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## Original article

## MMP-2 and TIMP-2 expression, quantitative analysis and biomechanical changes in scar hypertrophy after autologous free transplantation of rabbit oral mucosa and scrotal skin



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## ABSTRACT

This study aimed to investigate the long-term scar hypertrophy in the rabbit transplanted oral mucosa and scrotal skin with changed matrix environment, as well as the scar location expression, quantitative analysis of matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2) and biomechanical changes in the transplanted tissues. The split-thickness skin grafts were collected from the oral mucosae and scrotal skins of 30 male rabbits, and prepared into reelpipes for autologous transplantation into the rabbit back muscular tissues. Samples were collected to carry out elastic tensile mechanical detection and histological observation. The maximum longitudinal tensile displacement of scrotal skin before 8 weeks of transplantation was greater than that after 8 weeks of transplantation ( $P < 0.05$ ). The expression intensities of MMP-2 and TIMP-2 in the oral mucosa and in scrotal skin at 2 W time point were higher than those at  $T_0$  time point ( $P < 0.05$ ). The expression quantities of TIMP-2 in oral mucosa and scrotal skin during 8–24 W were higher than those of MMP-2 ( $P < 0.05$ ). At 8 W time point, the TIMP-2/MMP-2 ratio in scrotal skin was higher than that in oral mucosa ( $P < 0.05$ ). MMP-2 and TIMP-2 expression in normal oral mucosa and scrotal skin is weak, but their expression is remarkably up-regulated after 2 weeks of transplantation, revealing that scar formation was related to the high expression of MMP-2 and TIMP-2. At the 8th–24th weeks, the AOD values of TIMP-2 in oral mucosa and scrotal skin are apparently higher than those of MMP-2; moreover, the TIMP-2/MMP-2 ratio in scrotal skin at the 8th week was higher than that in oral mucosa, which can well explain the earlier scar formation in scrotal skin than in oral mucosa, and it also suggests that the different expression levels between TIMP-2 and MMP-2 may account for the important cause of scar formation.

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## 1. Introduction

Hypospadias is a common congenital malformation of male reproductive system. The incidence rate of hypospadias is about 0.3% (Barbagli et al., 2017; Zheng et al., 2017; Horiguchi, 2017; Nelson et al., 2005; Li et al., 2003; Baskin, 2008; Sugarman et al., 2015). The main clinical manifestations are chordee, dorsal

prepuce covering the glans penis and abnormal urethral external orifice located at any position of the ventral side of the penis. The farther the ectopic urethra is from the head of the penis, the more serious the urethral defect and the curvature of the penis. According to the abnormal position of external urethral orifice, it can be divided into subcoronal type, midshaft type, and penoscrotal type. According to the different positions of the abnormal external urethral orifice, reasonable and effective repair methods of hypospadias should be selected. At present, oral mucosa free transplantation is frequently used to reconstruct part of the urethral inner wall in hypospadias patients; at half a year after the survival of the one-stage mucosal free transplantation, the two-stage urethral anastomosis will be completed to reconstruct the urethral defect. For the transplanted mucosa in the changed matrix environment, effective evaluation on its scar formation, as well as the scar location expression of the matrix metalloproteinase-2 (MMP-2)/tissue inhibitor of metalloproteinase-2 (TIMP-2) and

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biomechanical changes in the transplanted tissues is lacking. Particularly, the erection phenomenon in postoperative patients has resulted in great changes in the retractable length of the reconstructed urethral wall; it has not been objectively pointed out in research so far about whether such a phenomenon and the long-term scar hypertrophy will affect the elastic tensile fatigue or even fracture of the reconstructed urethral wall, and the long-term retreat of the external orifice of reconstructed urethra. Therefore, we had designed to prepare the rabbit oral mucosa into the clinically consistent reelpipe form, which was transplanted into the rabbit back. In addition, a scrotal skin reelpipe was also designed as control, so as to compare their histological changes, long-term scar formation, as well as the protein localization expression, quantitative analysis of MMP-2 and TIMP-2 and biomechanical changes in the transplanted tissues after transplantation.

## 2. Materials and methods

### 2.1. Experimental animals

A total of 30 male 6-month-old New Zealand rabbits (weight, 3.0–4.4 kg) were provided by Beijing Longan Laboratory Animal Cultivation Center (animal quarantine certification: No. 1103231911000003). At 6 months after birth, the immature rabbits were transferred to raise in the Plastic Surgery Hospital Laboratory Animal Center for experiments. The feeding conditions were as follows, relative humidity of 40–55% and feeding room temperature of 24–27 °C; all male rabbits were raised in individual cages, and all fodders, drinking water and padding had conformed

**Table 1**  
Experimental apparatus.

Name	Manufacturer	Model
Dewaterer	Wuhan Junjie Electronics Co., Ltd	JT-12S
Embedding machine	Wuhan Junjie Electronics Co., Ltd	JB-P7
Pathological microtome	Leica, Germany	RM2235
Frozen machine	Wuhan Junjie Electronics Co., Ltd	JB-L7
Slide spreading machine	Kedi Instrument Equipment Co., Ltd, Jinhua, Zhejiang Province	KD-P
Slide warmer	Changzhou Guohua Electric Appliance Co., Ltd	DB-B2
Oven	Bonsi Instrument Technology (Shanghai) Co., Ltd	101-1BS
Shaking table	Scilogex, USA	SK-R1807-E
Mixing machine	Scilogex, USA	MX-S
Inverted microscope	Nikon, Japan	NIKON CI-S
Imaging system	Nikon, Japan	NIKON DS-U3
Double-column bench tensile tester	INSTRON, USA	5967

**Table 2**  
Experimental reagents.

Reagent	Manufacturer	CAS/article number/species
Absolute ethyl alcohol	Sinopharm Group Chemical Reagent Co., Ltd	64-17-5
Xylene	Sinopharm Group Chemical Reagent Co., Ltd	1330-20-7
EDTA antigen retrieval solution	Beijing Zhongke Wanbang Biotechnology Co., Ltd	NA
Perhydrol	Sinopharm Group Chemical Reagent Co., Ltd	7722-84-1
Bovine serum albumin V	Solarbio	A8020
PBS	Beijing Zhongke Wanbang Biotechnology Co., Ltd	NA
DAB developer	Beijing Zhongke Wanbang Biotechnology Co., Ltd	NA
Neural gum mounting medium	Beijing Zhongke Wanbang Biotechnology Co., Ltd	NA
Hematoxylin	Shanghai Bogu Biotechnology Co., Ltd	PT001
Eosin	Shanghai Bogu Biotechnology Co., Ltd	PT001
Ammonium hydroxide	Shanghai Aladdin Science and Technology Co., Ltd	13767-16-3

to the feeding conditions formulated by Beijing Laboratory Animal Control Committee. All rabbits were allowed for 7 days of observational feeding before experiment, and the laboratory animal welfare ethical review application was signed.

