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Successful engraftment of epithelial cells derived from autologous rabbit buccal mucosal tissue, encapsulated in a polymer scaffold in a rabbit model of a urethral stricture, transplanted using the transurethral approach

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ABSTRACT

Background: A pilot study reported an autologous buccal mucosal cell transplant in humans through the trans-urethral route using the buccal epithelium expanded and encapsulated in scaffold—hybrid approach to urethral stricture (BEES-HAUS), a minimally invasive approach to treat urethral stricture. Although successful outcomes were achieved in that study, for further validation, it is essential to prove that the transplanted buccal epithelium was engrafted over the urothelium through histological examination of the urethra, harvested post-transplant, which is infeasible in humans. Herein, we report the successful creation of an animal model of urethral stricture and the engraftment of epithelial cells derived from autologous buccal mucosal tissue, encapsulated in a thermo-reversible gelation polymer (TGP) scaffold, transplanted by trans-urethral route.

Methods: An animal model of urethral stricture was created in Japanese white male rabbits using electrocoagulation. Buccal tissue was harvested from the rabbits and subjected to enzyme digestion, followed by 5–7 days of in vitro culture in conventional two-dimensional (2D) culture and in a 3D platform of thermo-reversible gelation polymer (3D-TGP) culture. The cells harvested from the groups were mixed and encapsulated and transplanted with TGP, by transurethral catheterization. Fourteen days later, the urethra was harvested and subjected to histological examination. The buccal biopsy tissue, cells after digestion and cells post-culture were also subjected to histological examination. Urethrogram and endoscopy images were recorded at different time points.

Results: The stricture was successfully created, with the coagulated area markedly stenosed. Histological staining of the cells after in vitro processing showed that the cells grew with native epithelial and rounded cell morphology in 3D-TGP while they differentiated into fibroblast like-cells in 2D culture. Histological staining of the urethral tissue after transplantation revealed the engraftment of the

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transplanted buccal mucosal cells, with stratified squamous epithelium over the specialized stratified urothelium in the urethrotomy site.
Conclusion: We used histology to prove the successful engraftment of TGP-encapsulated buccal mucosal epithelial cells in an animal model of urethral injury with healing of the injured tissue. The model of urethral stricture and cell therapy, using a transurethral approach, recapitulates the previously reported BEES-HAUS approach and lays the foundation for larger multi-centric translational clinical studies.

1. Introduction

Urethral stricture is the narrowing of the urethral lumen due to ischaemic spongiofibrosis, which may have different aetiologies such as trauma, infection and inflammatory disorders. Conventional treatment approaches include dilatation, excision, primary anastomosis of the damaged segment with uniting of the two healthy ends, direct internal vision urethrotomy (DVIU) and substitution urethroplasty [1]. DVIU is less invasive and easier to perform than the other methods, but the procedure has a long-term success rate of only 20% [1]. In substitution urethroplasty, several types of grafts are employed, with buccal mucosa commonly used. Its success rate is 81% at 45-month follow-up. The complication rate is 12.5%, especially during penile urethroplasty, and urinary fistula, graft contracture and graft failure are common hurdles [2]. Novel potential treatment options include regenerative approaches, especially cell therapy-based approaches [2,3]. Minced buccal mucosal graft urethroplasty has been earlier reported [2] in this regard. Vaddi et al. reported a pilot clinical study using the BEES-HAUS [4] (buccal epithelium expanded and encapsulated in scaffold—hybrid approach to urethral stricture) technique which is a minimally invasive approach that showed successful outcome among four of the six patients in their study [4]. Reproducing the in vitro culture and transplantation processes is essential for training clinicians when applying the BEES-HAUS technique in large-scale multi-centric clinical studies, for approval as a routine clinical procedure. In addition, validation requires proving that the transplanted buccal epithelial cells engrafted over the urothelium contributed to the healing and regeneration in the stricture region. This can be done by histological staining, but doing so is infeasible in humans. Furthermore, a major hurdle is the lack of proper animal models which can accurately mimic the pathological situation seen in humans with a true stricture and a full-thickness ischaemic spongio-fibrosis, in which the BEES-HAUS technique can be tried before large-scale clinical translation. Therefore, we report herein the successful creation of urethral stricture in a rabbit model, which potentially could mimic urethral stricture in humans, following the protocol of Shinichi et al. [5] and the successful transplantation of autologous in vitro cultured rabbit buccal mucosal cells using the BEES-HAUS approach [4].

