Histological and immunohistochemical changes in the rat oral mucosa used as an autologous urethral graft

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Abstract

Purpose: The purpose of this study was to determine the histological and functional (immunohistochemical) changes that take place in oral mucosa grafts implanted in the rat urethra.

Methods: Urethroplasty was performed in 26 male Wistar rats weighing 250 g. All animals received autologous oral mucosa urethra grafting under general anesthesia. Samples were analyzed 10, 20, 30, 40, 50, 60, 90, and 120 days after surgery using light and scanning electron microscopy and immunofluorescence for the determination of the expression of epithelial markers (pancytokeratin, cytokeratin 1, 4, 13, and filaggrin).

Results: Grafted oral mucosa tissues were subjected to significant histological changes from the beginning with the formation of a well-developed epithelium whose structure was comparable to the native urethra from day 60 of the surgical implant. The immunofluorescence analysis demonstrated that the cytokeratin expression profile tended to mimic the pattern of the native urethra. These data suggest that the oral mucosa is able to efficiently transdifferentiate to the urethral environment.

Conclusions: The efficient transdifferentiation process of the grafted oral mucosa at both the histological and immunofluorescence levels, and the absence of local complications confirm the clinical usefulness of this type of tissues for the repair of the urethra.

Finding an appropriate donor site for urethroplasty procedures has always been a challenge. Currently, one of the tissues that is most currently used as a urethral graft is the oral mucosa [1–5]. Different experimental studies in animals [6] demonstrated that the use of oral mucosa grafts was associated with low complication rates. However, the structural and functional differences between the epithelium of the graft and the epithelium of the tissue where the graft is implanted [7] could lead to complications and functional impairment.

In this context, several reports have identified important structural differences between the oral mucosa and the urethra, especially regarding the number of cell layers and the epithelial organization. Adding to this, recent studies describe the existence of histological changes in patients...
undergoing oral mucosal grafts for urethral reconstruction in two stages (Bracka surgery) [8]. Finally, a published work in which oral mucosa was used during the urethroplasty procedure in rabbits [9] showed that there was a complete integration of the grafted oral squamous epithelium with the recipient urethral epithelium, and the histological characteristics of the graft did not significantly change.

To our knowledge, most of the previously published studies focused on the description of the major macroscopic and microscopic changes that take place in the implanted graft and on the assessment of the functionality of different urethroplasty techniques on the urinary flow [10]. However, none of them describes the key immunohistochemical protein expression changes that occur during the biointegration process and the histological and immunohistochemical changes that take place in grafts and recipient urethral tissues. Particularly, the study of specific oral mucosa markers such as cytokeratins and filaggrin (a cytokeratin-associated cytoskeleton fiber) has not been carried out to this date. Since the expression of these epithelial differentiation markers is tissue-specific and may vary among different epithelia, a time-course sequential expression study is in need to determine if protein expression tends to mimic that of the grafted urethra.

In this work, we have analyzed the histological and immunohistochemical protein expression changes that take place after urethral reconstructions using oral mucosa grafts in Wistar rats in order to identify the structural, histological and functional changes that may happen in the urethra at increasing follow-up periods to determine if these changes vary with time.

1. Materials and methods

1.1. Animals

We used a total of 29 (26 urethroplasties included in the study) male Wistar rats weighing 250 g provided by Harlan® laboratories and maintained in the Experimental Unit of the University Hospital Virgen de las Nieves (FIBAO).

In this study, two animals were used as controls. In these cases, the urethra was ventrally incised (incision of 0.4 cm of length from about 3 mm) and primarily repaired using 7/0 Safil quick® suture stitches without implanting any graft tissue. The rest of the rats were subjected to surgical intervention as described below. After a follow-up period of 10, 20, 30, 40, 50, 60, 90 or 120 days post surgery, one group of animals (3 rats per group) was euthanatized under general anesthesia for histological analysis. Both control animals were euthanatized after 30 and 90 days.

This study and its procedures were approved by the Institutional research and ethics committees, and the animal care and use committee. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) regarding the care and use of animals for experimental procedures.

