30 Evolutions in Hypospadiology and Current Status of Tissue Engineering

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Abbreviations

ECM	Extracellular matrix
P(LLA-CL)	Poly(L-lactide-co-caprolactone)
PCL	Polycaprolactone
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
SIS	Small intestine submucosa

30.1 Introduction

Hypospadias is characterized by a deficiency in the development of all the layers of the urethra and the surrounding corpus spongiosum and occurs due to incomplete fusion of the urethral folds, typically between 8 and 20 weeks of gestation. The term "hypospadiology" was introduced by John W. Duckett Jr. 4 decades ago, during a renaissance in urethral reconstruction and hypospadias surgery [1]. It is a broad term that incorporates all the available knowledge in the field and the long list of techniques over more than a century of continuous development. The sheer number of procedures described for hypospadias points towards the challenges in achieving perfect reconstruction and also reminds hypospadias surgeons that every case of hypospadias is unique. Surgical success is dependent upon accurate correction of penile curvature, tubularization of the urethra, reconstruction of the glans, and adequate skin coverage, ultimately aiming towards cosmetic and functional normalcy. Attempts to restore the anatomy of the urethra and correct the curvature of the penis have progressed through transitions in the philosophies of treatment, from single stage to multi-stage repairs and from use of local tissues to distant autologous grafts. While in distal hypospadias, representing at least 70% of all hypospadias cases, the urethral plate is usually amenable to single stage repairs, proximal hypospadias and re-do hypospadias repairs present a challenge for even experienced hypospadiologists. When preputial tissue is available, flaps or grafts of this skin might be used for the urethroplasty. When penile skin is inadequate, skin from non-hair bearing areas and buccal mucosa represent the most common sources of autologous graft tissue used to form the neourethra, almost invariably involving multi-stage surgery. However, even staged repairs are associated with significant complications and reoperations are necessary in >50% of boys undergoing surgery [2]. Furthermore, a urethra constructed from buccal mucosa or non-genital

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skin grafts may not develop in the same way as a native urethra over longer period of follow-up. In boys with severe hypospadias, particularly those who have undergone multiple repairs using autologous tissue, the options for urethral replacement are limited, especially for long defects. Additionally, in other boys who have less severe varieties of hypospadias, use of extragenital skin or mucosa may not be desirable due to donor site morbidity.

Concepts in hypospadiology are evolving constantly with the ultimate aim of the "perfect" technique, that is, the right operation for the right patient at the right time. Tissue engineering is emerging as an attractive solution to the conundrum where there is a paucity of ideal local tissue for urethral reconstruction. It eliminates the need of harvesting autologous tissues and the associated morbidity related to donor site harvesting. Further, it has the potential to offer readymade, prefabricated constructs that can be used for urethral replacement directly. Importantly, tissue-engineered grafts can also be designed to reproduce the structural, mechanical, and biological characteristics of the urethra by adjusting their composition. They can be made to bear the stretch during passage of urine and erection as well as avoid overdistension that may compromise their integrity and barrier function. An ideal tissue-engineered urethra has the following characteristics—(a) biocompatible, (b) multilayered, consisting of different type of cells (epithelial cells, fibroblasts, and smooth muscle cells), (c) effective barrier against the metabolites present in urine, (d) elastic to allow distension during voiding and stretching during erection, and (e) resistant to manipulative forces and during surgery, particularly suturing. Mimicking the native urethra, a tissue-engineered urethra should be fairly adaptable to voiding pressures, thus avoiding deformation and the potential for overdistension and diverticulum formation. Tissue engineering has progressed over the last three decades, with much research having been focused on finding suitable biomaterials and cells that can be tailored to meet the properties of the native urethra.

30.2 Biomaterials for Urethral Tissue Engineering

Three types of biomaterials are used to tissue engineer the urethra: (a) autologous cells, (b) acellular biomaterials or scaffolds (polymeric or extracellular matrix derived), and (c) autologous cells seeded on the scaffolds. The cell-only constructs are too fragile to bear transportation or handling and therefore are impractical for surgical use. So, acellular and cell-seeded scaffolds have undergone the most testing in animal and clinical studies for urethral regeneration. Scaffolds provide a mechanical framework for tissue regeneration. They promote three-dimensional movement of cells and may be degraded in the process. They can be classified as per their origin (natural or synthetic) and biodegradability (biodegradable and non-biodegradable).