2. Materials and methods

All of the experiments were done after review and approval by the Institutional Animal Care and Use Committee of the National Defense Medical College, Saitama, Japan. The study population consisted of three Japanese white male rabbits weighing 2.5–3.5 kg. Fig. 1 shows a schematic illustration of the complete procedure from urethral stricture induction to transplantation.

The animals were anesthetized with intramuscular ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg). They were placed in the dorsal position, and retrograde urethrography was carried out (Fig. 2). The urethra was injured by electrocoagulation with spherical monopolar electrocautery, wherein a spherical electrode was inserted into the bulbar urethra from the external urethral meatus and used for 30 s of electrocoagulation at 40 W [5]. Retrograde urethrography and urethroscopy observations were carried out to confirm the injured urethral segments immediately and after 14 days to confirm the stricture's creation (Fig. 2). Buccal tissue was harvested from the rabbits' buccal mucosa on the 14th day after urethral stricture was inducted. The buccal mucosal tissues were transported to the cell-processing laboratory using a TGP polymer-based transportation cocktail reported by Rao et al. [6].

In the laboratory, the tissue was weighed, and a portion was sent for histological examination. Then, the remaining tissue was subjected to cell processing (Fig. 3), employing the protocol of Vaddi et al. [4], with modifications suggested by Katoh et al. [7]. Enzymatic digestion was performed using 1% P/S, 50 μg/ml gentamycin (GM) and 0.25 μg/ml amphotericin B (Amp B) in Dulbecco's phosphate-buffered saline (DPBS), after which the tissue was incubated at 4 °C for 30 min. Then, 15 ml of 1000 PU/ml Dispase II in D-MEM was added, and the tissue was incubated at 4 °C overnight (15–19 h). After 15–19 h, the epithelial layer was peeled off and minced into tissue bits less than 1 mm in diameter. The tissue bits were then digested using 1 ml Accutase incubated at 37 °C for 15 min. The digested sample was passed through an EASY-strainer™, and he flow-through was collected in a 50-ml tube. Centrifugation was performed at 400 g * 5 min * 20 °C. The supernatant was discarded, and the cell pellet was used for cell counting using the Trypan blue dye exclusion method. After the cells were counted, a small portion of the cells was sent for histological examination. Half of the remaining cells was cultured using a culture medium containing 10% foetal bovine serum (FBS), 1% P/S, 50 μg/ml GM, 0.25 μg/ml Amp B, 5 μg/ml insulin and 10 ng/ml epidermal growth factor (EGF) in D-MEM/Ham's F-12. This group was termed the two-dimensional culture (2D) group. The other half of the obtained cells using the TGP scaffold was termed the three-dimensional TGP (3D-TGP) culture. For this culture, 400 μL of TGP—reconstituted in D-MEM high-glucose medium with 1%P/S, 50 μg/ml GM and 0.25 μg/ml Amp B (for 10 ml) at a 1:9 ratio—was added into the centrifuge tube containing the digested cells, mixed well and placed in ice for the TGP to liquefy so that the cells would be immersed in the TGP. Then, 200 μL of cold-reconstituted TGP suspension was added to the centre of each well of a 24-well culture flask and allowed to solidify. The 400 μL of TGP with cells was overlaid over the previously added TGP alone in the culture well and was allowed to solidify. Both groups were cultured for 5–7 days, after which the cells in the 2D culture were harvested using Accutase and centrifuged at 400 g * 5 min * 4 °C to obtain the cell pellet. The culture plates from the 3D-TGP group were refrigerated at 2–8 °C and diluted with 2 ml of cold saline to take the TGP out of the gel phase. Then, the contents were centrifuged at 400 g * 5 min * 4 °C to obtain the cell pellet. A portion of the cells was sent for histopathological examination. The 2D and 3D-TGP cell pellets were mixed into 5 ml of TGP reconstituted in 0.9% normal saline for transplantation.