1.2. Surgical procedure for urethral reconstruction

Urethral reconstruction using oral mucosa grafts was performed under general anesthesia by induced by intraperitoneal injection of a mixture of 100 mg/kg ketamine, 0.05 mg/kg atropine and 1 mg/kg acepromazine.

The surgical technique was carried out as follows (Fig. 1): 1) removal of an oral mucosa graft of approximately 0.4 × 0.3 cm size from the animal’s lower lip; 2) repair of the mucosa defect using 7/0 Safil quick® suture; 3) urethral catheterization with a 3F venous catheter and fixation to the glans; 4) penis skin circular incision approx. 2 mm below the coronal sulcus, sliding the skin to the base of the penis to expose the urethra; 5) longitudinal ventral urethral incision of 0.4 cm in length from about 3 mm below the sulcus, in a proximal direction; 6) onlay implant of the oral mucosa graft patch on the ventral urethral face defect using 7/0 Safil quick® suture stitches on both sides of the urethral incision; the grafted area was labeled using non-absorbable stitches to facilitate the identification of the grafted tissue after the follow-up period for histological study; 7) repositioning the penis skin to the coronal sulcus to cover the urethra and the graft, and reparation of the skin using the same suture material. The skin suture was placed distally to the graft so that no extra suture material was placed on the graft; 8) the catheter was removed from the urethra.

The same surgical technique was carried out in the two control animals, but no oral mucosa graft was implanted. In addition, the urethral tissue allocated proximal and distally to the operated area of each animal was also used as controls. The oral mucosa tissue excised from these sham animals was used as control tissue for the histological and immunohistochemical analyses.

1.3. Evaluation by light and electron microscopy

Immediately after euthanasia, the rat penis was dissected and tissue samples corresponding to native urethra (controls) and grafted tissue were fixed in 4% buffered formaldehyde for histological analysis. The grafted area of the urethra was identified by the presence of the marker stitches proximally, distally and at the lateral margins of the graph and a biopsy was taken of this area to include the graft and the native urethra.

Samples were dehydrated and placed in paraffin. Tissue sections of 4 μm thickness were stained with haematoxylin and eosin for histological examination using a light microscope. In each case, the structure of the epithelial and stromal layers was analyzed, and the following parameters were recorded: integrity of the epithelium, number of cell layers, type of epithelium, presence of cell apoptosis or
necrosis, and presence of inflammatory infiltrates. The urethral areas allocated proximally and distally to the graft were used as control tissues.

For scanning electron microscopy, samples were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 24 h. Then, samples were washed twice in 0.1 M phosphate buffer (pH 7.2) at 4 °C, dehydrated in increasing concentrations of acetone (30%, 50%, 70%, 95% and 100%), and completely dried by using the critical point method. Once dried, tissues were covered with gold and analyzed in a scanning electron microscope FEI Quanta 200 using the high vacuum mode.

1.4. Evaluation of markers of epithelial differentiation by immunofluorescence

To evaluate the differentiation level of the urethral and oral epithelium, we analyzed the expression of pancytokeratin (PanCK), cytokeratin (CK) 1, 4, 13 and filaggrin in the different tissues using conventional immunofluorescence methods. Different authors previously demonstrated that these cytokeratins are expressed in native control oral mucosa and in other mucosal epithelia [11,12]. Tissue samples were deparaffinized in xylene, washed in alcohol and rehydrated with water. Then, nonspecific sites of antibody binding were blocked by incubation in bovine serum albumin. Once washed, tissue sections were incubated in pre-heated buffer citrate for antigen retrieval. Then, samples were incubated with primary antibodies for 2 h at room temperature, and then washed in PBS 1×. After that, a fluorescently labeled secondary antibody was applied and samples were washed again in PBS 1×. Finally, samples were mounted using DAPI and glass coverslips and analyzed in a Nikon Eclipse 90i fluorescence microscope. Positive and negative controls were used for each immunological reaction, and the urethral areas allocated proximally and distally to the graft were used as control tissues.