2.2. Experimental apparatus and reagents are shown in [Tables 1 and 2](#)

### Names and ratios of antibodies

- (1) Primary antibody: Anti-MMP-2 antibody, 1:400, antibody source: mouse monoclonal antibody, with the molecular weight of 72 kDa (abcam)
- (2) Primary antibody: Anti-TIMP-2 antibody, 1:400, antibody source: mouse monoclonal antibody, with the molecular weight of 72 kDa (abcam)
- (3) Secondary antibody: Goat anti-rabbit IgG, KPL, Article number: 074-1506, 1:200

## 3. Experimental methods

### (1) Experimental grouping

To investigate the long-term scar hypertrophy and biomechanical changes of the oral mucosa and scrotal skin, the 21 rabbits were randomly divided into different groups according to the sampling time points of 0 ( $T_0$ ), 2 (2 W), 4 (4 W), 8 (8 W), 16 (16 W), 20 (20 W) and 24 (24 W) weeks after surgery. The experimental group had utilized the rabbit oral mucosa, while the control group had adopted the rabbit scrotal skin; for each sampling time point, 3 samplings were repeated as controls. Among them, with obvious changes in TIMP-2, MMP-2 and biomechanics of 2 W, 8 W and 24 W were repeatedly taken for verification.

### (2) Autologous oral mucosa and scrotal skin free transplantation

#### Surgical procedure:

① General anesthesia: 30 min prior to the surgery, the male immature rabbits were given intramuscular injection of the mixed anesthetics at a dose of 0.4 ml/kg body weight prepared by ketamine and Su-mian-xin II (Xylazine Hydrochloride) at the ratio of 1:1. In addition, 0.2 ml/kg mixed anesthetics were further injected for maintenance anesthesia based on the intraoperative anesthesia condition. ② Preoperative preparation: 20,000 U penicillin sodium (0.12 g/kg) was prepared based on the immature rabbit weight, which was then mixed with 2 ml of 0.9% normal saline for intramuscular injection in the lower limb; skins in the scrotum and rabbit back were prepared; the surgical region was disinfected with iodine conventionally for three times, and covered with the sterile towel. ③ Surgery: the laboratory male rabbits were in supine position, bilateral scrotal skins and bilateral buccal mucosae were cut open along the labeled line based on the preoperative methylene

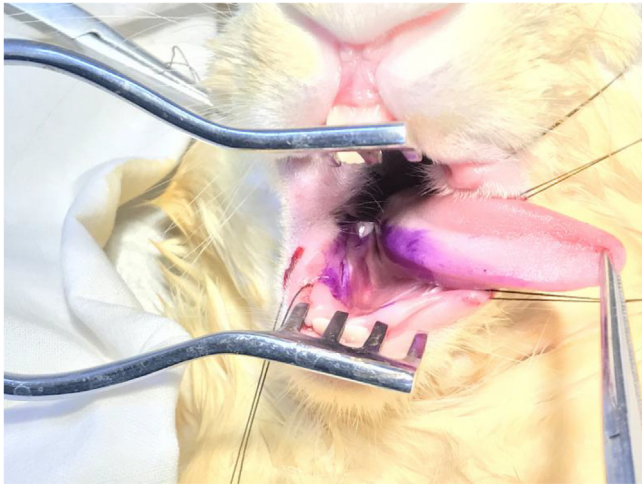


Fig. 1A. Oral mucosal incision in the rabbit.

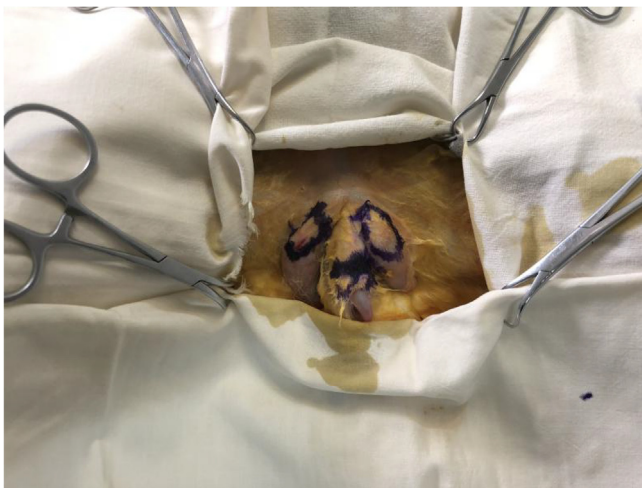


Fig. 1B. Skin incision of scrotum in the rabbit.

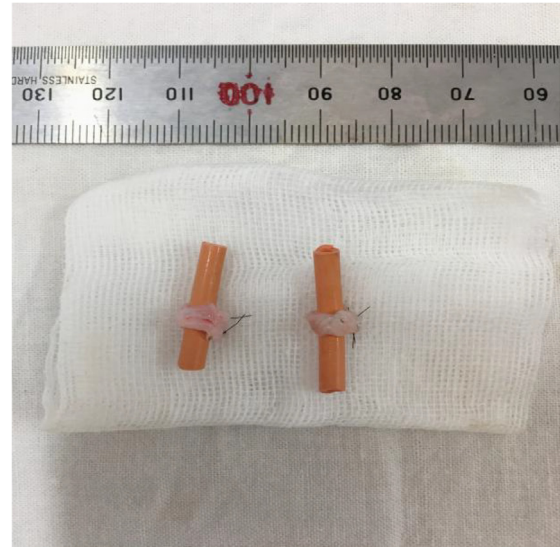


Fig. 1C. Reelpipes of buccal mucosa and scrotal skin.