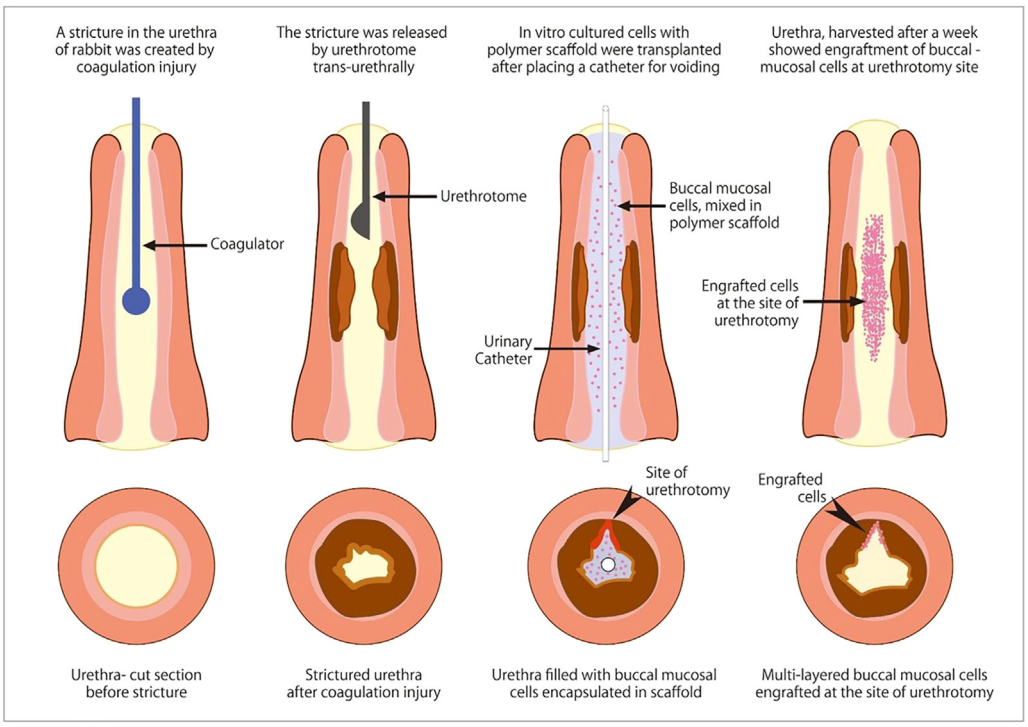

Fig. 1. Schematic illustration of the urethral stricture's creation, urethrotomy, TGP-encapsulated cell transplantation and post-harvest outcome.

