprehensive understanding of uterus anatomy is essential to successfully tissue engineer uterine tissue. As the uterus is an organ that supports vital functions such as embryo implantation, gestation, and labor, a profound understanding of uterine functions and uterine morphology is fundamental to creating tissue that replicates native tissue behavior and performance.

1.3.1 THE HUMAN UTERUS

The human uterus is a hollow, single, pear-shaped organ located within the pelvic cavity between the urinary bladder and the rectum, and it consists of several distinct anatomical parts (Figure 5). The fundus is the uppermost region of the uterus, connecting the Fallopian tubes, which are further connected to the ovaries. The corpus (body) constitutes the core and central part of the uterus, extending towards the isthmus, which is the lower and tapered portion. Following the isthmus, the cervix emerges, culminating in its connection to the vagina [59]. The uterine corpus, however, is the main structure targeted for tissue engineering for treating female infertility due to its central role in nurturing and supporting a developing embryo and fetal growth during pregnancy. The corpus has several layers, each with distinct functions, until it reaches the uterine cavity. The primary layers of the uterus corpus include the endometrium, myometrium, and perimetrium.

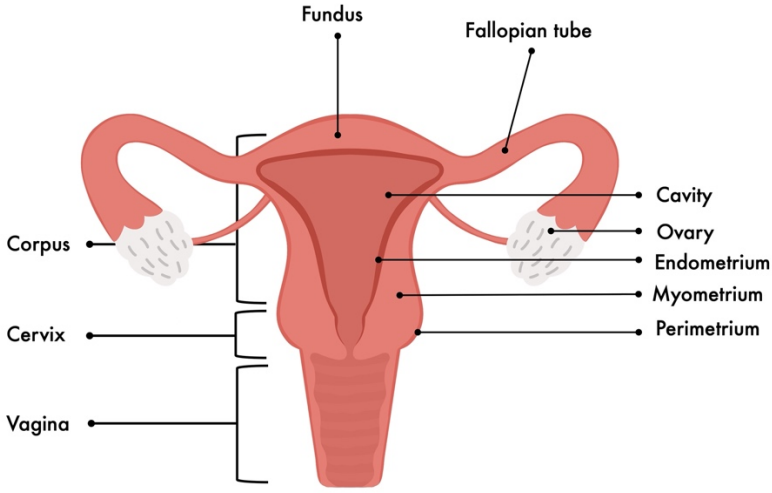

Figure 5. The human uterus anatomy

1.3.1.1 ENDOMETRIUM

The human endometrium is a dynamic layer capable of remodeling that undergoes over 400 cycles of regeneration throughout a woman's reproductive years [60]. It experiences cyclic changes in response to the hormones estrogens and progesterone secreted by the ovaries throughout the menstrual cycle [61]. The endometrium can be divided into two main layers (Figure 6). The stratum functionalis is the functional layer that undergoes changes during the ~28-day menstrual cycle. The layer thickens under the influence of estrogens to prepare for possible embryo implantation prior to ovulation. Around two weeks ovulation occurs, and in the absence of a pregnancy/placentation, progesterone levels have shown a decline because of corpus luteum demise, and the functionalis is shed (menstruation).

The second endometrial layer, the stratum basalis, remains relatively constant throughout this period and gives rise to the new functionalis under the influence of a new estrogen-dominant cycle after menstruation [62]. Hence, the basalis serves as the replenishing layer and harbors adult uterine stem/progenitor cells, believed to play a role in the cyclic regeneration of the functionalis layer [63-65]. The endometrium also consists of two epithelial cell populations, one that lines the outermost part of the uterine cavity known as the luminal epithelium (LE). The LE acts as the attachment site during the embryo implantation phase (Somasundaram, 2016), and it also serves as a protective barrier toward the uterine cavity against pathogens and infections [66].

The second epithelial type, known as glandular epithelium (GE) constitutes the endometrial glands located in the functionalis layer and plays an important role in the secretion of substances during the secretory phase supporting and facilitating a potential embryo. Such substances are composed of growth factors, cytokines, hormones, and glycoproteins [67]. The glands respond to the variety of hormones secreted by the ovaries throughout the menstrual cycle which impacts the secretory function of the uterine glands [68]. An additional cell type found within the endometrium includes fibroblasts, which, together with the extracellular matrix (ECM), provide structural support and are also known as the endometrial stromal compartment. [62, 69, 70]. Immune cells such as macrophages, uterine natural killer cells, and T and B-cells are also found within the endometrium and play a role in the immune response and, for instance, protection against foreign particles [71].

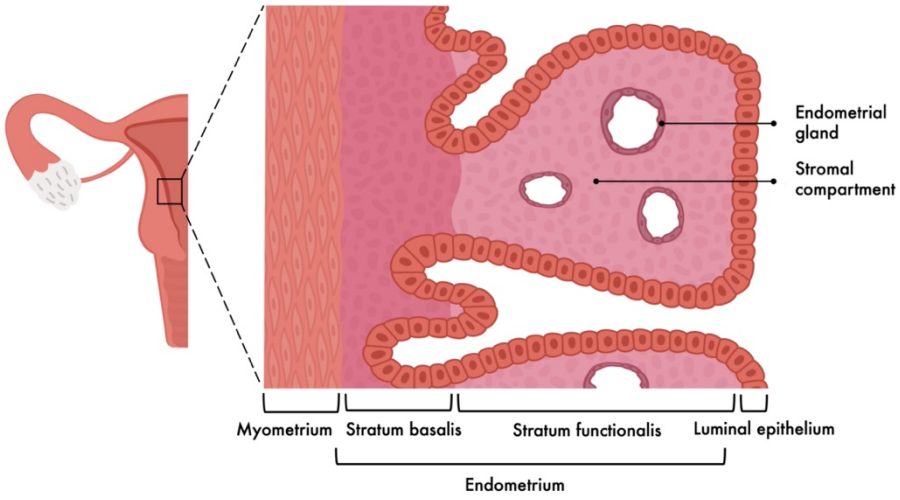

Figure 6. The endometrium and the endometrial layers

1.3.1.2 MYOMETRIUM

The myometrium is the thick muscle layer of the uterine corpus and is primarily composed of smooth muscle cells, also known as myocytes. The primary function of the myometrium is to be the structural barrier that protects the pregnancy and to contract and relax to enable labor and expel menstrual blood during menstruation. The myocytes contract and relax in response to electrical activity, characterized by depolarization (increased permeability of Ca+). Further, hormones, such as oxytocin and locally produced factors, such as prostaglandins influence the calcium release and contractions [72, 73]. The myometrium is organized into three layers of muscle fibers arranged in longitudinal (outer layer), mesh-like (middle layer) and circular (inner layer) directions (Figure 7). The middle intermediate layer contains a vascular zone that supplies oxygen and nutrients to the muscle cells [73, 74]. Further, the myometrium can expand to accommodate the growth of the fetus, which is contributed by both an increase in the number of cells (hyperplasia) and an increase in cell size (hypertrophy) [75].

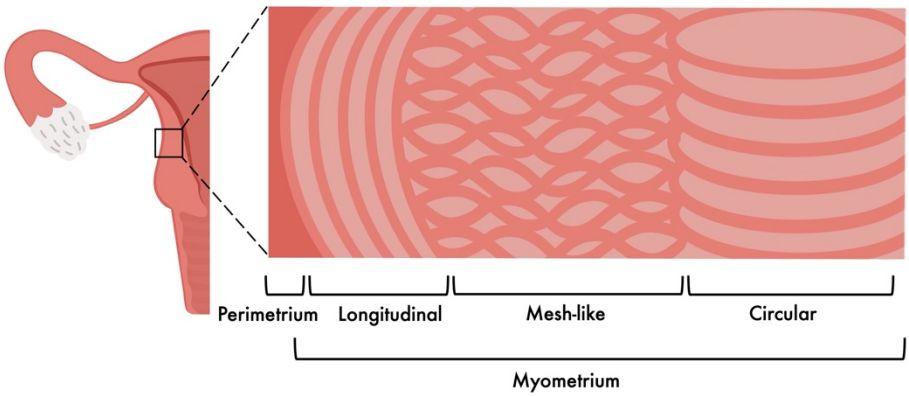

Figure 7. The myometrium and the muscle fiber layers

1.3.1.3 PERIMETRIUM

The perimetrium is the outermost layer of the uterus, and its main function is to provide structural integrity and support. It helps to maintain the overall shape and position of the uterus within the pelvic cavity, but it also acts as a protective layer that covers the uterus and separates it from surrounding structures. Moreover, the perimetrium attaches ligaments that hold the uterus in place within the pelvic cavity. The perimetrium mainly consists of mesothelium and connective tissue [76].

1.3.2 COMPARATIVE UTERINE ANATOMY TO ANIMAL MODELS

A robust understanding of the differences between animal models and humans is essential for ensuring that translational research is effective. Species-specific variations (Figure 8), such as cycle length, hormonal regulation, and mechanisms of pregnancy, must be considered and understood to ensure that strategies with the highest potential for clinical impact are investigated. This approach minimizes risks and maximizes the chances of successful outcomes when therapies are eventually applied to patients. As the UTx project achieved success through a comprehensive translational approach, it has set a valuable example and may serve as a future guide into how uterine tissue engineering protocols may be developed.

1.3.2.1 RAT

The rat uterus differs significantly from the human uterus. Rats have a bicornuate (two-horned) uterine shape and can handle the implantation of multiple embryos in each horn. Despite the anatomical difference in shape and size, humans and rats share common elements, including the endometrial layer, myometrium, and the perimetrium. However, differences emerge upon closer inspection. Rats exhibit a more distinct myometrial composition, characterized by well-defined longitudinal and circular muscle layers.

In contrast, the human myometrium presents as a less discrete and more interwoven muscle fiber network. These structural differences can play a role in distinct contraction profiles during labor [77]. The endometrial layer, however, is similar, featuring a luminal epithelium covering the endometrial surface and glandular epithelium forming tubular glands. However, distinctions lie in the shedding. Unlike humans, rodents do not experience menstruation. Instead, rats undergo the resorption of the endometrial functionalis layer at the end of each estrous cycle [78]. Rats have an estrous cycle lasting four or five days, and sexual maturity occurs at around six to ten weeks [79, 80].