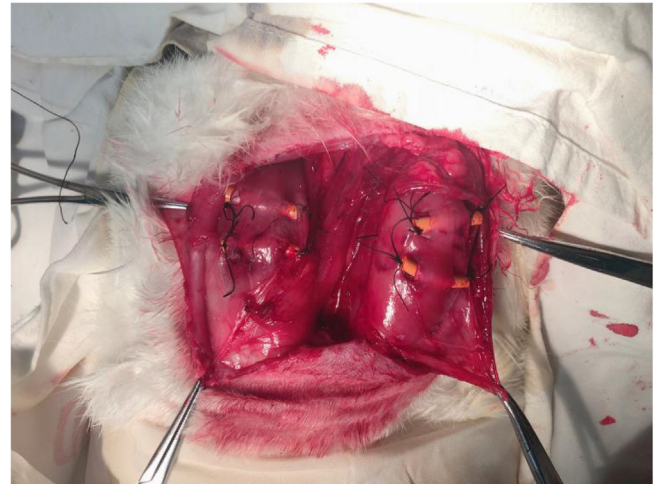


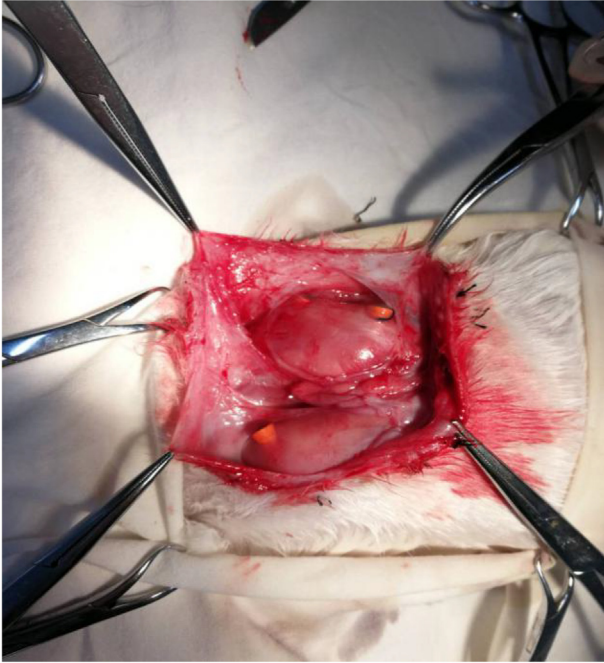
Fig. 1D. Reelpipes were fixed in the dorsal muscle tissue of the rabbit.

blue-labeled scrotal and oral mucosal incisions (Figs. 1A–1B), and the intact scrotal skin (length 20–25 mm, width 8–10 mm) and bilateral buccal mucosae (length 20–25 mm, width 8–10 mm) were collected. Afterwards, the mucosa and skin graft were pruned to the split-thickness skin graft, and the donor site was closed with the 3–0 stitches through interrupted suture. Then, the 12F sterile balloon-free silicone rubber catheter was tailored into the 20 mm segmented tubes, and the single-layer mucosa and scrotal skin were sutured onto the segmented tubes, respectively, with the 5–0 absorbable stitches to form the reelpipes (Fig. 1C). The laboratory rabbits were adjusted to prone position, later, the rabbit back skin, subcutaneous fat and rabbit back muscular tissues were cut open along the designed line, followed by blunt dissection of the muscular tissues to form the muscular bridge. Subsequently, the mucosal reelpipe was passed through the muscular bridge along the bottom layer of muscle, and its both ends were sutured with the 3–0 stitches; at the same time, the scrotal skin reelpipe was fixed at the contralateral muscular bridge (Fig. 1D), the wound surface was closed with the 3–0 stitches through interrupted suture, and the skin wound was covered with the chloramphenicol powder gauze for 3 days. ④ After surgery: the rabbits were given intramuscular injection of antibiotics in the lower limb for three days after surgery to prevent postoperative infection, and the surgical

wound was observed every day to examine whether there was complication.

### (3) Postoperative sampling and mechanical detection of specimens

I. Rabbit back muscular tissue samples were collected at  $T_0$ , 2 W, 4 W, 8 W, 16 W, 20 W and 24 W time points: the laboratory rabbits were given general anesthesia with mixed anesthetics according to the preoperative dose, afterwards, the animals were adjusted to prone position, and the rabbit back skin was prepared; the surgical region was disinfected with iodine conventionally for three times, and covered with the sterile towel. Then, the rabbit back skin and subcutaneous fat tissues were cut open along the previous surgical scar, and the mucosal and scrotal skin reelpipes were located in the muscular bridge (Fig. 2); part of muscular tissues and reelpipe were cut off completely, and the redundant muscular tissues were pruned (Figs. 3A and 3B). Afterwards, the fresh tissue specimens were immersed into the standard tube containing 0.9% normal saline at 25 °C, and sent to the mechanics laboratory within 30 min after sampling for biomechanical detection. After sampling, the laboratory rabbits were sacrificed through injecting excessive air via the rabbit ear vein.



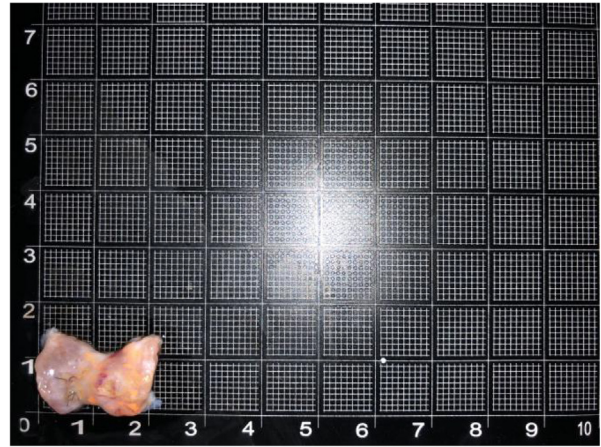
**Fig. 2.** Reelpipes were fixed in the muscle tissue, and no tissue necrosis and infection were found in the recipient area.



**Fig. 3A.** Complete removal of transplanted recipient tissue.



**Fig. 3B.** Removing the silicone tube and trimming excess muscle tissue.



**Fig. 4.** Trimming the width of both sides of the specimen to make it dumbbell-shaped.

II. Biomechanical detection: The fresh specimens were placed onto the flat surface to measure the changes in length, width and thickness. To guarantee that the specimen ruptured at the intersection between the vertical axis and the horizontal axis centers, the bilateral widths of the specimen should be pruned, so that it was dumbbell-shaped (Fig. 4). In addition, the both ends of specimen were fixed with the clamp, and tensile test was begun after measuring its effective tensile length; typically, the detection data included elastic modulus, tensile strain (displacement) at the maximum loading (mm/mm), tensile stress at the maximum loading (MPa), variation curves of length, width and thickness. One effective detection was completed when the specimen cross-section was completely ruptured.

#### (4) Hematoxylin-eosin (HE) staining and immunohistochemical (IHC) staining of the tissue paraffin sections

The tested tissue specimens at each time point were sampled at the sagittal plane and put into the specimen tube. Afterwards, the specimens were immersed and fixed in 10% neutral formaldehyde solution for 48 h to prepare the paraffin sections. Later, the sections were subjected to HE staining and IHC staining for MMP-2 and TIMP-2.

#### (5) IHC result judgement and AOD value detection under microscope

The presence of faint yellow or claybank in the parenchymal membrane or cytoplasm could be deemed as positive antigen expression, and the nucleus was blue. 5 fields of view (FOV) were randomly selected from each section under the high power lens (x20), and the image pro-plus software was utilized to measure the integral optical density (IOD) values and areas of MMP-2 and TIMP-2 in experimental group and control group. Finally, the average optical density (AOD) value was calculated as follows,  $AOD = IOD/area$ , and the MMP-2 and TIMP-2 protein expression difference was quantitatively analyzed.