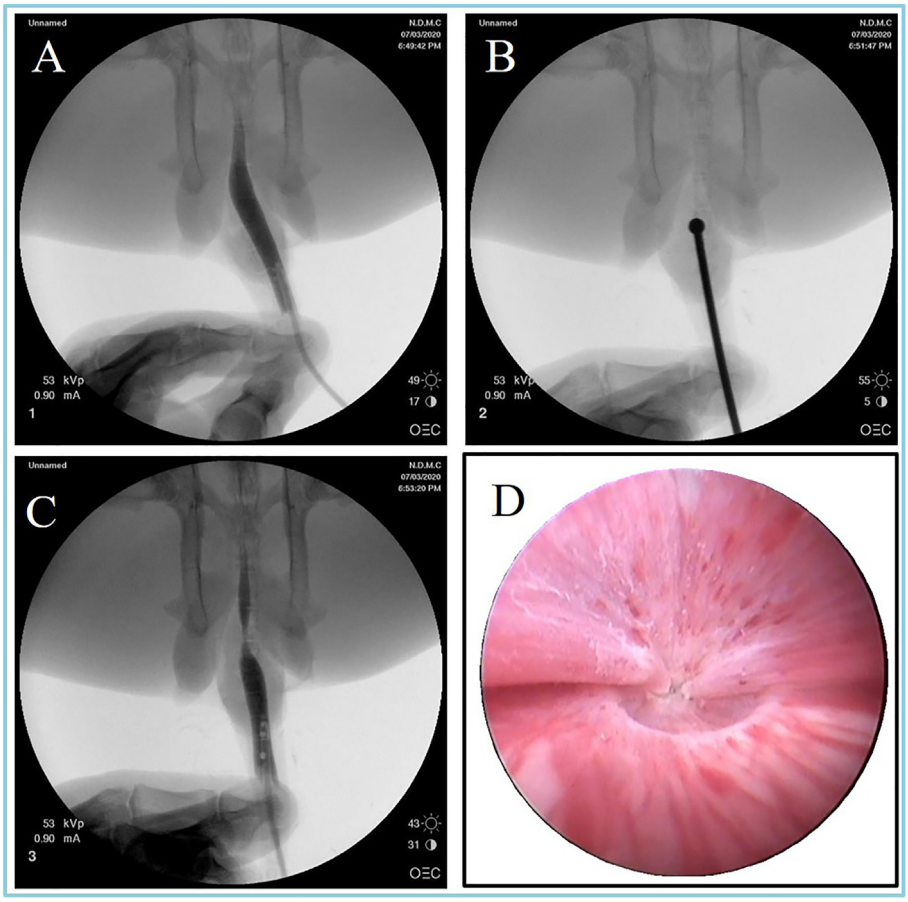

Fig. 2. A: Pre-stricture urethrogram; B: Electro-coagulator inside the urethra; C: Post-coagulation image showing the stricture - narrowing of the urethra; D: Endoscopy showing the stricture inside the lumen.

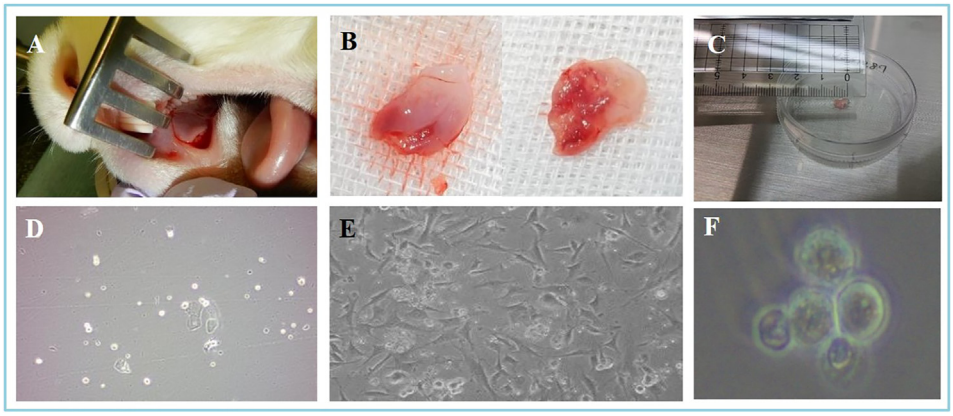

Fig. 3. A: Punch biopsy to harvest rabbits' buccal tissue to obtain mucosal epithelial cells; B: Harvested buccal mucosal biopsy tissues; C: Buccal tissue sample in the laboratory before processing; D: Cells immediately after digestion, before starting the in vitro culture. E and F: Cells during in vitro culture; E showing 2D cultured cells with fibroblast-like morphology (×10 magnification) and F showing 3D-TGP cultures exhibiting rounded cells and cells with epithelial morphology (×200 magnification).