30.2.1 Acellular Synthetic Polymeric Scaffolds

Scaffolds derived from synthetic polymers are easy to construct and may be biodegradable or non-biodegradable. The biodegradable scaffolds are synthesized from polymers such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), polycaprolactone (PCL), and poly(lactic-co-glycolic acid) (PLGA). The ester bonds in these polymers degrade by nonenzymatic hydrolysis, and their nontoxic degradation products are eliminated from the body in the form of carbon dioxide and water. Their rate of degradation depends on their crystallinity, molecular weight, and copolymer ratio which is useful for tailoring them to suit the needs of the tissue being reconstructed. Non-biodegradable scaffolds such as polytetrafluoroethylene and poly(ethylene terephthalate) are mainly used as temporary supports and hence have limited application as they frequently undergo calcification, shortening, and migration. A disadvantage of all synthetic polymers whether biodegradable or not, is that they lack the specific proteins on their surface that

interact with cells and facilitate adhesion. Hence, they require surface treatment to promote cell attachment.

30.2.2 Acellular Natural Polymeric Scaffolds

The natural polymers that have been used to make scaffolds are collagen, alginate, chitosan, hyaluronic acid, and silk fibroin. Collagen is the most abundant protein and forms a major part of the extracellular matrix (ECM). It is usually derived from animal sources (such as bovine or porcine skin) although recombinant human collagen is also available. However, it is antigenic and has a fast degradation rate limiting its use in urethral regeneration. Silk fibroin, on the other hand, is an excellent biomaterial which has low immunogenicity and hence generates less inflammatory response. When compared with small intestine submucosa (SIS) grafts or urethrotomy in male rabbits, silk fibroin scaffolds had a lower inflammatory response though the growth of epithelial cells and smooth muscle cells was the same [3]. Furthermore, it has a hydrophobic structure with strong intramolecular and intermolecular interactions, and is hydrolyzed by proteolytic enzymes. Its elasticity and shape memory are good for urological application and blending it with synthetic polymer can impart cell adhesion property to the latter. A composite scaffold of silk, keratin, gelatin, and calcium peroxide film allows high continuous oxygen delivery and also has antimicrobial activity while having similar regenerative capacity as SIS [4]. Natural polymers have integrin-binding peptide sequences and a surface topography that promotes cell adhesion as well as angiogenesis. The rate of degradation of difficult to control and transfer of pathogens is possible during their use.

30.2.3 Acellular Extracellular Matrix Scaffolds

Decellularization of allogenic and xenogenic tissues yields acellular ECM scaffolds that retain the biomechanical properties and structural integrity as well as bioactive growth factors. They degrade rapidly once implanted and the degradation products stimulate regeneration by *constructive tissue remodelling*. When the scaffolds have an optimized degradation that is synchronous with the growth of the cellular component, the resultant tissue has layered epithelia, organized smooth muscle cells, and better vascularization. On the other hand, non-degradable synthetic biocompatible scaffolds are associated with a non-functional remodeling that is associated with complications such as migration, calcification, and narrowing. The most common acellular ECMs used in urethral repair are SIS. It is obtained by mechanical removal of tunica mucosa and muscularis, and serosa from porcine small intestine. When used for urethral replacement, the results are similar to those obtained with skin and buccal mucosal grafts, achieving high rates of cell growth and angiogenesis. The degradation time is four to eight weeks and the degradation components are eliminated in urine. SIS has been used successfully during corporal incision and grafting for correction of chordee and demonstrates low tendency to break. However, the regenerative potential of SIS depends on age of donor and the part of small intestine used to derive it resulting in batch-to-batch variability. Another acellular ECM is bladder acellular matrix which has shown encouraging results in animal studies on urethral regeneration. All acellular ECM scaffolds have potential to cause inflammatory reactions due to residual nucleic acids and xeno-antigens.