The gestation length in rats varies from 22-23 days [80, 81]. Unlike humans, who typically give birth to one baby or occasionally twins, rats have more prolific reproductive capabilities. Rats may have between ten to twelve pups per litter [81]. These anatomical, cycle, and reproductive distinctions highlight the differences between rodents and humans. Nonetheless, rats are valuable animal models in reproductive research due to their cost-effectiveness, manageable size, short reproductive cycles, and fewer ethical concerns, which benefit reproductive studies and enable cost, and time-effective research outcomes. Furthermore, rats offer similarities in organ structure, immune responses, and physiological processes. Still, their remarkable regeneration capacity makes direct extrapolation to humans challenging [82].

1.3.2.2 SHEEP AND COW

Various large animal models, including sheep, pigs, horses, and cows, have been used to study gynecological diseases and infertility. Among these models, the sheep stands out as particularly advantageous due to its close resemblance to humans in anatomical size and physiological characteristics [83]. While pigs exhibit a large uterus size with elongated uterine horns like rodents, the cow and sheep uteri possess bicornuate shapes but with similarities to the human uterus in terms of structure and vasculature, despite their bicornuate shape. The sheep,

however, display a uterus size similar to the human, compared to the much larger cow uterus.

Notably, unlike humans, sheep, cows, and other ruminants display an endometrium which features a distinct anatomical region known as the caruncle. This is a non-glandular endometrial surface where embryo implantation specifically occurs. The caruncles consist of dense stroma covered by a simple luminal epithelium, while inter-caruncular areas contain a high number of uterine glands. [84-86]. The myometrium of the sheep uterus features an inner circular muscle layer and an outer longitudinal layer with rich vasculature separating the muscle layers [87]. The cow has an 18-24 day long estrus cycle [88], similar to the sheep, which is slightly shorter of 16-17 days. In contrast to the cow, the sheep is characterized by season-dependent ovarian cycles. However, this can be dependent on the breed and living climate. In general, sheep are characterized by having ovulatory cycles occurring during the fall and winter, which is known as the breeding season [89]. Sheep placentas share structural traits with human placentas, with a gestational period of approximately five months [83] compared with nine months in cows [90] and humans. Notably, the sheep and cow pregnancies are typically singleton or duplex, yielding offspring numbers comparable to humans.

Large animal models offer the advantage of representing the challenges encountered in human surgeries, offering insights into the viability and safety of regenerative techniques in a context that aligns more closely with clinical applications. Using sheep as an animal model presents the added advantage that, if organs or tissues are collected from slaughterhouses and from animals that were bred for food production, research may be conducted without the need for ethical review or purpose breeding of study animals. However, it is essential to consider that large animal models, like the sheep, require stricter ethical review, more resources, and specialized expertise. Thus, thoughtful considerations are necessary when utilizing sheep as a large animal model for research purposes.

1.3.2.3 NON-HUMAN PRIMATES

Non-human primates comprise a set of mammalian species, including monkeys and apes, that exhibit shared anatomical and physiological features with humans while maintaining their distinct identities. NHPs are usually used as the last step in translational research to ensure that scientific discoveries are thoroughly tested and validated before they reach clinical trials in humans [91]. Due to their remarkable anatomical resemblance to humans, the NHPs and particularly the

baboon, have emerged as a valuable model in reproductive research. Their uterine size and vasculature closely approximate that of the human [92]. The uterine shape of NHPs is generally simplex in larger species, whereas the shape in smaller NHP is usually duplex and consists of two uterine horns. However, variations exist among NHP species.

Non-human primates share similarities with humans in the physiological process of menstruation, with menstrual cycles having durations approximately 25 to 35 days, unlike many other mammals. Also, the mating activity is not restricted to a specific stage of the estrus cycle, as observed in many other mammalian species, and as in humans, it is more related to the social context. Furthermore, the reproductive hormones and ovulation mechanisms are also shared characteristics with humans [93]. The gestation times for NHPs vary from approximately five to eight months, depending on species [94]. The endometrium in NHPs closely resembles that of humans, featuring a functionalis layer with a single epithelial lining and a basalis layer containing progenitor and stem cells. Like humans, NHPs possess glandular epithelium within the endometrium [95]. Surrounding the endometrium is the myometrium, consisting of an inner circular later, a middle layer with smooth muscle fibers arranged randomly, and an outer longitudinal layer. A vascular network is present between the muscle layers, comparable to the human myometrial architecture [96].

Despite the numerous shared characteristics between the NHPs and the human uterus, significant challenges are associated with using these animals in research. Ethical considerations must be thoroughly evaluated and justified, as NHPs have a significant cognitive capacity and complex social behavior. The research conducted in NHPs must be morally justified, and today, many laws and regulations restrict the use of NHPs as research animals [91]. Additionally, specialized training, housing, and maintenance of these animals are required, which are not typically available at standard animal facilities.

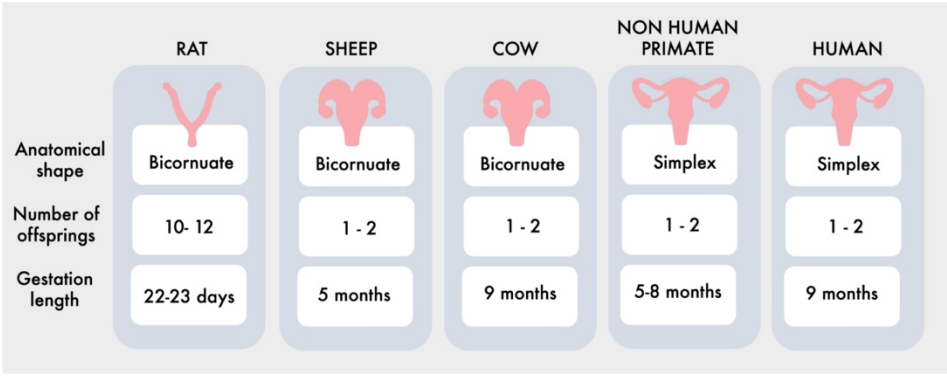

Figure 8. Diagramed illustration of differences between rat, sheep, cow, non-human primate, and human uterus in terms of size, number of offspring, and gestation length

1.4 TISSUE ENGINEERING

The term tissue engineering was introduced in the late 1980s [97] and represents a growing field that involves innovative techniques with the goal of cultivating tissues and organs and use them to restore, maintain, or improve tissue/organ function [98]. The general strategies involve using a scaffolding structure (building frame) in combination with different types of cells and growth factors that together form the tissue-engineered material [99]. In recent decades, tissue engineering and regenerative medicine have experienced significant advancements, offering a promising array of therapeutic products for addressing various medical conditions characterized by organ and tissue damage. Predominantly, products from musculoskeletal tissues like bone and ligaments have been clinically used, but also skin and heart valves were developed and successfully transplanted into human recipients [100]. However, the clinical translation has mainly been limited to simple, smaller, or thin structures, as the development of thicker tissues and more complex organs is limited by cell sourcing, culturing, vascularization, and hypoxia [101]. Despite the existing hurdles, the pace of progress in this field remains rapid. The knowledge gained from engineering simpler tissue structures contributes to the understanding of how more complex organs may be constructed. The successful achievement of this goal would indeed transform the landscape of modern medicine with increased availability of organs and tissues for transplantation.

1.4.1 SCAFFOLDS

The structural framework used in tissue engineering, often called “scaffold”, ideally aims to mimic the ECM. The ECM is the natural scaffolding for tissues and organs in the body and is composed of structural proteins such as collagen, and elastin and non-structural proteins such as fibronectin and laminin. Further components such as growth factors and integrins are fundamental components of the ECM [102] (Figure 9). Collagen is the most abundant protein that provides tensile strength (the force a material can resist when being stretched) and is usually crosslinked with elastin, another ECM protein, that confers elasticity in the tissue [103]. Fibronectin and laminin are found in greater abundance in the basement membrane and are involved in cell adhesion and migration mechanisms [11, 102]. Glycosaminoglycans (GAGs) are negatively charged unbranched polysaccharides that contribute to critical physiological functions by harboring growth factors and cytokines that regulate cellular growth, differentiation, and tissue homeostasis [104]. Hence, contradicting to what was long believed, that the ECM only had the function of providing structural support, it is now understood that it is also involved in the modulation and regeneration of tissues [105]. For tissue engineering applications, the scaffolds should ideally mimic nature’s own blueprint and possess a three-dimensional structure with the same attributes. Furthermore, these scaffolds should exhibit minimal immunogenicity, good biocompatibility, and appropriate biomechanical characteristics to be tolerated by the recipient following transplantation. Today, various methods are employed to acquire scaffolds from biological or synthetic sources, or a combination of these.

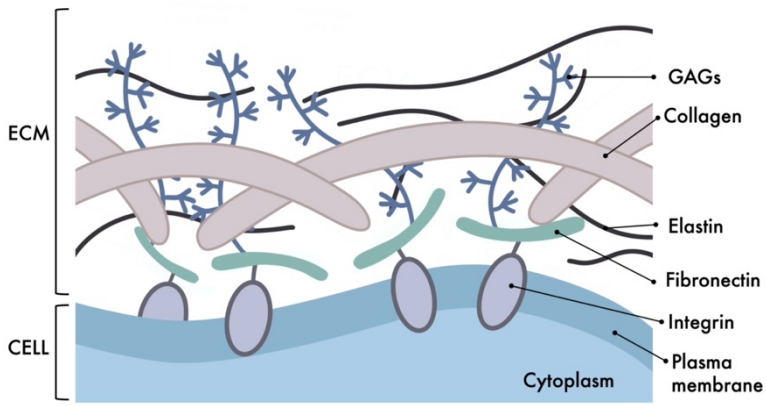

Figure 9. The extracellular matrix (ECM) and its components

1.4.1.1 BIOLOGICAL SCAFFOLDS

Biological and natural scaffolds are often derived from allogenic or xenogeneic ECM, often produced through a process known as decellularization. This procedure involves the removal of cellular components from a donor source tissue while preserving the ECM [106]. The benefits of using a biological scaffold in tissue engineering applications are that the scaffold closely resembles the native ECM, is biocompatible and that the host cells can remodel as required after engraftment. Furthermore, biological scaffolds can retain bioactive molecules (e.g., growth factors) from the source tissue, promoting tissue regeneration and guiding cell behavior [106, 107]. However, this type of scaffold has some limitations, including production batch variability, large-scale production challenges, and unclear quality control requirements for developing clinically approved scaffolds.