For the transplantation, anaesthesia was induced in the rabbits using intramuscular ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg). Urethrograms and video endoscopy were recorded (Fig. 4). Urethrotomy was performed using an A3558 urethrotome (Olympus, Japan). Fresh bleeding was observed. A catheter was fixed. The cells embedded in TGP were injected between the catheter and the urethral stricture site (Fig. 4G and H). The catheter was removed on the third day. After 2 weeks, a urethrogram was taken, the animal was sacrificed, and its urethra was harvested. The harvested urethra was sent for histological examination, specifically for haematoxylin and eosin staining.

3. Results

The urethral stricture induction was successful and was similar to ischemic-spongiofibrosis occurring in humans, as confirmed by urethrogram and video-endoscopy (Fig. 2). The urethral lumen at the coagulated site was markedly stenosed (Fig. 2D). Approximately $0.67-1 \times 10^6$ cells were used for the transplantation with TGP in the rabbit. The cells in the 2D group had several cells with a fibroblast-like morphology (Fig. 3E), while the cells in the 3D-TGP group were rounded, apart from cells that had the native buccal epithelial morphology (Fig. 3F). Histological staining of the buccal tissue biopsy and cells immediately after digestion was positive for native squamous epithelial cell morphology (Fig. 5A). Histological staining of the cells after tissue digestion and before the transplant exhibited the maintenance of the native buccal squamous epithelial cell morphology (Fig. 5B and C). We were able to confirm that the transplanted buccal squamous epithelial cells were successfully engrafted, with the histological staining of the harvested urethra showing their engraftment over the host urethral epithelium (Fig. 6), which was a specialized stratified epithelium, with the urothelium consisting of umbrella-shaped cells in the superficial layer [8] and the engraftment region exhibiting the native buccal squamous epithelial cell morphology consisting of a stratified squamous epithelium [9], similar to the buccal tissue biopsy (Fig. 5A) initially obtained from the rabbit, in one of the animals. Three days after the cell transplantation, the catheter was removed. Harvesting of the urethra 14 days post-transplant showed that no intact TGP was left behind inside the urethra.

4. Discussion

Tissue engineering is considered an alternative for buccal mucosal substitution urethroplasty for urethral reconstruction [3]. Some tissue-engineering approaches have been reported in earlier research. For instance, during liquid buccal mucosa graft endoscopic urethroplasty, mechanically minced buccal mucosa micrograft suspended in fibrin glue was injected during urethrotomies [4]. A biodegradable protein containing scaffold is used in tissue-engineered autologous oral mucosa grafts [10]. The risks of using biological scaffolds and their associated contamination warrant the development of procedures that employ synthetic scaffolds. Vaddi et al. [4] overcame the disadvantages of employing biological scaffolds by using a synthetic TGP scaffold in their BEES-HAUS approach, which they reported to be an advantageous and less invasive approach, in a human pilot clinical study. In that study, the authors used autologous buccal epithelial cells from a small buccal mucosal biopsy that were cultured in vitro, encapsulated in a synthetic 3D-TGP scaffold and implanted at the stricture site after a wide endoscopic urethrotomy. The procedure was successful in four of six patients [4], yielding more than a three-year recurrence-free interval. Proving the engraftment of transplanted buccal epithelial cells onto the urothelium becomes necessary to validate the technique, which can be done by simple histological staining as the buccal mucosal epithelium is a stratified, non-keratinizing squamous epithelium [9], while urothelium is a specialized stratified epithelium consisting of umbrella-shaped cells in the superficial layer [8]. Furthermore, in order to repeat recapitulate the technique and use it in larger multi-centric clinical studies, clinicians require animal models to practice the transplantation technique. Note that the lack of animal models for simulating human urethral strictures has impeded the proper clinical translation of regenerative and cell-based urethra therapies. The techniques used to create strictures, which usually comprise electrocautery or other electro-coagulation methods, do not yield reproducible strictures and cause complications [11,12]. The other animal studies on similar lines have proven the engraftment of minced oral mucosal tissue [2] in strictured urethra but not free oral mucosal epithelial cells, to our knowledge. We followed the method reported by Shinchi et al. [5] and successfully formed strictures. TGP-encapsulated cell transplantation was performed without hurdles. We grew the cells in both 2D culture and 3D-TGP using a modified BEES-HAUS in vitro culture procedure, especially in terms of overnight tissue digestion (Katoh et al. [6]), while Vaddi et al. employed a digestion protocol of 1 h and 15 min duration in the BEES-HAUS study [4]. In addition, the native epithelial morphology was maintained in the 3D-TGP during the culturing, as were the rounded cells, which could represent the progenitor