## 4. Statistical methods

The SPSS 23.0 statistical software was applied for statistical analyses. The Shapiro-Wilk test was employed to examine whether the measurement data had conformed to normal distribution, afterwards, the normally distributed data were expressed as mean  $\pm$  standard deviation ( $X \pm S$ ), whereas data conforming to

**Table 3**The size of oral mucosa and scrotal skin after transplantation ( $X \pm S$ )  $N = 60$ .

Transplanted tissue	Oral mucosa	Scrotal skin	t	P value
Width(mm)	4.08 ± 1.15	3.86 ± 1.15	0.704	0.485
Thickness(mm)	2.91 ± 0.99	2.61 ± 0.77	1.138	0.262
Length(mm)	10.17 ± 3.63	14.43 ± 3.41	-3.963	<0.001

**Table 4**Biomechanical changes of oral mucosa and scrotal skin after transplantation [M(Q25,Q75)]  $N = 60$ .

Transplanted tissue	Oral mucosa	Scrotal skin	U	P value
Elastic modulus (MPa)	0.78(0.65, 1.07)	1.61(1.19, 1.89)	97	0.001
Tensile displacement at maximum load (mm/mm)	0.85(0.60, 1.16)	0.58(0.44, 0.82)	131	0.009
Tensile stress at maximum load (MPa)	0.37(0.21, 0.79)	0.37(0.25, 0.50)	233	0.833

skewed distribution were expressed as median and quartile [M(Q25,Q75)]. For measurement data with normal distribution and homogeneity of variance, the independent sample *t*-test was utilized for inter-group comparison, otherwise, the Mann-Whitney *U* test was adopted for inter-group comparison. A difference of  $P < 0.05$  was deemed as statistically significant.

## 5. Results

### 5.1. Biomechanical detection results of rabbit oral mucosa and scrotal skin

A total of 60 groups of test specimens were collected in this study, including 30 rabbit oral mucosae and 30 rabbit scrotal skins.

- (1) Length after transplantation: difference between the length of oral mucosa (10.17 ± 3.63 mm) and scrotal skin (14.43 ± 3.41 mm) after transplantation was statistically significant ( $P < 0.05$ ), and the change in oral mucosal length was smaller than that of scrotal skin after transplantation (Table 3).
- (2) Elastic modulus (secant line-cursor): after transplantation, the elastic modulus of oral mucosa (0.78(0.65, 1.07)) was smaller than that of scrotal skin (1.61(1.19, 1.89)) ( $P < 0.05$ ). As for the tensile strain (displacement) at maximum loading: that of oral mucosa (0.85(0.60, 1.16) mm/mm) after transplantation was greater than that of scrotal skin (0.58(0.44, 0.82) mm/mm) ( $P < 0.05$ , Table 4).
- (3) Comparison of the maximum loadings of oral mucosa and scrotal skin before and after 8 weeks of transplantation: difference in the maximum loading of oral mucosa before and after 8 weeks of transplantation was not statistically significant ( $P > 0.05$ ); while that of scrotal skin before 8 weeks of transplantation (2.75(1.87, 3.74)) was smaller than that after 8 weeks of transplantation (4.77(4.19, 5.46)) ( $P < 0.05$ ). Difference in the maximum loading between oral mucosa and scrotal skin before 8 weeks of transplantation was not statistically significant ( $P > 0.05$ ); while difference in the maximum loading between oral mucosa and scrotal skin after 8 weeks of transplantation was not statistically significant ( $P > 0.05$ , Table 5).
- (4) The longitudinal tensile change per mm of oral mucosa at the first 8 weeks of transplantation was  $0.96 \pm 0.31$  mm, while that of rabbit scrotal skin was  $0.82 \pm 0.49$  mm, and the difference between the two groups was not statistically significant ( $P > 0.05$ ). The maximum tensile displacement of rabbit oral mucosa before and after 8 weeks of transplantation was not markedly declined ( $P > 0.05$ ), but the maximum longitudinal tensile displacement per mm of rabbit

**Table 5**Comparison of maximum load of oral mucosa and scrotal skin before and after 8 weeks of transplantation [M(Q25,Q75)]  $N = 60$ .

	Before 8 W	After 8 W	U	P value
Oral mucosa	3.11(2.50, 5.65)	4.73(3.42, 9.77)	39	0.180
Scrotal skin	2.75(1.87, 3.74)	4.77(4.19, 5.46)	21	0.016
P value	0.667	0.965		

**Table 6**Comparison of tensile displacement at maximum load of oral mucosa and scrotal skin before and after 8 weeks of transplantation(mm/mm)( $X \pm S$ )  $N = 60$ .

	Before 8 W	After 8 W	P value
Oral mucosa	0.96 ± 0.31	0.80 ± 0.33	>0.05
Scrotal skin	0.82 ± 0.49	0.45 ± 0.17	<0.05
P value	>0.05 < 0.05		

scrotal skin at the first 8 weeks of transplantation ( $0.82 \pm 0.49$  mm) was greater than that after 8 weeks of transplantation ( $0.45 \pm 0.17$  mm) ( $P < 0.05$ , Table 6).

### 5.2. AOD detection results of MMP-2 and TIMP-2 in rabbit oral mucosa and scrotal skin

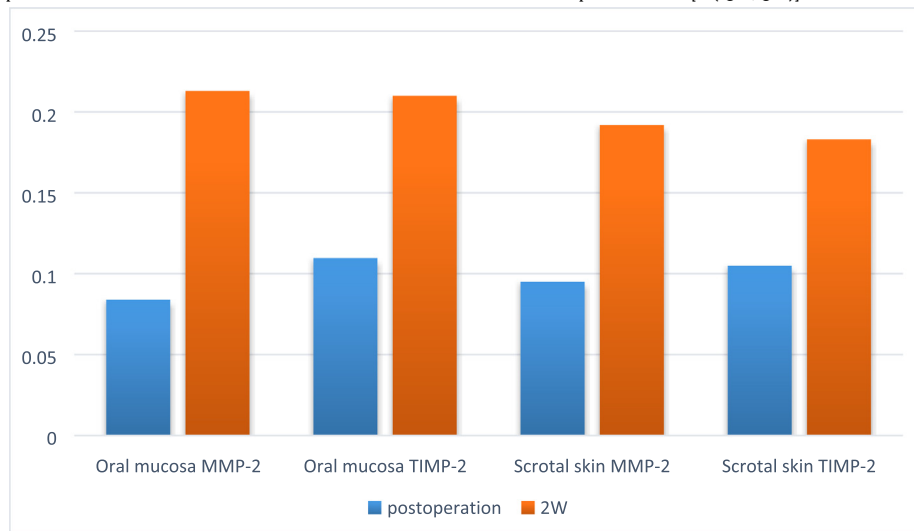
The expression intensities of MMP-2 and TIMP-2 in oral mucosa at 2 W time point ( $0.175 \pm 0.037$ ;  $0.176 \pm 0.035$ ) were higher than those at  $T_0$  time point ( $0.075 \pm 0.009$ ;  $0.094 \pm 0.0156$ ), and the difference between two groups was of statistical significance ( $P < 0.05$ ). The expression intensities of MMP-2 and TIMP-2 in scrotal skin at 2 W time point ( $0.169 \pm 0.027$ ;  $0.171 \pm 0.015$ ) were higher than those at  $T_0$  time point ( $0.075 \pm 0.009$ ;  $0.094 \pm 0.0156$ ), and the difference between two groups was statistically significant ( $P < 0.05$ ) (Table 7). The expression quantities of TIMP-2 in oral mucosa and scrotal skin at 8–24 W were higher than those of MMP-2 ( $P < 0.05$ ) (Table 8). At 8 W time point, the TIMP-2/MMP-2 ratio in scrotal skin (2.25 (2.11, 2.35)) was greater than that in oral mucosa (1.55 (1.45, 1.62)), and the difference between two groups was of statistical significance ( $P < 0.05$ ); however, at 16–24 W, difference in the TIMP-2/MMP-2 ratio between two groups was not statistically significant ( $P > 0.05$ ) (Table 9).