1.4.1.2 SYNTHETIC SCAFFOLDS

Synthetic scaffolds have become popular since they can originate from diverse sources. However, the common characteristic shared among them is that the synthetic materials used often mimic the structural composition of the ECM. Common materials used for crafting synthetic scaffolds encompass a range of polymers [108], including e.g., polyethylene glycol (PEG) [109], poly-L-lactic acid (PLA) [110], poly-lactic-co-glycolic acid (PLGA) [57], Polycaprolactone (PLC) [111]. These materials may be shaped into the desired physical appearance, but synthetic polymers may also be used to formulate viscous-like scaffolds, i.e., hydrogels. A hydrogel is a three-dimensional (3D) network with the capacity to absorb large amounts of water and can be chemically or physically cross-linked to resemble structural attributes of ECM, which makes it suitable for the proliferation and growth of cells [112-114]. Synthetic scaffolds offer the advantage of allowing precise design and modification to meet the unique requirements of different tissues and organs. This includes the ability to control parameters such as porosity, mechanical strength, and degradation rate. In contrast to biological scaffolds, synthetic materials exhibit greater consistency between batches and can be manufactured with a high degree of accuracy, which facilitates scalability, mass production and compliance to regulatory authorities [105]. However, synthetic scaffolds have certain drawbacks, such as limited inherent bioactivity and the need for additional adjustments to encourage cell attachment and tissue regeneration. Moreover, they may not fully replicate the complexity and biochemical composition of the natural ECM [56]. The choice between synthetic and biological scaffolds depends on the objectives of the target

tissue and organs. The research presented in this thesis focuses on the use of biological scaffolds derived from decellularized tissues.

1.4.2 DECELLULARIZATION

The primary objective of the decellularization is to eliminate cellular components and donor-specific elements, which reduces the risk of an immune response upon allogenic or xenogeneic transplantation [115]. The decellularization process varies depending on the target tissue, including characteristics such as thickness and morphology. Therefore, it is important to optimize the most suitable method for the target tissue [116]. For this purpose, chemical/enzymatic and physical approaches are common methods [117] (Figure 10). Systems for decellularization using chemicals and enzymes can usually be separated into two distinct methods: perfusion, which is usually used for whole organ decellularization, where decellularization reagents are perfused through the organ vasculature. Second, immersion or agitation, where organs or tissue fragments are submerged in the decellularization reagents [118]. Physical approaches such as temperature changes using freeze-thawing, or high hydrostatic pressure lyse cells inside the tissues. Commonly, various methods can be combined to effectively remove cells as various tissues have several layers with different characteristics [115]

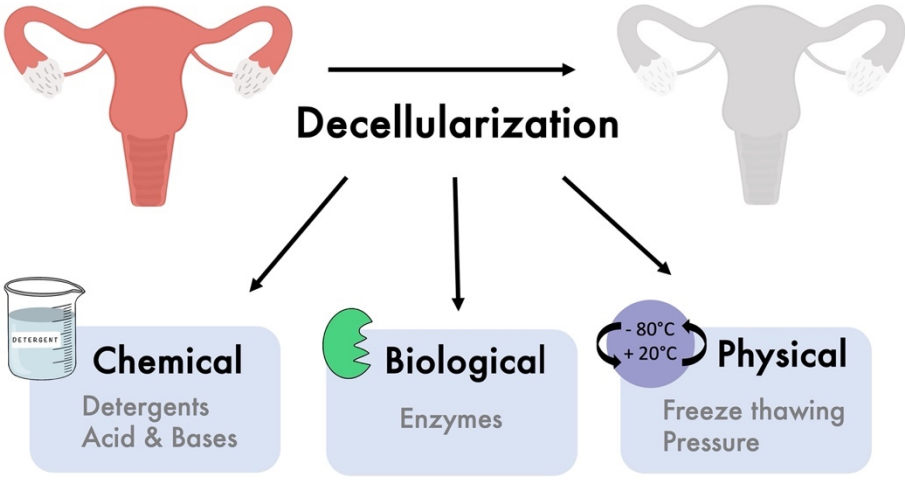

Figure 10 – Methods for decellularization

1.4.2.1 CHEMICAL AND ENZYMATIC DECELLULARIZATION

Various chemicals have been evaluated for the decellularization process. Among a broad range of substances, detergents are the most used decellularization agents. They main function by disrupting the cell membrane and protein bonds and can be classified based on their charge [116]. Ionic detergents such as sodium dodecyl sulfate (SDS) and sodium deoxycholate (SDC) are effective as they effectively break and solubilize protein bonds between the cells and the ECM. Non-ionic detergents such as Triton X-100 are milder than ionic detergents and are sometimes used as a rinsing agent to remove remnants of SDS. However, Triton X-100 it is often used as a decellularization agent, but it requires longer exposure time as it acts on breaking the protein and lipid bonds. The zwitterionic agents have both ionic and non-ionic properties and maintain the structural integrity of the ECM well [119-121]. However, remnants of cytoplasmic compartments are often found, which can be detrimental to the immunogenicity of the scaffold when transplanted [122, 123]. It is important to note that most surfactant-treated tissues typically require thorough rinsing with solutions like phosphate-buffered saline (PBS) and water to remove cell component residuals and remaining surfactants. Additionally, enzymes can be used as part of a decellularization protocol, either alone or in combination with surfactants. Applied enzymes usually target residual nucleic acids or peptide bonds that are common in the ECM cell attachment [115]. The choice of decellularization agents depends on the target tissue and the intended use of the ECM. Different tissues have distinct compositions, requiring a varying level of chemicals. Hence, its crucial to explore various combinations of decellularization agents and optimize protocols to achieve high removal of DNA components while minimizing the ECM damage. Additionally, when considering adopting and translating the protocols for use in other animal models, evaluating the protocol's effectiveness is important, as outcomes may vary depending on the species [13, 124].

1.4.3 RECELLULARIZATION

Recellularization represents a fundamental concept in tissue engineering. It involves the strategic introduction of specific cell types so that the scaffolds become repopulated, and ultimately, provide tissue/organ functionality. Each organ possesses a unique composition and function that need to be considered during the tissue engineering process. The cells used need to meet the organ-specific cellular characteristics and be robust enough to withstand a prolonged cell culture and proliferation process. For large bioengineered constructs or for whole organs, it will also be important to reconstruct the vascular structures, as the provision of oxygen and nutrients facilitated by these vascular networks is

crucial for tissue survival and functionality [115, 125]. In addition to the choice of appropriate cellular sources, the methods employed for cell delivery are equally important. Various strategies are available, including methods such as cell injection [13, 107, 126] or seeding onto the scaffold material [127]. Additionally, dynamic recellularization techniques, which involve the application of physical forces like agitation [128] or perfusion [129, 130] are frequently employed to enhance cell integration and distribution within the scaffold [131]. Further techniques, such as coating of scaffolds to enhance the recellularization have been employed. However, the recellularization process remains a major challenge in tissue engineering as many scaffold types are problematic to effectively populate with cells. Yet, recellularization serves as an important step in tissue engineering, enabling the revival of biological functionality in artificial tissues and organs. The careful selection of cell sources with the application method is central to a successful tissue engineering application.

1.4.3.1 CELL SOURCES FOR RECELLULARIZATION

The choice of cellular source is a fundamental decision that can profoundly influence the success of the tissue or organ reconstruction. Among the options available, mesenchymal stem cells (MSCs) and tissue-specific cells emerge as the two primary choices. Furthermore, the cell donor source needs to be considered, e.g., if autologous cells should be used (patient derived cells). One of the fundamental objectives to use bioengineered construct is to make it personalized to eliminate the need for immunosuppressive drugs. However, for most organs, autologous cells cannot be harvested and cultured easily, and therefore, bone marrow-derived stem cells, or other types of stem cells, such as fetal or embryonic stem cells are used [107, 132, 133]. Umbilical cord blood derived MSCs are not only easy to collect, but it has also been suggested that they have a low risk of an allogenic immune response [134]. Further, induced pluripotent stem cells (iPSCs) are another promising stem cell type that could facilitate the use of patient-derived cells, as they can regain the capacity to differentiate into a desired cell type [125, 135].

Additionally, if the concept is to construct a complete whole organ that is missing, e.g., a bioengineered uterus for MRKH, the cell source must be derived from somewhere else. Unlike autologous cells, allogenic cells are often harvested in larger quantities from young, healthy donors [131]. Nonetheless, one disadvantage of using allogenic cells includes the risk of a potential immune reaction towards them after engraftment. However, one can also use an approach where the primary goal of the cell source is to stimulate and support the body's natural regenerative process. In that case, MSCs are favored due to their

therapeutic and immunomodulatory properties. It is suggested that they exert their immunomodulatory actions through cell-to-cell interaction with immune cells [136, 137] and through paracrine activity by secreting diverse factors such as cytokines, chemokines, and growth factors [138-140] that could potentially skew the immune response towards a pro-regenerative state [141]. However, the eventual choice or strategy depends on the aim and function of the tissue-engineered construct, and the use of cells requires careful evaluation of immunogenic considerations and clinical aspirations.

1.4.4IMMUNE RESPONSE

The immunological evaluation of transplanted biomaterials is critical in both research and clinical applications since it can predict graft degradation or successful tissue regeneration [142]. Hence, understanding how the immune system responds after engraftment of the foreign material is critical for developing successful tissue engineering protocols. Even if decellularized materials were traditionally considered immune-privileged due to the close genetic homology of ECM molecules between species, recent findings have shown that ECM fragments within the scaffold post-decellularization can act as immunoreactive damage associated molecular patterns (DAMPs). The immunoreactivity of a scaffold is further potentiated by aggressive decellularization agents [122, 123, 142]. Surprisingly, a limited number of studies have investigated the immune response following decellularized tissue transplantation, and this complex issue remains largely unexplored. However, it is known that the response follows a typical pattern (Figure 11). Initially, the innate immunity responds within hours to tissue injury by recruiting inflammatory neutrophils to the injury site [143]. They signal to facilitate material degradation by secretion of cytokines and growth factors, which recruit macrophages and monocytes which phagocytose apoptotic neutrophils and other dying cells in the tissue [144]. Macrophages are important throughout the tissue healing process and play a central role in re-establishing the tissue balance [145]. They achieve this by removing cellular debris, reshaping the ECM, and synthesizing a multitude of cytokines and growth factors [143, 146]. Cytokines then modulate the outcomes of macrophage differentiation, and they can become pro-inflammatory (M1) or anti-inflammatory (M2) macrophages. The interplay between these two phenotypes can either promote fibrosis or facilitate tissue repair [147, 148].