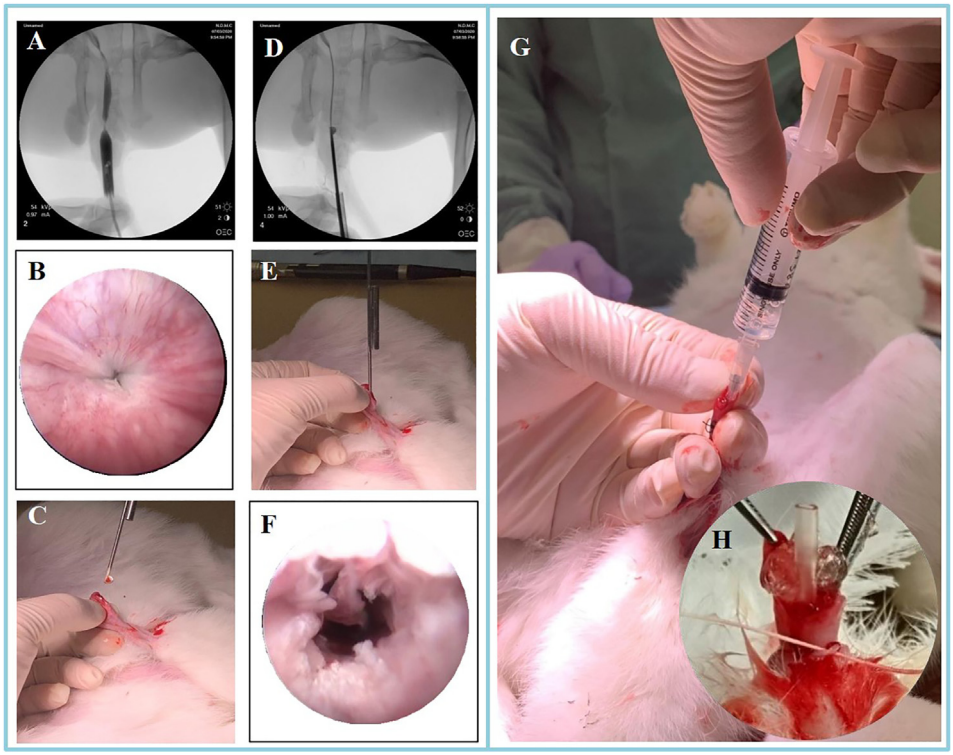

Fig. 4. A – Urethrogram confirming the stricture before urethrotomy; B- Video endoscopy of the stricture before urethrotomy; C – Urethrotome; D – Urethrogram showing the urethrotome inside urethral lumen; E– Urethrotomy underway; F- Urethral lumen after urethrotomy, showing released fibrous adhesive bands; G – Transplantation of the cells encapsulated in TGP. H (Insert) Gel-cell mix spilling through meatus from the urethral lumen between urinary catheter and the lumen.