30.2.4 Cell-seeded Scaffolds

For urethral defects up to 0.5 cm, acellular scaffolds promote healing and facilitate repair but for larger lesions, cells are required on the scaffolds. These cells may be harvested from the urinary tract or other sources. Both urothelial cells and oral mucosal cells can produce a stratified epithelium for urethral reconstruction, although, urine-derived stem cells can differentiate into urothelial as well as smooth muscle cells. The cells for

seeding may be obtained by invasive or non-invasive methods. Invasive methods including open bladder biopsy harvest a small number of cells only and additionally require general anesthesia besides causing donor site morbidity. Bladder washings is a non-invasive method that is safe and easily reproducible. When seeding cells on a scaffold, it must be remembered that the proliferation of cells is greatly affected by the mechanical properties of the scaffold. Multiple cell types can be cultured at the same time. In fact, coculture is found superior to the culture of individual cell types highlighting the importance of paracrine signaling. The cell-seeded scaffolds can be fabricated to form tubular constructs that look like urethra and are lined on the inside by urothelial cells. They can be bio-functionalized using exogenous trophic factors and suitable microenvironment in a bioreactor to stimulate differentiation of the construct for functional maturation.

30.3 Approach to Urethral Tissue Engineering

30.3.1 Selecting the Type of Construct

Cell-only constructs are not suitable for urethral reconstruction due to lack of mechanical strength. So, tissue-engineered urethra must be derived from either acellular matrices or cell-seeded matrices. For acellular matrices to be successfully epithelized, the defect must be small and the urethral bed must be vascular. They are not suitable for patients with extensive spongiofibrosis, recurrent strictures, and long defects. Autologous cell-seeded matrices are useful in these situations. The cells are harvested from a tissue biopsy and then expanded in a culture after which they are seeded into the matrix. Urothelial as well as non-urothelial cells such as keratinocytes may be used for seeding. The matrix in then implanted to replace the urethral defect.

SIS is unsuitable for use as a tubularized construct and is associated with fibrosis and luminal obstruction [5]. When used as an onlay patch, four-layer SIS has less shrinkage than single-layer SIS although they have similar re-epithelization and neovascularization [6]. For long and complex urethral defects, tubular cell-loaded constructs are superior to onlay grafts. Bladder acellular matrix when combined with silk fibroin has excellent revascularization, a property that is desired in patients with failed hypospadias repair [7].

30.3.2 Natural vs Synthetic Biomaterials

The structure and properties of a synthetic scaffold can be altered based upon the site of implantation. Such precise tuning is not possible for natural polymeric and ECM based scaffolds because of the variability of the source. However, the ECM proteins that facilitate adhesion and ingrowth of cells are absent in synthetic scaffolds, so, they cannot be used without surface treatment. Recently, hybrid or composite scaffolds have been developed, which combine a synthetic polymer with a natural matrix [8]. PLGA microfibers can be attached onto the abluminal surface of bladder acellular matrix to produce a hybrid scaffold.

30.3.3 Electrospinning

Electrospinning is one of the latest techniques that has evolved in the last few years and helps to design, produce, and characterize the 3D scaffolds for in vitro as well as in vivo culturing. It can produce biomaterials with nanoscale properties. Such materials have a high porosity and spatial interconnectivity suitable for tissue engineering. Using electrospinning, it is possible to add cells and proteins within the scaffold with high efficiency. Natural as well as synthetic materials can be electrospun. Zhang et al. fabricated a nanofiber scaffold of collagen type I and poly(L-lactide-co-caprolactone) (P(LLA-CL)) using coaxial electrospinning technique and reported satisfactory programmed biodegradation and tensile strength [9]. Wei et al. prepared nanofiber

scaffolds of polycaprolactone (PCL), collagen, and silk fibroin which allowed adhesion and proliferation of oral mucosal cells to produce a seeded scaffold for urethral reconstruction [10].

30.3.4 3D Bioprinting

3D Bioprinting deposits cells and biomaterials in a manner similar to an inkjet printer to prepare a construct with predefined architecture. The deposited substances are called bioink. 3D Bioprinting was used to produce a spiral scaffold in which urothelial cells and smooth muscle cells were applied on the inner and outer layer of the scaffolds to produce a urethral construct with mechanical properties of the native rabbit urethra [11]. Recently, multichannel coaxial extrusion system has been developed, which allows bioprinting of circumferential multilayered tubular structures [12]. Using this technique, tubular constructs of urethra have been developed using human urothelial cells and human bladder smooth muscle cells.