The innate response is followed by actions by the adaptive immune system, which is initiated between a few days and one to two weeks after engraftment [149]. The

major players in the adaptive immune response are the different subgroups of T-cells, such as CD4+, CD8+, and regulatory T-cells, which exhibit both anti- and pro-regenerative properties, respectively. They possess the capability to release a diverse array of cytokines and growth factors, which can exert either beneficial or inhibitory effects on tissue healing [150]. An array of additional immune cells and cytokines also play crucial roles in tissue regeneration, including B-cells, dendritic cells, mast cells, eosinophils, interferon-gamma (IFNγ), tumor necrosis factor-alpha (TNFα), interleukin (IL) -4, IL-10, IL-13 [143, 151]. Furthermore, the immune response may be subject to modulation by the cells used in the recellularization phase of decellularized tissue. For example, it has been known that MSCs have immunomodulatory effects and paracrine functions, rendering them an attractive choice to be combined with decellularized tissue for immune response modulation and improved regenerative outcomes.

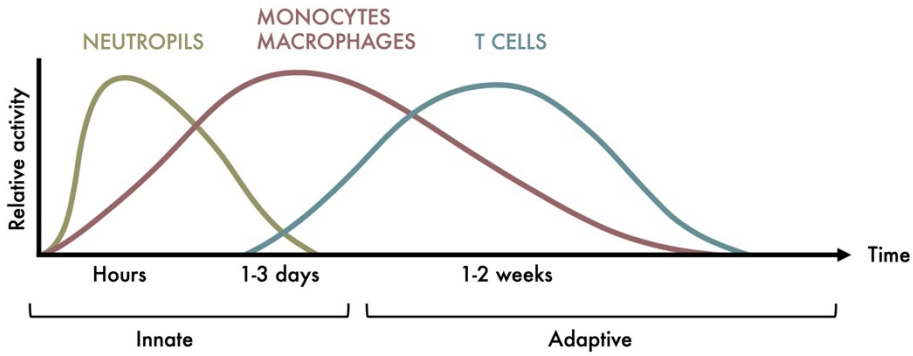

Figure 11. The immune response over time following tissue injury

1.4.5 PROGRESS IN UTERINE TISSUE ENGINEERING

In the pursuit of bioengineering uterus tissue for infertility treatment, extensive research spanning over a decade has been performed that investigated the development of uterine scaffolds with additional testing in various animal models, predominantly in the rat model [12]. In 2014, three separate research groups, including our own, collectively published a total of seven decellularization protocols for scaffold production. These protocols effectively decellularized uterine tissue, employing detergents like SDS, Triton X-100, and Dimethyl sulfoxide (DMSO) or by controlling the hydrostatic pressure [14, 16, 152]. Since then, several protocols have undergone continuous refinement,

involving numerous *in vivo* assessments using the rat model. A few studies have developed decellularization protocols for larger animals than rodents, including for the rabbit [153, 154], pig [129] and sheep [124, 155], and the human uterus [156]. Additionally, the decellularized materials have been rigorously tested and evaluated with and without the addition of cells, exploring various aspects of their performance. Various recellularization techniques such as injection [13, 124], perfusion [14, 15, 130] and seeding on top of the scaffold [129] have been tested using mainly MSCs, endometrial and epithelial cells. Further, several transplantation studies have been performed in rodents, where the scaffolds have been evaluated *in vivo* with variations in the post-transplant follow-up duration ranging from seven weeks to four months [13-16, 58, 129, 141, 153, 157]. A great number of these studies have also evaluated pregnancy outcomes with reported live births after transplantation of tissue engineered uterine tissue (Table 1) [13-15, 58, 157]. Despite these promising transplantation results, including live birth, more detailed evaluations of the scaffold functionality are needed, such as confirmation of implantation sites at the scaffold area. Nevertheless, it is crucial to bear in mind that these studies typically have employed small-sized grafts that may not faithfully mimic the clinical context in humans. Consequently, *in vivo* studies were clinically relevant sized grafts, and large animal models that have a uterus of similar size to humans become essential to validate the clinical significance and efficiency of these promising bioengineering techniques.

Table 1. Table modified and used with permission from the publisher and the authors [12] Summary of studies using whole organ decellularization of the uterus. DC; Decellularization, RC; Recellularization

UTERUS WHOLE ORGAN DECELLULARIZATION						
Species [reference]	DC	RC	Cell type	Graft size	Transplantation	Pregnancy
Mouse [58]	SDS	N/A	N/A	5 x 2 mm	7 weeks	Yes
Rat [152]	Triton-X100 + DMSO or SDC	N/A	N/A	10 x 15 mm	Not tested	N/A
Rat [16]	Triton-X/SDS or high hydrostatic pressure	N/A	N/A	15 x 5 mm	51 days	Yes
Rat [14]	SDS	Perfusion	Rat neonatal, endometrial cells, MSCs	15 x 5 mm	90 days	Yes
Rat [13]	Triton-X100+DMSO or SDC	Injection	Rat GFP labelled MSCs	10 x 5 mm	9 weeks	Yes
Rat [157]	SDS	N/A	N/A	15 x 5 mm	11 weeks	Yes
Rat [15]	SDS	Perfusion	GFP labelled MSCs	10 x 5 mm	90 days	Yes

Rat [122, 123]	Triton-X100 + DMSO or SDC	N/A	N/A	10 x 5 mm	3 months subcutaneous	N/A
Rat [141]	Triton-X100+DMSO	Injection	GFP labelled MSCs	20 x 10 mm	4 months	N/A
Rat [130]	High hydrostatic pressure	Static culture	Rat endometrial stromal cells	N/A	N/A	N/A
Rabbit [153]	Triton-X100 + SDS	N/A	N/A	5 x 5 mm	90 days, xenograft (rat)	N/A
Rabbit [158]	Triton-X100 + SDS	N/A	N/A	N/A	N/A	N/A
Pig [154]	Triton-X100 + SDS	Seeded on scaffold	Stromal, epithelial cells	5 mm biopsy punch	N/A	N/A
Sheep [155]	Triton-X100+ DMSO or SDS, SDS+Formalin	N/A	N/A	10 x 5 mm	10 days, xenograft (rat)	N/A
Sheep [107, 124]	SDC, SDS, Triton-X100+SDC	Injection	Sheep fetal MSCs	0.3 - 0.5 mm rings / 5 mm biopsy punch	N/A	N/A
Human [156]	SDS	N/A	N/A	10 x 5 mm	10 days, xenograft (rat)	N/A

2 AIM

This thesis project aimed to form a basis towards further development of novel infertility treatments using uterus tissue engineering strategies in a preclinical setting. The thesis project particularly aimed to investigate the transplantation outcomes using large grafts in the rat, and in the clinically relevant sheep model. The aim also included to evaluate if the developed scaffold production protocols could be translated to other large animal models (the cow and the baboon) to investigate whether the methods may be relevant for future applications in investigations of human uterine tissue engineering.

More specific aims of the four papers included in this thesis are:

I. To compare the regenerative outcomes and the immunological consequences following a large uterus tissue replacement using a decellularized tissue graft with or without MSCs in the rat.

II. To investigate the feasibility of applying the same uterus tissue engineering methods that were developed in the rodent model on the larger sheep model.

III. To translate our established sheep uterus decellularization protocol to the cow model and validate its cross-species protocol independence and relevance for future human application.

IV. To translate the established decellularization protocol from the sheep and cow to the non-human primate model (baboon) and confirm its effectiveness on a human-like uterus.

3 MATERIAL AND METHODS

3.1 ANIMAL WORK

3.1.1 ETHICS

Research protocols for Papers I and II followed the ethical guidelines outlined in the animal ethics applications reviewed and approved by the animal welfare committee at the University of Gothenburg (no. 2228/2019). Additionally, an import permit for importing and working with non-human primate tissue (Paper IV) was granted by the same committee (no. 2465/2020). No animal ethics was required for Paper III, as the cow uteri were collected as byproducts from animals processed for food production at a local abattoir.

3.1.2 ORGAN RETRIEVAL

The biomaterials used in this thesis originate from donor organs collected from animals. There are several factors that affect the quality of the donor organ which must be considered before retrieval, including donor age, health, and animal strain. To reduce research variability, all retrieved organs should be of similar quality to ensure consistent decellularization results and scaffold composition. To avoid further batch-variability, it is important to handle all organs the same way after organ retrieval. In in Papers I-III, this was conducted by flushing each organ after cannulation with ice-cold PBS supplemented with lidocaine and heparin to remove existing blood before long-term storage at -20°C. In Paper IV, organs were acquired from a separate institution, making it unclear to us what the donor age was and how the organs had been handled prior to our reception at our institution.

For Paper I, the outbred rat strain Sprague Dawley was used for both organ retrieval and recipient transplantation procedures. All organ donors were 8-10 weeks old with a weight between 140-180g. The rat uterus was isolated under gas anesthesia where the vascular network to the uterus was dissected free. Anesthesia was employed to use the advantages of working with pulsating arteries, ensuring a more controlled dissection process, and minimizing potential complications. All extended vascular branches from the aorta to the vena cava not leading to the uterus was ligated and cut. The animals were euthanized through cardiac incision under anesthesia. After complete organ isolation, the aorta was cannulated and connected to a perfusion system that

allowed a controlled perfusion of the entire uterus with various solutions. In the case of larger animal models, the uterine vasculature is sufficiently large to enable the direct retrieval of organs from recently deceased animals (Table 2). In Papers II and III, we isolated uteri from female sheep and cows, respectively. Age of donor animals in these studies spanned from 9-12 months for sheep, and 12-24 months for cows. Each uterus was carefully dissected free from surrounding fat and connective tissue, but with a particular attention to preserving the uterine arteries on both sides of the uterine horns. As explained for the isolated rat uteri above, the uterine arteries in these organs were also cannulated which enabled whole organ decellularization through vascular perfusion.

Table 2. Overview of animal models and organ retrieval methods

Paper	Animal	Organ retrieval	Transplantation	Graft size
I	Rat	Surgical isolation under anesthesia	Yes	2x1 cm
II	Sheep	Surplus material from a slaughterhouse	Yes	3x2 cm
III	Cow	Surplus material from a slaughterhouse	No	N/A
IV	Baboon	Surplus material imported from the USA	No	N/A

3.1.3 GRAFT TRANSPLANTATION

To investigate the regenerative potential of the biomaterials, *in vivo* evaluation is essential. In Paper I, rats served as the experimental recipient animal model, while in Paper II, it was the sheep. The transplantation procedures are explained in greater detail in each presented paper later in this thesis. In brief, the animals were operated on under gas anesthesia by a large abdominal wall to access and expose the uterine region. A well-established uterus patch transplantation model was used. Only the vascularized mesodermal side of the recipient uterus horn was left intact, giving the graft a U-shaped structure.