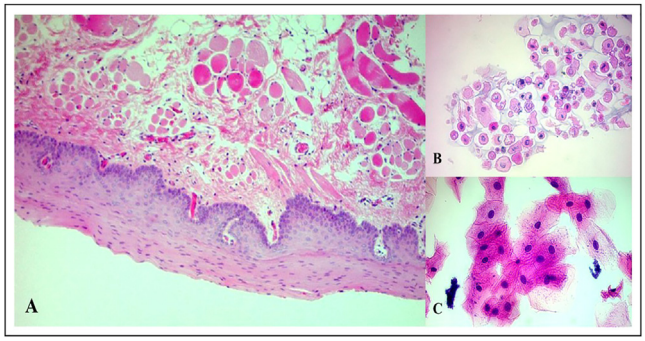

Fig. 5. Histological (Haematoxylin and eosin) staining images. A. Buccal tissue biopsy collected from rabbit before in vitro cell culture exhibiting squamous epithelial morphology. B. Buccal mucosal squamous epithelial cells immediately after in vitro processing. C. Cells after in vitro culture but before transplantation, which maintained the squamous epithelial morphology. All images at ×10 magnification.

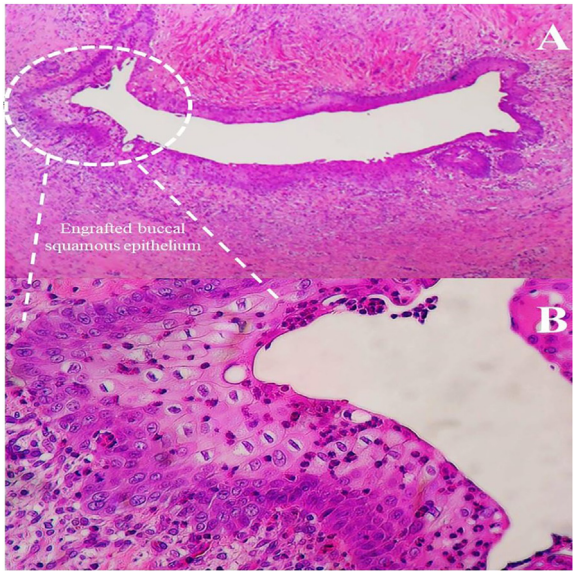

Fig. 6. A: Successful engraftment of autologous buccal mucosal squamous cells over the host urethral epithelial tissue in histological (Haematoxylin and eosin) staining of post-transplantation urethral tissue (×20) 14 days post cell-TGP transplant; B. Enlarged (×100) image showing multi-layered squamous epithelium of buccal mucosa, engrafted at the site of urethrotomy.

cells, but this requires further studies for confirmation. Regarding the animal model's creation, the urethral-stricture model captures what occurs in humans [4]. Procedurally, injecting TGP-encapsulated cells after fixing the catheter was easy to accomplish.

The successful engraftment was confirmed in one and in the other two rabbits we believe that unlike humans, rabbits could not be catheterized for a long period of time after treatment because they are prone to urinary tract stones, and the urinary catheter had to be removed as soon as possible, could be one of the reasons which could have marred the engraftment of the transplanted cells. Nevertheless, we believe that the success of one case is noteworthy and deserves to be reported. Further, whether the administration of oral mucosa cells will heal the stenosis in two weeks we do believe that though it is premature to evaluate it at two weeks post-operatively, since oral mucosa cell engraftment itself could be proven at two weeks postoperatively, this technique is worth recommended to be extended to larger studies.

5. Conclusion

We were able to efficaciously recapitulate an animal model of urethral stricture that resembles stricture occurring in humans. Post-urethrotomy transplantation of the TGP-encapsulated cells showed the engraftment of the transplanted buccal mucosal cells over the urothelium at the urethrotomy site, successfully yielding optimal healing of the damaged urethral lumen. Based on this experience, multi-centric human clinical trials in line with the earlier BEES-HAUS study are now considered feasible.

Ethics and animal rights

The animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European Communities Council Directive of 24 November 1986 (86/609/EEC) or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Further, the study complies with the ARRIVE guidelines.

Declaration of competing interest

Authors Dr. Abraham and Dr. Katoh are inventors of several patents on biomaterials including the one described in the manuscript.

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