### 5.3. Histological microscopic results of the specimen sections

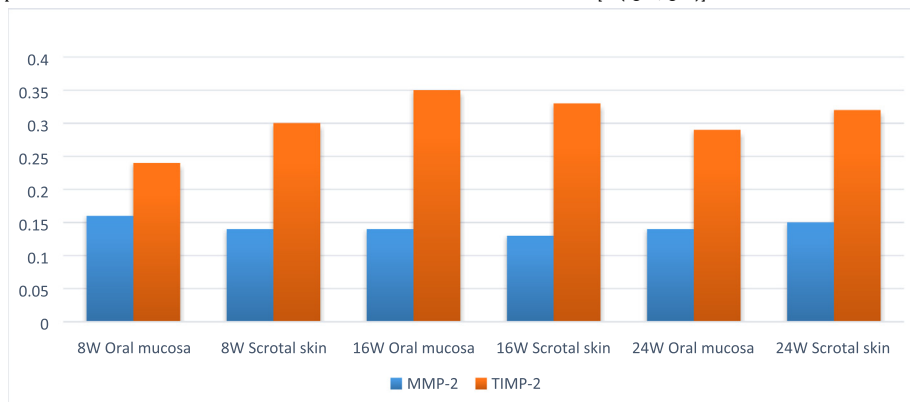
#### (1) Histological HE staining results

Generally, rabbit granulation tissues begin to occur within 2 days after mucosal or skin injury, which will become mature after about 10 days. The early wound tissue edema and hemor-

**Table 7**  
Expression of MMP-2 and TIMP-2 in oral mucosa and scrotal skin at Postoperation –2W[M(Q25,Q75)]N = 60.



**Table 8**  
Expression of MMP-2 and TIMP-2 in oral mucosa and scrotal skin at 8–24 W[M(Q25,Q75)].

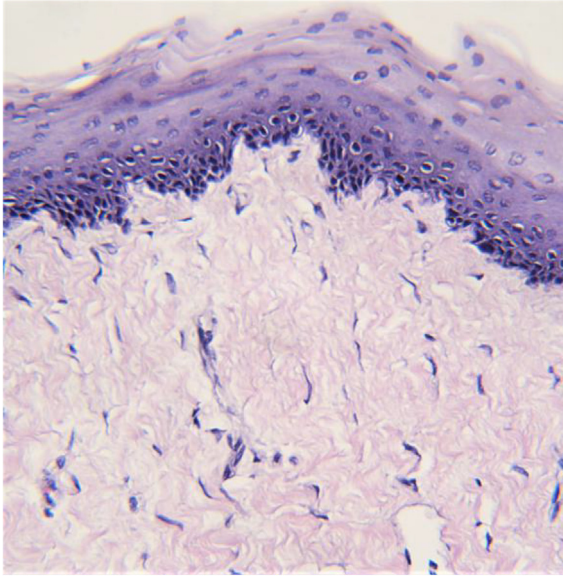


**Table 9**  
TIMP-2/MMP-2 of oral mucosa and scrotal skin at 8–24 W (M(Q25,Q75)).

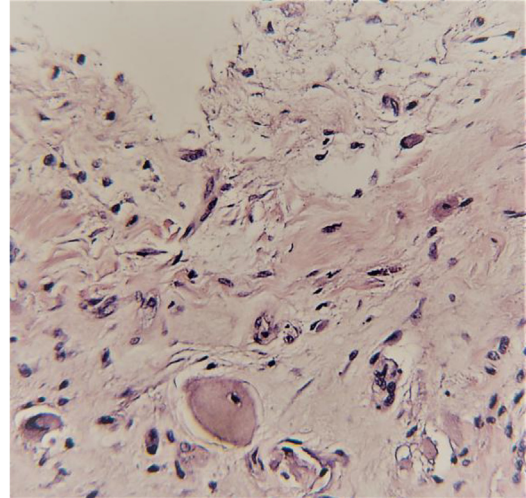
Transplanted tissue	Oral mucosa	Scrotal skin	P value
8 W TIMP-2/MMP-2	1.55(1.45, 1.62)	2.25(2.11, 2.35)	<0.01
16 W TIMP-2/MMP-2	2.45(2.22, 2.70)	2.54(2.30, 2.78)	>0.05
24 W TIMP-2/MMP-2	2.13(2.09, 2.17)	2.13(1.92, 2.31)	>0.05

rhage phenomena could not be observed due to the sampling time, so the exact time that the granulation tissues began to occur could not be determined. At  $T_0$  time point, intact mucosa could be observed in experimental group, with parakeratosis in mucosal stratified squamous epithelium, while keratinization could be observed in the scrotal skin epidermal squamous epithelium in control group (Figs. 5A and 5B). At 2 W, mature granulation tissues could be seen in experimental group, fibroblast hypertrophy was not obvious, collagen synthesis on wound surface was increased, and the collagens were arranged along the wound surface direction; a small amount of cell necrosis could be seen in the wound surface of control group, the granulation tissues had become mature, fibroblasts were increased, collagen synthesis was remarkably increased, and the collagens were mostly arranged along the wound surface direction (Figs. 5C and 5D). At 4 W, mucosal ulcer surface edema could be seen in experimental group, along with abundant newly formed capillaries, and increased collagen synthe-