Partial removal of the full-thickness uterine wall was then performed by scissors. The removed segment was then replaced with a size-matched uterus scaffold, with or without cells, by suturing it in place using 6:0-8:0 polypropylene sutures. In Paper I, a 2x1 cm uterus segment was excised from one uterine horn and replaced with either a decellularized or recellularized graft material. However, compared to previously reported studies [13-15], Paper I replaced a larger portion of the uterus wall with the tissue-engineered uterus materials Figure 12 A). In Paper II, a 2x3 cm section was excised from each uterine horn (i.e., two grafts per animal). One graft was decellularized sheep uterus tissue without added cells, and the other graft had been recellularized with MSCs (Figure 12 B). After the uterus patch transplantation, the muscular layer and abdominal wall were ligated and closed in separate layers. All animals receive postoperative analgesics and antibiotics following directions and doses for each species. In Paper I, euthanasia was performed at three distinct time intervals: two weeks, one month, and four months after transplantation. In paper II, the sheep underwent a second round of surgery two weeks post-transplantation. At this time point, a biopsy was collected from each graft using the same surgical methods as the initial operation. The sheep were euthanized six weeks post-transplantation so that the grafts could be assessed. Subsequently, in both Papers I and II, the collected materials from the endpoints were subjected to further analyses.

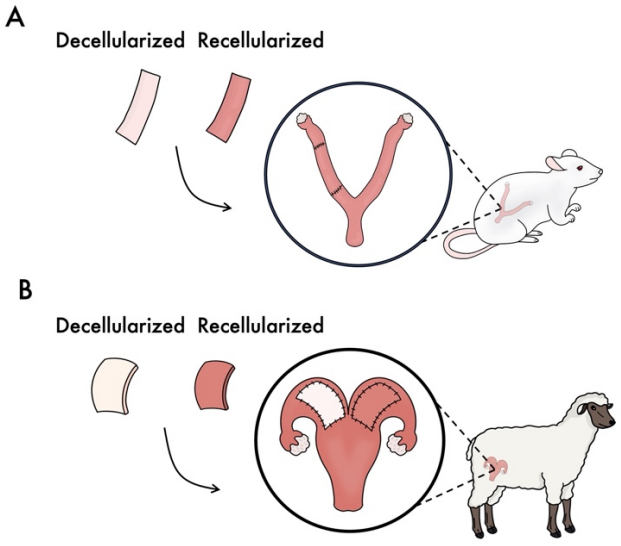

Figure 12. Illustration of the (A) rat and (B) sheep transplantation model

3.2 DECELLULARIZATION

As described earlier, decellularization can be performed in various ways. The, the methods used in this thesis include the perfusion of detergents followed by several washing steps with different solutions (Figure 13, Table 3). For example, the rat uterus decellularization protocol in Paper I was established in a previous publication [152] and briefly, includes a continuous organ perfusion with 4% DMSO and 1% Triton-X100 for 4h, respectively. The organs were then perfused with deionized water (dH20) to remove cell remnants (16h). This 24h cycle was then repeated another four times. The decellularized organs were then sterilized using perfusion of peracetic acid (peroxyacetic acid) for 1h in room temperature, and then perfused with sterile PBS. Each uterus was cut into 2x1 cm patches by removal of the cervix and uterine vessels, and stored in -20°C until further use. In Papers II, III, and IV, a different decellularization protocol, as compared to that of Paper I. For all three species, the protocol previously established for the sheep model was used [107, 124]. Briefly, each organ was first perfused with EDTA overnight to remove remaining blood remnants. SDC was then perfused for 8h followed by dH20 for 16h. An enzymatic step then followed by perfusing DNase I (40UI/mL) at 37°C for one hour. A washing step was then used for 48h, before a second cycle of the same steps were conducted. In paper IV, an additional third cycle was performed due to some detected tissue differences between the animal models and to ensure the complete removal of cell remnants. After decellularization, all organs were sterilized by the 0.1 % peracetic acid perfusion for 1h. The organs were perfused with PBS until a neutral pH was reached and then frozen down at -20°C until further use.

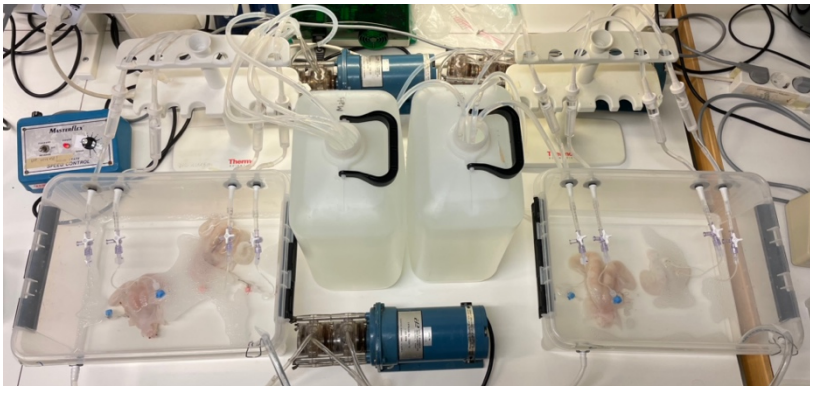

Figure 13. Picture of perfusion system including sheep uteri during decellularization

Table 3. Methods for decellularization and cell types used for each animal model. DC: decellularization, RC: recellularization

Paper	Animal	DC method	RC cell type
I	Rat	4% DMSO + 1 % Triton X-100	Rat MSCs
II	Sheep	2% SDC + DNAse I	Fetal sheep MSCs
III	Cow	2% SDC + DNAse I	Fetal cow MSCs
IV	Baboon	2% SDC + DNAse I	Rat MSCs

3.3 RECELLULARIZATION

Recellularization is the process of reintroducing cells into the decellularized tissue. In this thesis, the method for recellularization was performed by multiple sequential injections into the scaffold using a syringe with a fine steel tip (30G). Briefly, cells were seeded and expanded under normal cell culturing conditions until the desired quantity was obtained. Prior to recellularization, each scaffold was treated with matrix metalloproteinases (MMPs) 2 and 9 to precondition the scaffolds. MMPs are a type of enzyme that have degradation and remodeling properties of the ECM and are highly expressed by various cell types including macrophages, lymphocytes, fibroblasts, endothelial cells [159]. It was shown previously by our group in the rat model that preconditioning the scaffolds with MMPs enhanced the recellularization efficacy [107]. Hence, the decellularized scaffolds were incubated at 37°C with MMPs 2 and 9 for 24h. In Paper I, rat MSCs were used for the recellularization ($20x10^6$ cells/patch), and in Paper II, fetal sheep MSCs were used for recellularization ($200x10^6$ cells/patch). The recellularized materials were incubated for two weeks on a slow-moving rolling board to allow media access to the entire scaffold and to let the cells acclimatize to the scaffold and initiate cell migration and colonization before transplantation (Figure 14). In Papers III and IV, cow and rat MSCs were used, respectively, to evaluate the recellularization efficacy *in vitro* by transferring $1x10^6$ cells to each scaffold segment (0.5 cm^2). The recellularized biopsies were incubated for three and fourteen days in trans-well cell culture devices under standard culturing conditions.

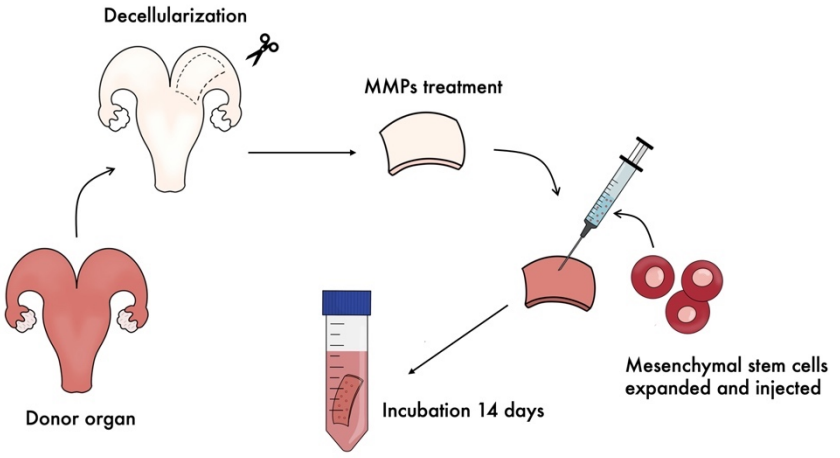

Figure 14. Schematic illustration of the recellularization process and scaffold production

3.4 HISTOLOGY

3.4.1 FIXATION

In Papers I-IV, all obtained biopsies underwent a standardized process for histological assessment. Initially, the biopsies were fixed using formaldehyde, followed by tissue dehydration in alcohol baths with increasing concentration to allow for a complete tissue penetration of paraffin wax. The paraffin embedded biopsies were then sectioned for histological evaluations. For papers II and IV, biopsies were also fixed in glutaraldehyde and subjected to sputter-coating for scanning electron microscopy (SEM).

3.4.2 HISTOCHEMISTRY

In all papers, the conventional hematoxylin and eosin (H&E) staining technique was used to assess the effectiveness of decellularization, and to enable examination of the general morphology. Furthermore, in Papers III and IV, additional staining protocols were employed to evaluate the ECM components after decellularization, including the Verhoff van Geison (VVG; elastin), Masson Trichrome (MT; collagen) and Alcian Blue (AB; GAGs) staining. Stained slides were analyzed in a light microscope and a slide scanner.