30.4 Clinical Results of Tissue-Engineered Grafts

Both animal and human studies have assessed the feasibility of using tissue-engineered grafts for urethral regeneration. The results of these studies have been encouraging; however, it was noted by Versteegden et al. in a recent meta-analysis of preclinical and clinical studies that the results obtained in animal models did not translate into the human subjects [13]. This difference is likely due to the design of preclinical studies. The animal models usually had a healthy urethra which provided a suitable environment for the uptake of the graft whereas most of the patients who require such grafts usually have a scarred and fibrotic region where the graft is placed.

Most of the clinical studies on the use of tissue-engineered grafts for urethral replacement have been performed in adults with urethral strictures. The use of tissue-engineered urethra in children is still considered experimental. The earliest studies were performed in the early 1990s and involved use of autologous urethral epithelial cells mounted on petroleum gauze or polytetrafluoroethylene tube and had dismal results [14, 15]. In 1999, Atala et al. reported the use of bladder submucosal, collagen-based inert matrix for urethral repair in four boys with history of failed hypospadias surgery [16]. The collagen matrices were obtained from cadaver bladders after processing to render them noncellular and nonimmunogenic. They were used in an onlay fashion over the urethral plate and the length of the neourethra ranged from 5 to 15 cm. At 22 months, except for one boy who had a subglanular fistula, the remaining three boys had a successful outcome. Much later, in 2009, Li et al. used gelatin sponge in combination with tissue from prepuce or urethral plate to reconstruct the urethra, supported by a local flap [17]. The mean repaired length was smaller, about 3.4 cm and over a follow-up period of 2–24 months, none had a fistula or a stricture although slight penile curvature was noted in one patient.

Raya-Rivera et al. reported use of cell-seeded tubularized PGA:PLGA scaffolds in five boys with posterior urethral defects [18]. Autologous epithelial and muscle cells were obtained from a tissue biopsy and were expanded in culture before being seeded. After posterior urethroplasty, urethral biopsies at 3 months revealed a normal appearing architecture in the engineered grafts which remained functional for up to 6 years of follow-up. Long-term results of this type of graft are not yet known. In the next year, Fossum et al. reported long-term follow-up of 8 boys with severe hypospadias, who underwent urethroplasty with autologous urothelial cell-seeded acellular dermis [19]. At 7.25 years of follow-up, all had a good cosmetic appearance and all but one had bell shaped uroflowmetry curve. Though this study lacked a control group and had a small sample size, it indicated that the cell-seeded matrices have a good long-term outcome. Subsequently, Orabi et al. performed a pilot study of 12 patients with hypospadias (distal in six, mid-shaft in four, and proximal in two) who underwent a repair with four-layer prefabricated SIS as an onlay graft [20]. These boys were either

circumcised or had a failed previous repair. After a mean follow-up of 23 months, six patients had a successful repair, three had urethrocutaneous fistula requiring closure, and remaining three had complete disruption or stricture. The authors identified graft infection as the main cause of graft failure.

Till now, there is lack of well-designed studies with a control group on use of engineered urethra. The series reported thus far are retrospective reviews with a small sample size. The results are no better than those with the current techniques of repair which underline the need for further refinement in technology.

30.5 Future Outlook

The evolution of biomaterials for urethral regeneration began with non-biodegradable materials which were very soon replaced by biodegradable scaffolds. However, the transition of biodegradable materials from preclinical to clinical studies failed the expectations. Composite materials involving natural and synthetic components improved the mechanical and biological properties suited for urethral regeneration. As the technology evolved further, the last decade saw a rise in "smart" biomaterials which respond reversibly to temperature, pH, light, and ionic strength. These responses include gelation, reversible adsorption, collapse, and alteration between hydrophobic and hydrophilic states. Further, incorporation of peptides into the structure of the smart biomaterials can create 3D scaffolds for synthesizing scaffolds that closely mimic the native ECM.

Thermo-responsive polymers change volume or phase with change in temperature. They are precipitated above a lower critical solution temperature, becoming hydrophobic while they remain hydrated and hence hydrophilic below the critical temperature. Shape-memory polymers change shape in response to a stimulus such as heat. Similarly, electroconductive polymers may be useful to regenerate electrically active tissue such as muscle. A collaborative effort between the clinics and the laboratory will pave way for the advent of biomaterials with successful bench-to-beside application.

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