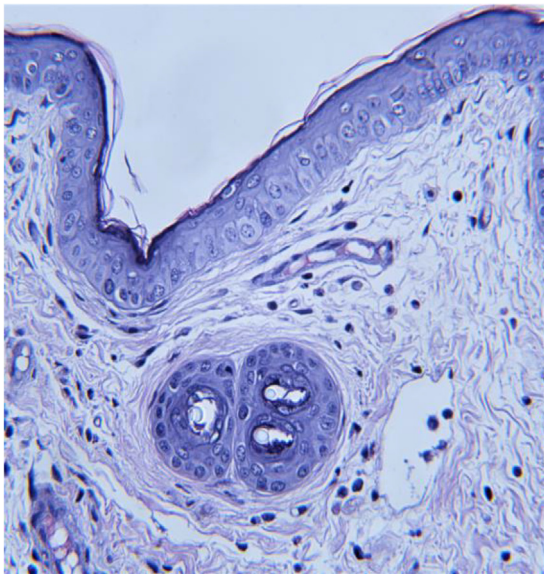
sis, but no obvious inflammatory cell leakage or covering of squamous epithelium on the wound surface was observed; in the control group, severe ulcer surface edema could be seen, few new capillaries were formed, and a small amount of lymphocyte infiltration could be observed (Figs. 5E and 5F). At 8 W, mild ulcer surface edema could be seen in experimental group, along with sparse capillaries, and a small amount of neutrophil infiltration; in the control group, the collagens were mostly arranged in a staggered way, and wound surface scarring could be observed (Figs. 5G and 5H). At 16 W, wound surface scarring could be seen in experimental group, the collagens were mostly arranged along the wound surface direction in a wavy manner, together with a small amount of lymphocyte infiltration; the wound surface in control group had been scarred, the capillaries had disappeared gradually, the collagens were irregularly arranged, and a small amount of lymphocyte infiltration could be seen (Figs. 5I and 5J). At 24 W, scar tissues were formed on the wound surface of experimental group, the collagens were arranged along the wound surface direction in a wavy manner, accompanying with sparse tissue cells and blood vessels; in the control group, the wound surface had formed the scar tissues, the collagens were arranged in a staggered way, with sparse tissue cells and blood vessels (Figs. 5K and 5L). Scar tissues could be observed in the rabbit oral mucosa at the 16th week after transplantation; while those could be seen in rabbit scrotal skin at the 8th week after transplantation.



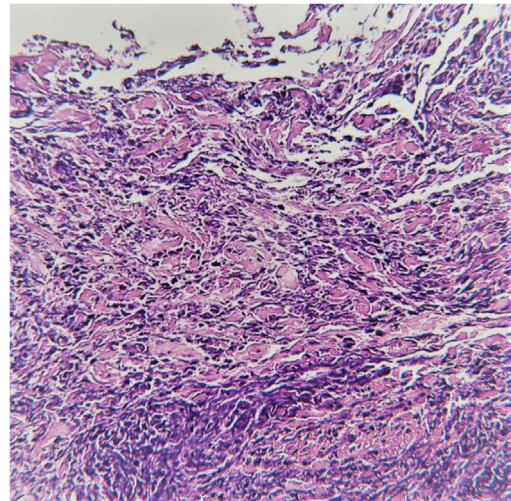
**Fig. 5A.** T<sub>0</sub>: Oral mucosa HE staining (×100).



**Fig. 5C.** 2 W: Oral mucosa HE staining (×100).



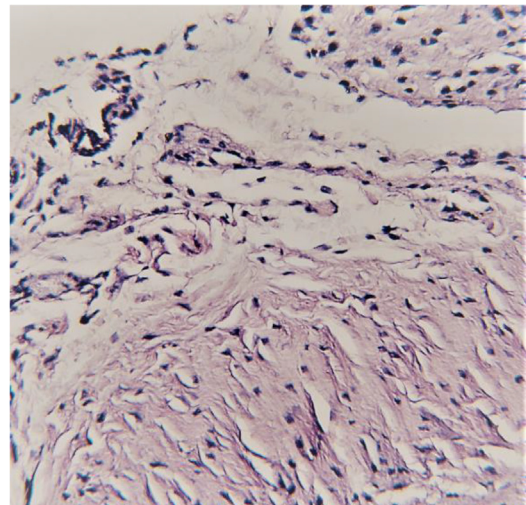
**Fig. 5B.** T<sub>0</sub>: Scrotal skin HE staining (×100).



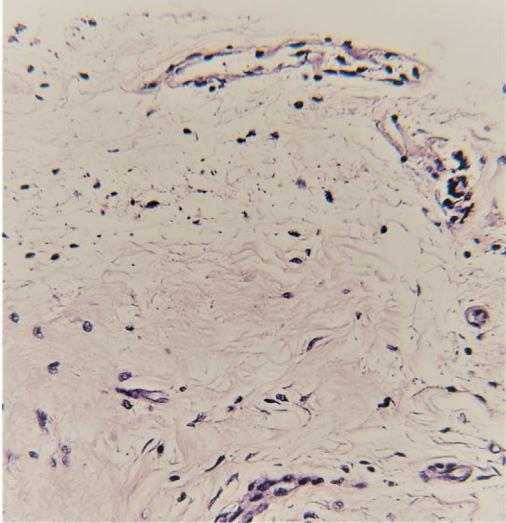
**Fig. 5D.** 2 W: Scrotal skin HE staining (×100).

## (2) Anti-MMP-2, anti-TIMP-2 IHC staining results

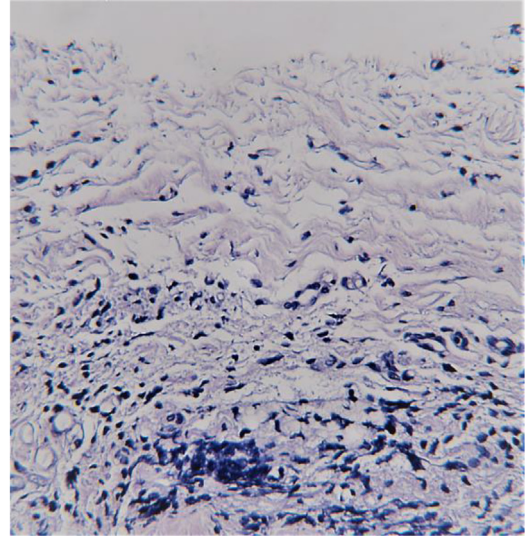
At T<sub>0</sub>, MMP-2 was weakly expressed in normal mucosa, which was mainly distributed in the lamina propria and manifested as the faint yellow stained particles (Fig. 6A); while TIMP-2 was also weakly expressed in normal mucosa, which was mainly distributed in the lamina propria and manifested as the faint yellow stained particles (Fig. 6B). At T<sub>0</sub>, MMP-2 was lowly expressed in normal scrotal skin, which was mainly distributed in the corium layer and manifested as the faint yellow stained particles, while the cutaneous appendages and other structures were not stained (Fig. 6C); TIMP-2 was also weakly expressed in normal scrotal skin, which was mainly distributed in the corium layer and manifested as the faint yellow stained particles (Fig. 6D). At 2–4 W, MMP-2 expression in the mucosa was located in the cytoplasm of fibroblasts, keratinocytes and endothelial cells, and it was also expressed in some cell membrane, which manifested as the clay bank stained