3.4.3 IMMUNOHISTOCHEMISTRY

To identify and visualize specific proteins within the tissue biopsies, immunohistochemistry was used. This method uses specific antibodies to detect the target protein, which is then visualized either by a fluorescent-labeled secondary antibody against the primary antibody, or by a colored enzymatic substrate visualized by light microscopy. In Paper III and IV, 4′,6-diamidino-2-phenylindole (DAPI), was used to visualize the DNA before and after decellularization. Additionally, DAPI was used in Papers I and II as a counterstain to specific immunofluorescent staining with antibodies to visualize the nucleus in individual cells. Immunofluorescent staining of specific uterine tissue compartments after transplantation was performed with antibody markers for smooth muscle cell actin (myometrium), vimentin (stromal compartment), wide spectrum cytokeratin (luminal epithelium and glands), estrogen receptor alpha and progesterone receptor. All fluorescent staining was visualized with a fluorescent microscope. To identify infiltrating immune cells for immunological evaluation in Papers I-III, various immune cells were identified using antibodies against CD45 (lymphocytes), CD4 (helper T-cells), CD8 (cytotoxic T-cells), FoxP3 (regulatory T-cells), CD68 (macrophages), CD86 (M1-macrophages), and CD163 (M2-macrophages). The cells were imaged, and different fields were manually counted to give a quantitative estimation of the average number of cells per mm^2.

3.4.4 SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) is a type of advanced microscopy that uses a beam of electrons to examine the surface of specimens at a very high magnification. Thus, examination by SEM can thus provide high-resolution images that are useful to visualize structures at a nanoscale level. In Papers III and IV, we wanted to evaluate the microarchitecture after decellularization to ensure proper removal of cellular material and visualize ECM morphology and architecture. Thus, the collagen fibers were imaged and their thickness measured for comparison between experimental groups.

3.5 ECM EVALUATION

Evaluation of the ECM after decellularization is essential to assess the quality of the scaffold integrity, given that the decellularization process can potentially harm the ECM components. While staining and microscopy techniques offer visual insights into the ECM preservation, quantitative methods are equally

valuable for precisely measuring crucial ECM proteins after decellularization. This was extensively done for the scaffolds used in Paper I and II in earlier studies [107, 124]. However, the scaffolds used in Paper III and IV underwent a detailed analysis that included the quantification of specific ECM components, including elastin, collagen (soluble and insoluble), and GAGs. The quantification was based on colorimetric kit-based methods, meaning that the targeted protein is colored, and the concentration is proportional to the measured absorbance. The total protein concentration was measured using the bicinchoninic acid assay (BCA), which is a copper-based method causing a change of color. The concentrations for all these ECM components were assessed using a plate reader that measures the absorbance at different wavelengths for each sample which is then compared to standard samples with a known concentration. The residual donor DNA content in the scaffolds was also determined. In both Papers III and IV, Qiagen's DNA extraction kit DNeasy Blood & Tissue was used to extract even small, visually imperceptible traces of DNA from homogenized decellularized tissue samples. Subsequently, a spectrophotometer was utilized to determine the DNA concentration.

3.5.1 BIOACTIVITY

Evaluating the bioactivity of decellularized tissue is important because it can determine whether the material can retain biological cues and signaling molecules that can potentiate the therapeutic outcomes. The chorioallantoic membrane (CAM) assay is a valuable method for assessing the angiogenic potential of a biomaterial and was applied herein in Papers III and IV. In brief, this assay used a fertilized chicken egg as the graft recipient The grafting procedure is conducted by creating a small opening in the eggshell to expose the chorioallantoic membrane on which a biopsy of the decellularized material was placed. The eggshell was then sealed and placed in an incubator to allow a normal chick embryo development. After the incubation period at the end of the second trimester (embryonic day 14), the blood vessel formation around the inserted graft on CAM was examined and quantified. This provides insights into the ability of the material to promote angiogenesis. Additionally, since the developing chick embryo is sensitive to toxic agents, the assay acts as a biotoxicity control.

3.5.2 MECHANICAL TEST

The decellularization process can alter the ECM properties, making mechanical testing essential to ensure that the tissue closely matches the mechanical characteristics of the native tissue. It also profoundly impacts how

stem cells differentiate and migrate when they integrate with the biomaterial. Since the mechanical tests were conducted and presented in earlier published studies for the scaffolds used in Paper I and II, this thesis includes mechanical tests for the scaffolds constructed in Paper III. This evaluation was conducted on excised rings from the decellularized tissue that then were mounted and pulled to breaking point in a mechanical testing machine (Zwick/Roell Z1.0, Zwick, Ulm, Germany). Hence, the tissue samples are subjected to a gradually increasing force while monitoring the applied load until the material reaches its breaking point. The resulting data was then analyzed, yielding crucial parameters such as the tensile strength, maximum load and modulus so that characteristics e.g., the scaffold stiffness and elasticity could be determined. Due to the anatomical difference of the produced baboon uterus scaffolds presented in Paper IV, and the scarcity of the material, mechanical testing was omitted in that study.

3.6 GENE EXPRESSION ANALYSIS

To gain insights into the gene activity, which dictates the protein production in the analyzed tissue, a digital droplet polymerase chain reaction (ddPCR) instrument was used. This enables the precise quantification of the expression levels of selected genes and provide informative data on the cellular activity and tissue specificity within the scaffold before and after transplantation. First, the total messenger RNA (mRNA) needs to be extracted from homogenized tissue samples using a kit. The isolated mRNA is then reversed transcribed by an enzyme (reverse transcriptase) to create complementary DNA (cDNA). By using labeled complementary DNA strands (probes) for selected genes, the starting amount of the cDNA, and ultimately also the mRNA, can be quantified after several PCR cycles, which, in theory, doubles the DNA quantities of the selected genes for each cycle. Rather than measuring the PCR amplification real time (quantitative PCR), the ddPCR measures the fraction of positive and negative duplicates and can determine absolute copies rather than giving a relative gene expression measurement. This also gives the ddPCR a significantly higher sensitivity, which is a major advantage when analyzing tissue-engineered constructs with a low cell density. In Paper II, ddPCR was used to evaluate several genes involved in uterus function and regeneration after transplantation, including the genes for progesterone receptor (*PGR*), estrogen receptor alpha (*ESR1*), vascular endothelial growth factor (*VEGF*), von Willebrand factor (vWF), tumor necrosis alpha (*TNFA*), interferon-gamma (*IFNG*), fibroblast growth factor 2 (*FGF2*), and homeobox A10 (*HOXA10*).

The peptidyl-prolyl cis-trans isomerase H (*PPIH*) gene was used as the reference gene.

3.7 FLOW CYTOMETRY

Fluorescence-activated cell sorting (FACS) is a method used to analyze separate individual cells based on their size and fluorescent properties, typically after labeling with a specific antibody conjugated with a fluorochrome. In Paper II, this method was used to identify and quantify the number of circulating immune cell populations at various time points post transplantation. Briefly, blood samples were collected from the experimental animals, both before transplantation and at the intervals two, seven, 14 days, and six weeks following transplantation. These blood samples underwent processing through a density gradient medium, facilitating the isolation of lymphocytes through several centrifugation steps. Subsequently, the lymphocytes were fixed and stained with immune cell-specific antibodies and FACS sorted in a BD FACSMelody™ (Becton Dickinson and company, Franklin, NJ, USA), machine. The analyzed cell population encompassed monocytes, macrophages, NK-cells, CD4 and CD8 T-cells, and T-regulatory cells. The quantitative assessment of these cell populations resulted in an estimation of the systemic immune response dynamics for each animal during the whole experiment. In Paper I, a different approach was used to evaluate the systemic immune response. Rather than quantifying the immune cells, a Firplex^{TM} analysis was conducted. This is a commercial immunoassay using flow cytometry to identify and quantify multiple cytokines and chemokines in a single sample using specific antibodies. These biomolecules are secreted by the cells involved in the immune response, and, thus, can provide valuable information on the systemic immune response mechanisms following transplantation. In Paper I, isolated rat plasma was used to analyze interleukin (IL) -1β, IL-2, IL-4, IL-5, IL-9, IL-10, IL-12p70, IL-13, IL-A17, motif chemokine ligand 1(CXCL1), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein (MCP1), macrophage inflammatory protein (MIP) 1α and 1β, interferon-gamma (INFγ) and tumor necrosis factor-alpha (TNFα).

3.8 STATISTICAL ANALYSES

Statistical analysis for all data was processed using the GraphPad Prism software. To assess the distribution of the acquired datasets, the Shapiro-Wilk

test was used. If the data exhibited a normal distribution, comparisons between the two groups were conducted using a t-test or unpaired t-test. In cases of multiple group comparison with normally distributed data, one-way ANOVA with Tukey's corrections was applied. Two group comparisons were performed using the Mann-Whitney U (non-parametric test) test for datasets of two groups that did not pass the normality test. In instances involving multiple group comparisons with non-parametric data, the Kruskal-Wallis test was utilized, followed by Dunn's post-hoc test, which corrects for multiple group comparisons.

4 RESULTS

4.1 PAPER I

This study thoroughly investigated the immune response following the transplantation of decellularized uterus tissue with or without recellularized tissue with mesenchymal stem cells (MSCs). The macroscopic evaluation of the grafts revealed a notable influx of infiltrating immune cells. Since the MSCs used for the recellularization were labeled with a green fluorescent protein, we also noted that they were no longer present after the initial fourteen days post-engraftment. No macroscopic differences were observed between the study groups during tissue harvesting. However, the histological and microscopic observations showed that the recellularized grafts had a more organized and improved myometrium after four months of engraftment compared to only decellularized tissue. The endometrial compartment showed no major differences between the study groups. However, few glandular structures were detected, and the luminal epithelium was well regenerated. When analyzing the immune response, there was a significantly lower number of infiltrating leukocytes in recellularized grafts, especially during the first month post-engraftment. The population of cytotoxic T-cells was decreased at all time points in the study. Additionally, a consistent trend towards a higher density of anti-inflammatory regulatory T-cells (FOXP3+) was observed in the recellularized grafts throughout the experiment. When it came to infiltrating macrophages, no significant differences were noted between the study groups in terms of the total number of macrophages, and nor for the pro-inflammatory M1 subtype. However, there was a significant increase in the anti-inflammatory M2 subtype at the fourteen-day time point in the recellularized grafts. In the analysis of pro-inflammatory and anti-inflammatory cytokines, some noteworthy findings emerged. The pro-inflammatory cytokines did not show significant differences between the groups, except for IL-1 and IL-17, which were at higher quantities four months after transplantation. In contrast, the anti-inflammatory cytokines exhibited a trend towards having higher levels in the recellularized grafts, particularly for IL-10 at the fourteen days and one-month time points and IL-12 at the three days and four months.

In summary, the results point conclusively towards that the MSCs have an immune-modulating role following decellularized uterus tissue transplantation. More specifically, the MSCs contributed to a reduction in

immune cell infiltration, particularly during the first month, but also by skewing the immune response towards a regenerative state. This shift was marked by the reduction of cytotoxic T-cells and an increase in regulatory T-cells and M2 macrophages accompanied by elevated levels of anti-inflammatory cytokines such as IL-10, IL-12, and IL-13 in the cellular grafts. Overall, these findings suggest that MSCs have the potential to establish a pro-regenerative immune microenvironment in conjunction with decellularized tissue after transplantation.