The evaluation of the results was done by determining the presence of positive signals at two levels: 1) location of the fluorescent signal: uniform in all epithelial layers, signal in basal layers only, signal in suprabasal layers only, and 2) intensity of the signal: (−) no expression, (+/−) slightly positive, (+) positive, (+++) very positive, (++++) strongly positive. All assessments were made by two independent histologists and, in case of disagreement, by a third party.

2. Results

2.1. Surgical procedure of urethral reconstruction by oral mucosa grafts

Good tolerance to the anesthetic procedure was found in 27 animals, and 2 rats died during anesthesia. These 27 animals corresponded to the 2 control rats and 25 animals in which an oral mucosa patch was grafted in the urethra.

Postoperatively 25 rats were free from complications (no evidence of urethral or meatal stenosis, penile curvatures or abnormal scarring). There were two complications: a local infection which led to graft loss 48 h after surgery in one rat (this animal was excluded from the study and replaced by a new animal that did not show any complications), and an urethral fistula occurred in one rat that apparently did not affect the final analysis of the sample, corresponding to a 30-days animal.

2.2. Evaluation by light microscopy

As shown in Fig. 2, histological analysis of the oral mucosa grafted into the urethra demonstrated adequate levels
of biointegration of the oral tissues in the implant sites. There was no evidence of tumor, immune rejection or necrosis after 10, 20, 30, 40, 50, 60, 90 and 120 days of surgical reconstruction.

After 10 days of grafting we observed a proper integration of the graft, although the epithelium of the graft had around 10 cell layers, whereas the native oral mucosa had between 15 and 30 cell layers. In the stroma, a slight inflammatory infiltrate composed of lymphocytes and neutrophils was observed, and a large number of newly formed blood vessels were detected. Histological analysis at day 20 showed adequate levels of integration in all tissue layers, with a stroma rich in collagen fibers and fibroblasts accompanied of abundant blood vessels, and a stratified squamous epithelium that was histologically similar to that of the oral mucosa. 30 days after the intervention, we observed well-structured stromal and epithelial tissues, whose morphology and structure were still similar to those of the oral mucosa control. In comparison to the native urethral epithelium, the graft showed more acidophilic cytoplasm and the epithelial cells formed a stratified squamous epithelium with two or three cell layers. However, at 40 days after the surgery, we observed that the epithelial cells of the grafted tissue became cubic-shaped, with the number of cell layers progressively decreasing as compared to the native oral epithelium. The same phenomenon was observed at postoperative day 60. The histological evaluation at 90 days demonstrated the proper integration of the oral mucosa grafts into the recipient urethral wound-bed, with an epithelium whose structure was very similar to the control native urethra. In the same way, the structure of the stromal tissue was identical to that of the native urethra. Similarly, the evaluation at 120 days post-implantation showed that the biointegration of the graft was complete, with the formation of a urethral-like epithelium and numerous blood vessels.

2.3. Evaluation by scanning electron microscopy

The histological evaluation of control oral mucosa and urethra samples using scanning electron microscopy (SEM) showed that the most superficial epithelial layer of both tissues was structurally different. As shown in Fig. 3, the epithelium corresponding to oral mucosa showed clear signs of cell desquamation and substitution of the desquamated cells by inner cell layers (Fig. 3A). In contrast, the urethral epithelium did not show any evidence of epithelial cell desquamation (Fig. 3B).

The SEM analysis of the grafted urethras showed that the oral mucosa epithelial cells tended to maintain their phenotype during the first 50 days, with the presence of...
surface differentiation patterns in these cells. As shown in Fig. 3D and E, the transition area between oral mucosa epithelial cells and urethral epithelial cells was detectable during the first 50 days post-grafting. However, from day 60 on, no structural differences could be detected, and the transition area between oral mucosa and urethral cells was not detectable using SEM.

2.4. Evaluation of markers of epithelial differentiation by immunofluorescence

The analysis of several epithelial differentiation markers revealed the existence of significant differences in the expression patterns between native oral mucosa samples and control urethra tissues (Table 1). In the case of oral mucosa controls, we found positive expression of PanCK, CK 1, 4, 13 and filaggrin. However, control urethral tissues only expressed low amounts of CK13, with very low expression of PanCK and CK4 (Fig. 4).