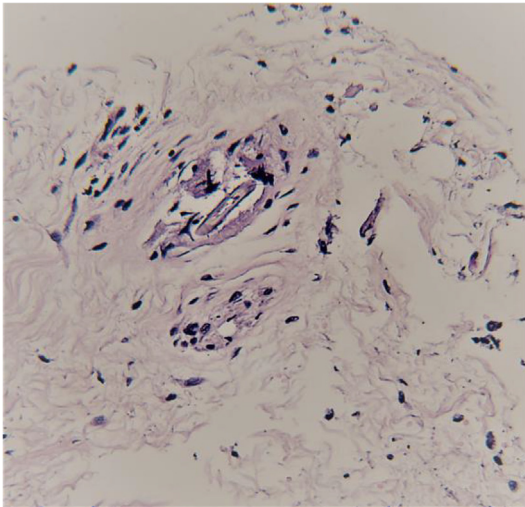
**Fig. 5E.** 4 W: Oral mucosa HE staining (×100).



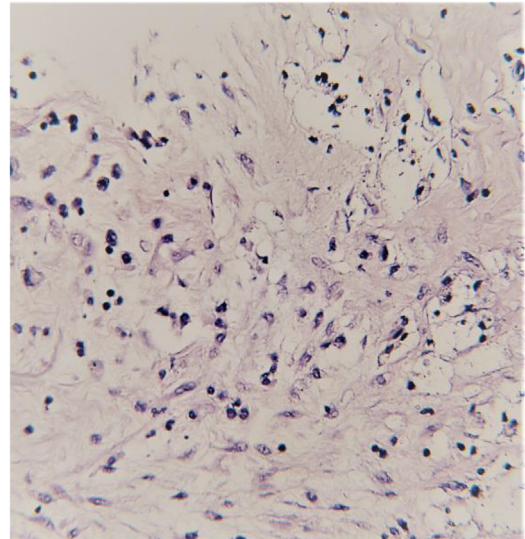
**Fig. 5F.** 4 W: Scrotal skin HE staining ( $\times 100$ ).



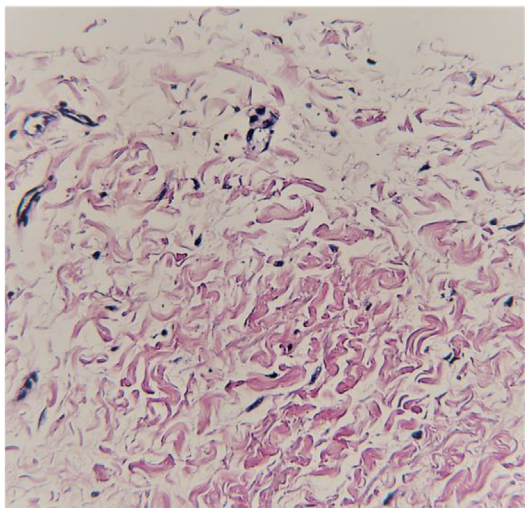
**Fig. 5I.** 16 W: Oral mucosa HE staining ( $\times 100$ ).



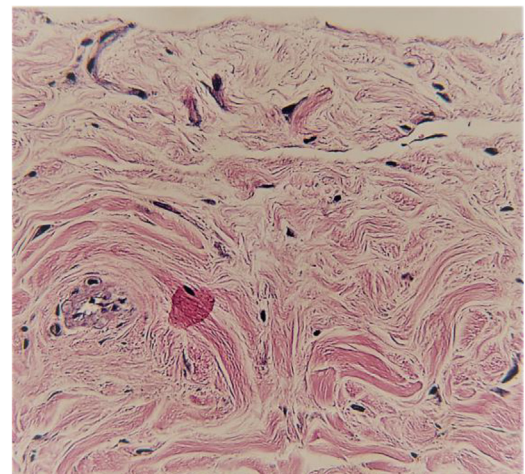
**Fig. 5G.** 0.8 W: Oral mucosa HE staining ( $\times 100$ ).