4.2 PAPER II

In this study, we extended our previous research by using the sheep animal model, which is more clinically relevant, in terms of uterine size, than the rat to assess the feasibility of bioengineered uterus patches for uterine tissue replacement. These transplantation experiments represent a progress toward clinical applications but also highlighted ongoing bioengineering challenges. During the preparation of the tissue-engineered grafts, we achieved a high recellularization efficiency with sheep fetal mesenchymal stem cells (SF-MSCs). However, we found a large variability within the study groups when assessing the regenerative outcomes following transplantation. Some grafts in both study groups exhibited a high degree of regeneration with well-structured uterine layers, while other grafts showed poor outcomes. A novel histological scoring system was established for uterus regeneration that differentiated the grafts based on the regenerative outcomes to evaluate potential mechanisms indicating positive and negative outcomes. The classification scores range from “0”, indicating minor regeneration, to "3” representing regeneration comparable to the normal tissue. In the group treated with decellularized grafts, outcomes varied, with one graft (1/7) achieving a regeneration equivalent to normal tissue, while one graft (1/7) displayed only “minor” regeneration. Additionally, two grafts (2/7) in this decellularized graft group were classified as “partial” regeneration and three (3/7) exhibited “substantial” regeneration.

The recellularized group also showed a diversified outcome, with one graft (1/8) reaching “normal” tissue regeneration, and another (1/8) displaying “minor” regeneration. Five grafts (5/8) achieved “partial” regeneration. The average score for the decellularized group was 1.57, while the recellularized group averaged 1.38. Although no significant differences were observed between the two study groups, there were apparent distinctions in regenerative capabilities among the individual grafts. As a result, we stratified the grafts

into two categories: those with minor regenerative outcomes (scores 0 and 1; n=9) and those with major regenerative outcomes (scores 2 and 3; n=6). Our subsequent data analysis was based on these new study groups.

We found that the luminal epithelial layer recovered consistently in all graft types. Elastin and collagen staining suggested similarities to native tissue in the scaffolds that were considered well-regenerated. No major difference was seen in the vascularization between the grafts, and the gene expression analysis showed a pattern similar to the histological findings. Interestingly, FACS analysis of circulating immune cells revealed an obvious discrepancy between the animals that had grafts with better regeneration and those with poorly regenerated grafts. It was particularly the T-cell subpopulations that showed notable differences where the naïve CD4+ T-cells were significantly higher in the major regenerative outcomes, and in contrast, the activated and effector memory CD4+ T-cells were significantly lower compared to the minor regenerative group exhibiting higher levels of activated T-cells.

Overall, the MCSs did not seem to play an equally important role in the sheep model compared with the rat model. However, our results suggest that the T-cells play an important role in the therapeutic outcome. The best-performing grafts exhibited remarkable regeneration, reaching a level comparable to native uterus tissue within six weeks of transplantation. Although the spread of regeneration among the two groups was difficult to explain, the potential for regeneration was seen in both groups and further studies will be conducted to better understand the underlying mechanisms.

4.3 PAPER III

In this study, we validated the cross-species adaptability of the larger cow uterus using the decellularization protocol originally developed for the sheep uterus. Furthermore, the effects of the previously established MMPs treatment were extensively evaluated. The decellularization process effectively removed DNA content, reducing it to less than 1% of the initial concentration after SDC perfusion. Histological and quantitative analyses using MT, VVG, and AB staining demonstrated the preservation of crucial ECM structures. However, the remaining ECM after decellularization exhibited a reduction in ECM molecule concentrations (e.g., collagen, elastin, and GAGs). No further reduction in protein concentrations was observed after the enzymatic preconditioning using MMPs. Moreover, the MMPs did not adversely affect

the collagen fiber size, which remained similar in thickness compared to the native tissue. A trend toward an increase in the number of stimulated blood vessels was observed in both the decellularized and MMPs treated groups, however, no significance was observed due to a large variation in the intra-group comparison. The results from the mechanical tests in of this Paper III revealed that MMPs did not have an adverse impact on the tissue. However, the decellularization process led to a reduction in both the maximum load and the work required to break the samples. Interestingly, the modulus, which quantifies the tissue stiffness, was less affected by the process. Evaluation of *in vitro* recellularization efficiency in decellularized and MMP-treated uterus tissue showed a significantly higher number of infiltrating cells in the MMPs treated tissue compared to the decellularized after three days of culture. However, the beneficial effect of MMPs plateaued after fourteen days of culture. The evaluation of the immunogenicity following MMPs treatment encompassed the quantification of six types of graft resident immune cells (CD45+, CD4+, CD8+, CD68+, CD86+, CD163+) fourteen days after subcutaneous engraftment in rats. The evaluation indicated that MMPs preconditioning did not adversely affect the scaffold immunogenicity *in vivo*, emphasizing its applicability for future studies.

4.4 PAPER IV

The validated decellularization protocol used for sheep and cow was further translated to the non-human primate model using the baboon uterus. However, the baboon uterus required a small adjustment to the protocol with the addition of one decellularization cycle. The baboon uterus exhibited a thicker myometrial compartment compared to previous animal models, which was ultimately successfully decellularized with a DNA content of less than 1% of the initial concentration after the extra perfusion cycle. Histological analysis showed well-preserved ECM structures, and quantitative analyses demonstrated a reduction in collagen, elastin, and GAGs after decellularization. The MMPs treatment did not affect the ECM in terms of molecule concentration, and no visible difference was observed in the histological evaluation. However, the impact of MMPs treatment appeared more pronounced when collagen fiber size and angiogenesis were evaluated. A significant reduction in the collagen fiber size was observed after MMPs treatment compared to the native control and decellularized tissue. The angiogenesis evaluation using the CAM assay showed elevated levels in the number of blood vessels in both study groups, but only a significant increase

was observed in the decellularized tissue compared to the native control. The *in vitro* recellularization showed similar levels between the decellularized and MMPs treated tissue after three days of culture, with a significant increase observed after fourteen days of culture. However, the cells were mainly distributed in the superficial layers of the tissue and around injection sites inside the tissue.

5 DISCUSSION

The aim of this thesis was to conduction basic studies for further experimental work to address current treatment challenges for uterus-related infertility with the creation of a bioengineered uterus tissue graft. This visionary concept involves the development of a "personalized graft", which may present numerous advantages compared to the currently available transplantation procedures, including for UTx. Among the most significant benefits is the potential to eliminate the shortage of donor organs and bypass the logistical challenges of sourcing compatible organs. Importantly, this personalized approach could reduce the need for prolonged use of immunosuppressive medications, often accompanied by unwanted side effects, e.g. infections, hypertension, nephrotoxicity, diabetes, and occurrence of certain malignancies [160]. Nevertheless, it is important to note that the idea of a tissue-engineered personalized uterus is a goal, although the technology is still in its early stages. Substantial challenges, including achieving full functionality, replicating complex physiological processes, and ensuring long-term safety, must be addressed [161]. Hence, the aim to construct a whole bioengineered uterus that can be used in a UTx setting is a long-term goal. However, short-term goals include strategies to create uterine tissue-pieces that can help infertile women with a partial uterine defect or women with severe uterine scarring that risks uterine rupture during pregnancy. A tissue-engineered patch may, in these cases, be used to replace the damaged tissue after a cesarean section or removal of fibroids to stimulate a scar-free regeneration and reestablish fertility [162]. Furthermore, applying such partial strategies will bridge the knowledge gap between the production of less complex tissues, and the ultimate objective of creating fully functional organs. Thus, the potential benefits of this innovative approach could reshape the landscape of obstetrics, gynecology, and reproductive medicine.

Animal models played a significant role in the successful UTx research program that resulted in the world's first birth of a child [3, 32]. We have followed the same translational progress in this thesis by applying the most relevant animal models for preclinical research, including the rat, sheep, cow, and baboon (Papers I-IV). Our research group began the uterus tissue engineering research using the rat [13, 152], which still acts as an important research model for certain research questions [122, 123]. However, established protocols were also evaluated in the sheep [107, 124], which has been

considered one of the most important animal models for developing UTx protocols and for team training of the procedure at a new center [43, 163].

The primary focus of this thesis centers around the advancement of a patch repair strategy (Paper I and II) and the establishment of a species-independent decellularization protocol (Paper III and IV). This approach aims to create decellularized uterine tissue constructs with the goal of regenerating functional portions of the uterus. The partial patch repair strategy serves dual purposes since the refined techniques for cell sourcing, scaffold design, and bioengineering methods also validate the feasibility of constructing more complex tissues, offering valuable insights into the tissue engineering field in general. Initial testing of a tissue-engineered uterus patch was conducted by our research group using the rat model in 2014-2016 [13, 152], where fertility was restored in two out of three tested uterine constructs. This concept was confirmed by two additional labs around the same time [14, 16]. Thus, together, they provide convincing proof-of-concept that decellularized tissue-derived constructs are promising biomaterials for uterus repair. However, these three studies used small-in-size transplants, which may not properly represent the human situation where significantly larger grafts will be needed. Additionally, neither of the studies looked at the immune system response following engraftment of these constructs. Given that the immune response plays a critical role in both tissue regeneration and the rejection of transplanted biomaterials, it was vital to conduct additional studies. Padma et al., concluded from a subcutaneous transplantation, that using a mild, yet efficient detergent protocol using Triton-X100 for the decellularization process was immunologically advantageous, in contrast to the more aggressive SDC treatment, due to a reduced DAMP content in the scaffolds [122]. Padma et al., also found that when the donor DNA levels were below 1% of its original content, the immune response was low [123]. Based on this knowledge, we assessed the systemic and local immune response after transplanting significantly larger constructs to the uterus in the rat model, where the grafts were developed by the mild decellularization technique. We also examined the potential immunological effects of including MSCs in the scaffolds (Paper I). Scaffolds recellularized with MSCs were shown to have immune-modulating and pro-regenerative effects by the contribution of reduction of leukocyte infiltration with a skewed immune response with induction pro-inflammatory of regulatory T-cells and M2 macrophages. Further, the myometrial regeneration was more developed and similar to the native tissue. Future studies in the rat model would include further evaluation of the regeneration after transplantation of larger constructs and evaluations of pregnancy

outcomes. Moreover, further investigations of the immune response following such transplantation could potentially reveal important mechanisms for successful graft implantation. However, it is essential to recognize the limitations of rodent models, especially when smaller graft sizes are used, as rodents possess faster self-regenerative capacities compared to larger animals [164].