The epithelial differentiation analysis of urethral tissues reconstructed using oral mucosa grafts demonstrated a gradual turnover of the cell differentiation process (Table 1). Initially, 10 and 20-days samples showed negative expression of CK4 and filaggrin (Fig. 4). Nevertheless, after 30 days of urethral grafting, CK4 and filaggrin were detected, accompanied by CK1 and CK13 positive expression (Fig. 4).

After 40 days post implantation, a significant decrease of the cytokeratin expression pattern was observed for PanCK, CK4 and CK13. In the case of CK1 and filaggrin expression, our results showed that the oral mucosa tissues grafted between 40 and 50 days tended to express lower levels of CK1 and low amounts of filaggrin. From day 60 after the surgical procedure, the grafted tissues were completely negative for both CK1 and filaggrin. Finally, 120-days samples showed slight expression of CK4 and 13 and negative expression of PanCK, CK1 and filaggrin (Table 1, Fig. 4).

<table>
<thead>
<tr>
<th>Postoperative time</th>
<th>PanCK</th>
<th>CK4</th>
<th>CK13</th>
<th>CK1</th>
<th>Filaggrin</th>
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Table 1 Protein expression of different epithelial markers in controls and grafted tissues after different times from the surgical procedure.
3. Discussion

The use of oral mucosa to repair different congenital and acquired defects of the urethra has been of great interest [13], and different grafting techniques have been previously described [14,15]. In the present work, our results demonstrated a progressive histological change in the morphology of the oral mucosa epithelium grafted to the urethra, which becomes indistinguishable from the native uro-epithelium. One possibility is that repair may have occurred by shrinkage of the operative defect and replacement by native uro-epithelium. However, we think that these changes should not be interpreted as graft shrinkage or disappearance, but as structural changes in the grafted tissues during the study period, since all grafts were properly labeled by using suture markers in the grafts limits to ensure their future identification during the follow-up period. While the grafted tissues were initially formed by a typical stratified oral mucosa epithelium with clear signs of epithelial desquamation, these tissues tended to progressively acquire phenotypes and functions that are characteristic of the epithelium of the native urethra. In addition, the procedure was very well tolerated by all animals, suggesting that this may be a good model for the study of urethral tissue grafting.

When the implanted tissues were histologically analyzed, we found that the oral mucosa graft tends to properly adapt to the urethral environment with the absence of relevant complications. Initially, slight signs of inflammatory reaction were detected, but these tended to progressively disappear. In addition, the grafted tissues tended to develop a high number of newly-formed blood vessels, with stromal collagen enrichment with abundant fibroblasts in the connective tissue. All these factors are very important for the success of a grafting tissue reconstruction, and suggest that both the graft and the recipient sites properly adapted to the new situation and tended to adequately integrate. In this case, our analysis showed that the transition area between both epithelia (the oral mucosa and the urethral epithelia) tended to progressively disappear, and the surface of both epithelia finally became indistinguishable. These results are in agreement with previous work by Souza et al., who described that the inflammatory process tended to completely disappear after 6 weeks of the implant in a model of oral mucosa urethroplasty in rabbits [9]. In this study, the authors noted that the junctional area of the urethral and oral tissues was still evident after 6 weeks. We hypothesize that the junctional area could have become histologically indistinguishable after a longer follow-up period, as was observed in our study. In addition, previous work published by Mokhless et al. also showed that oral mucosa tissues grafted into the urethra in humans tended to properly integrate, and that these tissues eventually show several histological changes, including focal keratinization and epithelial hyperplasia.