Therefore, the translation to larger animal models that have a uterine size similar to the human and thereby are more clinically relevant than rodent models became imperative. Hence, we applied the established decellularization techniques for the sheep uterus [107, 124] and assessed clinically relevant sized grafts *in vivo* (Paper II). This study was exploratory, where we wanted to evaluate the potential of using the sheep as a large animal model, hence the short transplantation time of six weeks. The outcomes of this study highlighted the challenges of translating results from smaller to larger animal models. Among these challenges, the analysis of sheep tissue was constrained due to the limited availability of diagnostic tools specifically designed for sheep, including antibodies and primers. However, the performance of the engineered grafts exhibited a varying degree of regeneration within the study groups, making it difficult to establish conclusions with major scientific value. Whereas, in the rat model (Paper I), the beneficial influence of the MSCs on the immune system was evident; this effect was not obvious in the study in sheep (Paper II). However, in context with the findings in Paper I, the regenerative outcomes reported in Paper II also seemed reliant on the immune system, in particular the different subgroups of T-cells which seem to play an important role, where higher proportions of circulating activated CD4+ cells were observed. However, the mechanisms and exact function of different T-cell subsets is largely unknown and seem to vary between tissues. Moreover, it is suggested that they appear to have both pro- and anti-regenerative effects and sub-populations. For example, it is known that activated T-cells are associated with a greater risk of rejection following kidney transplantation [165] and that subset of CD4+ and CD8+ T-cells can inhibit regeneration in the context of bone [143, 166]. We must also consider that the variability in results between Paper I and II may also stem from differences in the cell source used for the recellularization as in In Paper II, the MSCs used were obtained from an immunologically different, allogenic fetus. Since the sheep are genetically more diverse than rats, variations in the immune response may have been affected by a greater genetic allograft variation compared with the rats used in Paper I. Nevertheless, it is essential to acknowledge additional limitations in Paper II, such as the transplantation of

decellularized and recellularized graft types into the same individual, which presented challenges for the analysis of the systemic immune response. These limitations will be considered for future studies. Proposed refinements involve employing the same graft type into a single individual for a more precise evaluation of the systemic immune response. Additionally, advancements could encompass the utilization of a protective adhesive barrier to prevent graft adhesions, thereby mitigating potential negative impacts on regeneration.

As our research group continues to evaluate and improve our biomaterials in using both the rat and the sheep model, it is worth noting that parallel efforts are made in translating these applications further to accelerate a potential clinical introduction. Hence, in Papers III and IV, we tested the applicability of our sheep uterus protocol for decellularizing different donor species, as this is a key challenge for comparing studies and advancing the translation [124, 156]. When the same decellularization protocol was applied to the significantly larger cow uterus (Paper III), our results confirmed the successful removal of donor DNA and the preservation of the ECM architecture. These results suggested that the protocol should also be applicable to the non-human primate uterus (Paper IV) which is similar to the human uterus in terms of size and tissue composition. At the initiation of the study presented in Paper IV, it became evident that the myometrial layer was significantly thicker and denser compared with the myometrial composition in earlier tested animal models (rat, sheep, and cow). This did indeed seem to affect the tissue resilience to the decellularization chemicals, and in 2/5 of the decellularized organs, we detected donor DNA levels over 1% of its original content. Hence, we introduced an additional decellularization cycle compared to what was needed for the sheep and cow uterus. However, our protocol is time-efficient and significantly less aggressive than many other protocols published by other research groups for uterus decellularization, including studies made in the mouse [58], rat [14-16, 58, 157], rabbit [153, 154], pig [129], sheep [124, 155], and human [156]. The perfusion pressure is rarely discussed in published protocols and may explain the variety between protocols and perfusion times in published articles. It is crucial to ligate all leaking vessels to maintain the perfusion pressure and allow the decellularization reagents to reach all tissue compartments. It is possible that we were unable to identify and close all severed vessels in our baboon organs since they had been isolated by research staff not previously trained in dissecting uterine tissue. A non-identified leaking vessel might explain the need for the extra 8h SDC cycle in our baboon study, compared with our established protocol for the sheep and cow uterus (Paper II and III).

These findings hold significant importance as groundwork for forthcoming human studies and set a new standard for the field by facilitating cross-study comparisons, expediting clinical translation, enabling quality assurance considerations for the development of clinical grade tissue engineered products.

In the context of tissue engineering, regeneration is a fundamental concept to consider when designing and developing material to treat dysfunctional uterus tissue [11]. The uterus is one of the few organs with an outstanding regenerative capacity. E.g., the endometrium regenerates every menstrual cycle, which is essential for fertility [167], yet it is still to date poorly understood. Understanding the mechanisms and processes underlying endometrial regeneration may help us towards full tissue engineering of uterine tissue and provide us with clues on what cells and growth factors should be used during the construction [11]. The results in Papers I and II confirmed that our grafts hold the potential to regenerate into the fundamental tissue layers of the uterus. We did not conduct any pregnancy tests on these studies, which would provide important information on the functionality. Not only the implantation success of an embryo but also that the graft should withstand the mechanical forces and capability of a 20-fold size expansion during pregnancy [75]. Earlier studies in the rat showed that our grafts were strong enough to support pregnancy to full-term [13], but no implantation site with further placentation and with term pregnancy has yet been localized to the biomaterial, which would prove full functionality. Not only the mechanical aspects of the graft are essential, but also its biodegradability. The scaffold should degrade at an appropriate rate similar to the regeneration process [168]. This becomes more complex with the required increase in graft size, which therefore might need further scaffold manipulations. Investigations into crosslinking using genipin and procyanidins have been carried out in a uterus rabbit model. It was found that mechanical properties were enhanced without triggering host immune reactions [153]. However, the degree of enhancement varies with concentration and crosslinker type, suggesting its potential utility in future applications. Careful evaluations are required to balance mechanical support and tissue regeneration. Other approaches may include the use of hybrid scaffolds that are based on decellularized tissue, modified with hyper-branched PEGs, which allows the construction of a regulated degradation of the scaffolds [169, 170].

Scaffold material is typically regarded as just one of the fundamental elements in tissue engineering. The choice of cell source that should be used with the

scaffold is another consideration during the engineering phase. Mesenchymal stem cells hold promise for their ability to differentiate into various cell types, are easily obtained, and have shown regenerative and immunomodulatory capacities together with angiogenic effects [171], and is the reason why they were utilized in this thesis. However, other studies on uterus tissue engineering have used primary uterus cells [13, 14], endometrial stem/progenitor cells [154], and human MSCs [15]. The development of induced pluripotent stem cells might provide a useful source for uterine tissue engineering in future applications [172-174]. Further, it is important to consider the function of the cells, if they are used to promote tissue regeneration, or if the concept is to create a fully functional tissue *in vitro* [11], and it is likely that a combination of cell types will provide the best outcomes.

6 CONCLUSION

Overall, this thesis highlights the importance of a translational approach in tissue engineering and emphasizes the potential of using decellularized material to treat various conditions causing infertility.

Paper I — It was demonstrated that MSCs played an immune-modulating role by reducing the immune cell infiltration during the first-month post-transplantation. Furthermore, they initiated a graft milieu towards a pro-regenerative state characterized by the decreased density of cytotoxic T-cells, an increased density in regulatory T-cells and M2 macrophages, along with higher levels of anti-inflammatory cytokines. Overall, the findings suggest that MSCs can be used as a therapeutic cell source in conjunction with decellularized uterus tissue.

Paper II — By establishing a novel uterus regeneration scoring system, our findings demonstrated that the MCSs did not play an equally important role in the sheep model. However, there was a discrepancy in circulating T-cell subpopulations between successfully regenerated grafts and failing grafts, suggesting that the T-cell subpopulations play an important role in the therapeutic outcome. However, the remarkable regeneration found in the three best-performing grafts after just six weeks following transplantation provides compelling evidence that employing decellularized scaffolds for uterine bioengineering holds great promise for clinically relevant applications.

Paper III — Confirmed the effectiveness of our previously established decellularization protocol for use in the larger bovine uterus. The effective removal of DNA and preservation of essential ECM structures was shown. Further evaluations revealed that MMPs did not adversely affect the tissues in terms of protein content or immunogenicity *in vivo.* This study highlights the translational potential of our established decellularization protocol.

Paper IV Confirmed that the same uterus decellularization protocol used for the sheep and the cow could be adapted for the baboon uterus. The protocol generated bioactive, non-toxic, and three-dimensional uterus scaffolds and shows that the protocol holds great promise for future human tissue engineering studies. Importantly, our work sets a new standard for the field by facilitating cross-study comparisons, expediting clinical translation, and enabling quality assurance considerations for the development of clinically appliable tissue engineered medicinal products.

7 FUTURE PERSPECTIVES

In the context of human studies, only one investigation has explored uterine decellularization [156]. This study applied an extensive SDS protocol that demonstrated the feasibility of decellularizing human uterine tissue. However, this protocol was considerably more aggressive than the protocols used in this thesis and may show immunogenicity due to a likely high DAMP scaffold content [122]. Hence, these findings highlight the importance of evaluating and improving protocols further for human tissue and conducting additional transplantation studies in large animals to assess the safety and efficacy of generated tissue-engineered constructs. The design of future large animal studies could include improvements such as the use of an absorbable adhesion barrier to protect the grafts from adhering to themselves or to each other. Further, the inclusion of the same graft type in each animal could help to evaluate the systemic response depending on the graft type. Moreover, additional studies on investigating the immunological response to minimize immunogenicity and decipher the T-cell subgroup roles in tissue regeneration (Papers I and II) so that the therapeutic outcomes can be improved further with combinatory pharmacological and immune-modulating agents [143]. Throughout these studies, careful consideration should be given to the development and scalability of these methods to align with good manufacturing practices and adhere to relevant quality control regulations. The next step of our group's research is to translate our established decellularization protocol to *in vitro* studies of the human uterus while, in parallel, also investigating potential tissue engineering improvements using the rat and sheep models. Future studies will involve the collection of uteri from multi-organ donors, conducting human cell culture, and employing the decellularization protocols already established for large animals. Additionally, in depth-analyses of safety and immune response will be evaluated *in vivo.*

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