All these reports confirm that the oral mucosa is an adequate source of tissue for urethral reconstruction [8]. Moreover, our protein expression analysis using immunofluorescence demonstrated that the grafted oral mucosa sequentially acquired the characteristics of the native rat

![Fig. 4](image-url) Analysis of protein expression of several epithelial markers (CK13, CK1 and Filaggrin) on grafted tissues at different times of study and in oral mucosa and urethra controls. Magnification of each microphotograph is 100x (*), 200x (**) or 400x (***)
urethra. Initially, our analysis of the control tissues confirmed that native oral mucosa expressed PanCK, CK1, 4, 13 and filaggrin, with CK13 expression restricted to the cornified epithelial layer. However, control urethral epithelium was negative or showed very low expression of these markers, with slight expression of CK13 in all the epithelial thickness. When oral mucosa grafts were implanted in the urethra, these tissues tended to maintain some characteristics of the native oral mucosa, although the intensity of the expression of PanCK, CK4, 13 and filaggrin tended to decrease at day 10. This protein expression decrease could be associated with the initial inflammatory response of the host and the adaptation of the tissues to their new environment in the rat urethra. In this regard, the new situation of the implanted tissues would probably generate a hypoxic environment in the grafts during the first weeks, until a novel vascular network is formed. In addition, the reduction in the number of epithelial cell layers that we noted in these tissues was previously described by Leslie et al., who found that grafted tissues tended to form thinner epithelial linings with few cell layers after the urethroplasty with tunica vaginalis in rabbits [16].

Our sequential analysis of the grafted oral mucosa tissues at increasing periods of time revealed that the protein expression profile of the grafts tended to progressively reproduce the profile of the urethral epithelium. Thus, samples at day 20 and 30 still expressed high amounts of CK1, and recovered the expression of some epithelial markers such as filaggrin and PanCK, although the expression levels were lower than those of the oral mucosa controls. At the same time, the expression of CK13 in the grafted epithelium became more regularly distributed throughout all epithelial layers of the graft, being negative for the cornified layer. This distribution is more similar to the native urethra than to the oral mucosa. These results show that the grafted tissues at these time periods maintain certain expression profiles that are typical of the native oral mucosa, with a combined expression of urethral epithelium markers. All these data suggest that the implanted grafts may have an intermediate level of differentiation between the oral stratified epithelium and the urethral epithelium at this time of urethral reconstruction.

Finally, the long-term analysis of the oral mucosa tissues grafted at the urethral level demonstrated that the implanted tissues were able to adapt to the urinary tract after 60 days following implantation. From day 60 and during all the follow-up periods of the study, the analysis of the grafted tissues showed that the implanted epithelium was structurally similar to that of the native urethra, suggesting that this tissue efficiently transdifferentiated from an oral mucosa-like multistratified cornified epithelium to a non-cornified epithelium with a lower number of epithelial cell layers. This change in the number of cell layers is in agreement with previous reports in which urethroplasty with tunica vaginalis was described [16]. In this regard, transdifferentiation has been defined as a capacity of an adult mature cell or tissue to differentiate to another adult cell or tissue of a different lineage with no return to an undifferentiated progenitor cell stage [17,18]. According to our study, oral mucosa epithelial cells are able to efficiently transdifferentiate to urethral epithelial cells after 60 days following the implant, not only at the structural level, but also at the protein expression level. Most likely, the exposure of the implanted tissues to the new urethral environment and the paracrine signaling that the tissues receive at the urethral level could influence the process of transdifferentiation of the graft. The urinary environment is very different to that of the mouth, with different pH, enzymatic composition and mechanical stress, and all these factors probably influenced or induced the process of urethral transdifferentiation. These findings suggest that oral mucosa tissues grafted at the urethral level can efficiently transdifferentiate after 60 days. Caution should be taken before extrapolating these results to the human especially in cases in which a two-stage urethral reconstruction is carried out (i.e. inlay urethroplasty), since the situation of the grafts is different in our study using this technique and due to the different biology of the human and the rat.

Although these results should be confirmed in longer term studies in laboratory animals to determine the possible genetic changes that may occur over time, our data suggest that the oral mucosa implanted into the urethra in the rat model undergoes changes which make it indistinguishable from the native uro-epithelium. Nevertheless, one of the limitations of this study is the low number of animals in each study group. If the animals were analyzed in larger groups corresponding to fewer follow-up periods, the differences would probably be more evident.

The efficient transdifferentiation process of grafted oral mucosa at both the histological and the immunofluorescence levels and the absence of local complications support the potential clinical usefulness of this type of tissues for the repair of the urethra.

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