**Fig. 5J.** 16 W: Scrotal skin HE staining ( $\times 100$ ).

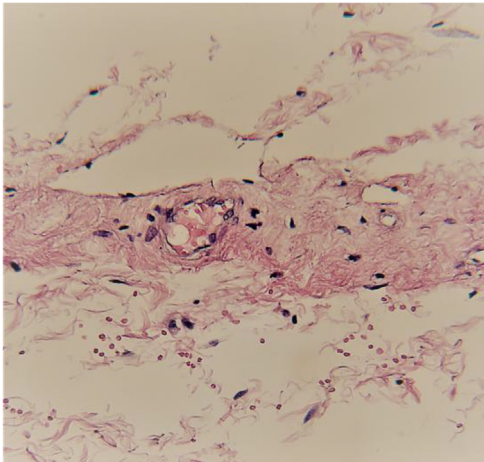


**Fig. 5H.** 8 W: Scrotal skin HE staining ( $\times 100$ ).

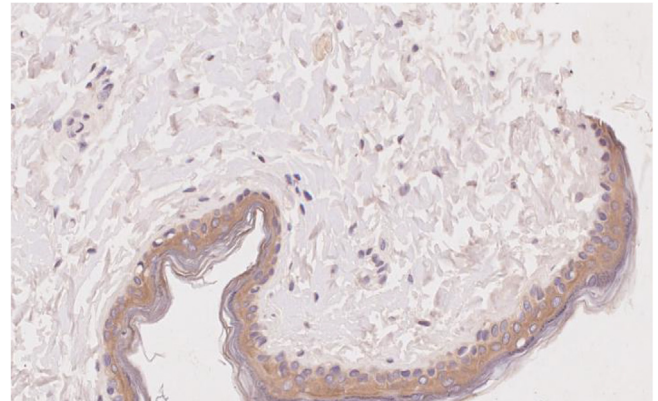


**Fig. 5K.** 24 W: Oral mucosa HE staining ( $\times 100$ ).

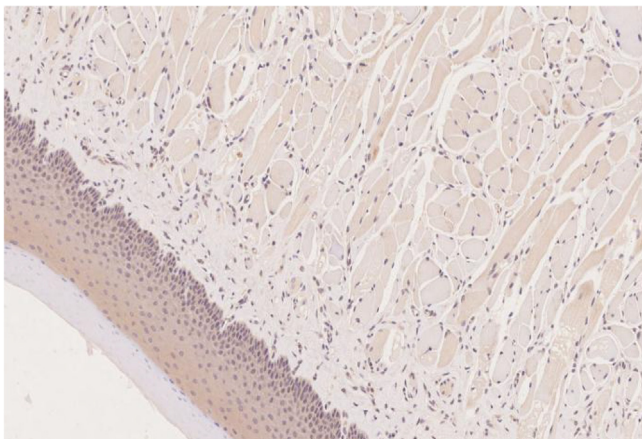




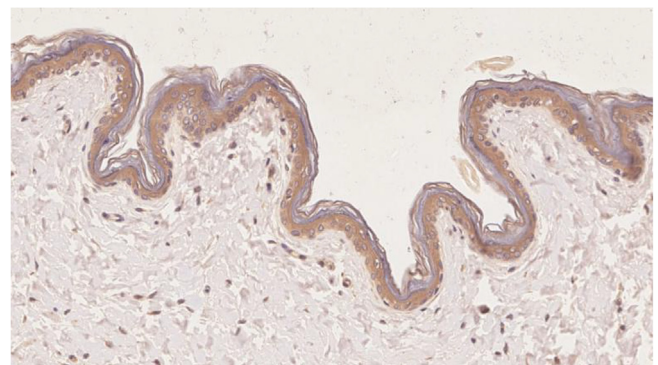
**Fig. 5L.** 24 W:Scrotal skin HE staining ( $\times 100$ ).



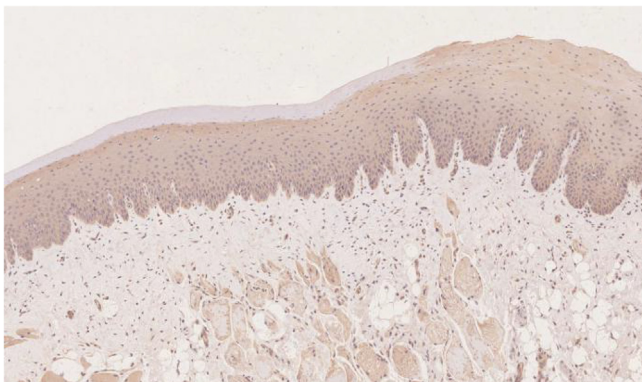
**Fig. 6C.** T<sub>0</sub>:MMP-2 expression in Scrotal skin, Immunohistochemistry ( $\times 100$ ).



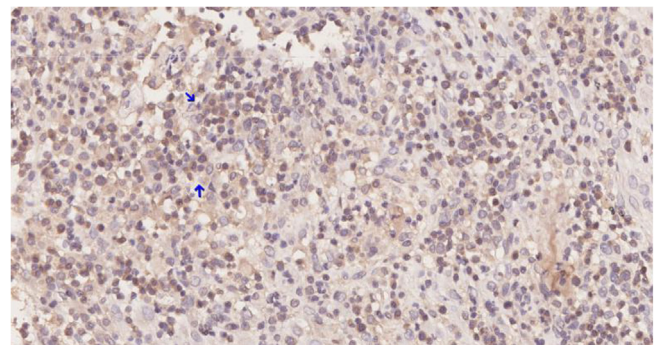
**Fig. 6A.** T<sub>0</sub>:MMP-2 expression in mucosa, Immunohistochemistry ( $\times 100$ ).



**Fig. 6D.** T<sub>0</sub>:TIMP-2 expression in Scrotal skin, Immunohistochemistry ( $\times 100$ ).



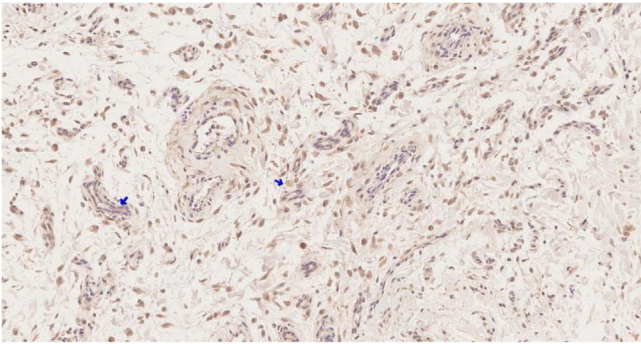
**Fig. 6B.** T<sub>0</sub>:TIMP-2 expression in mucosa, Immunohistochemistry ( $\times 100$ ).



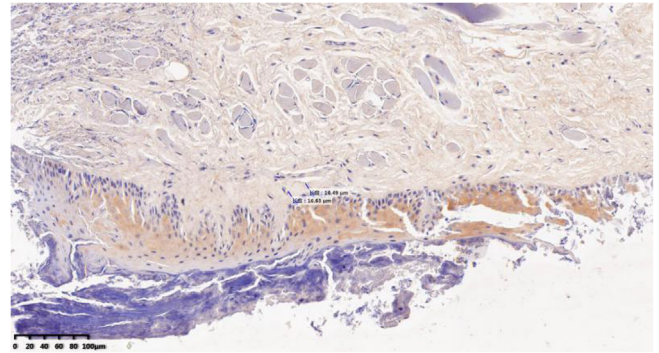
**Fig. 6E.** 4 W:MMP-2 expression in mucosa, Immunohistochemistry ( $\times 200$ ).

particles (Fig. 6E); by contrast, the positive expression of TIMP-2 was also located in the cytoplasm of fibroblasts, keratinocytes and endothelial cells in the mucosa, and it was also expressed in some cell membrane, which manifested as the clay bank stained particles (Fig. 6F). At 2–4 W, MMP-2 expression was located in the cytoplasm of fibroblasts, keratinocytes and endothelial cells in the scrotal skin, and it was also expressed in some cell mem-

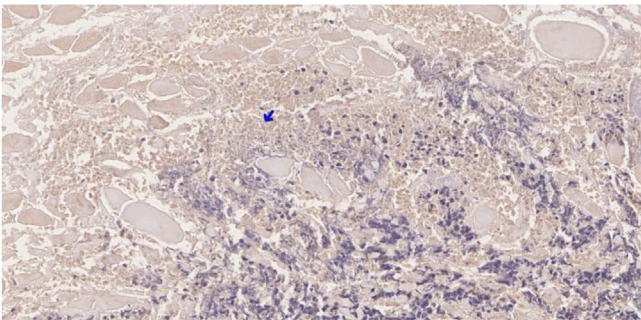
brane, which manifested as the clay bank stained particles (Fig. 6G); by contrast, the positive expression of TIMP-2 was also located in the cytoplasm of fibroblasts, keratinocytes and endothelial cells in the scrotal skin, and it was also expressed in some cell membrane, which manifested as the clay bank stained particles (Fig. 6H). At 8 W, the MMP-2 expression was mostly located in the cytoplasm of fibroblasts in the mucosa, and some was concentrated in the cytoplasm of keratinocytes and endothelial cells, which manifested as the clay bank stained particles (Fig. 6I); while the TIMP-2 expression was mostly located in the cytoplasm of fibroblasts, and some was concentrated in the cytoplasm of keratinocytes and endothelial cells, which manifested as the clay bank stained particles (Fig. 6J). At 8 W, MMP-2 expression was located in the cytoplasm of basal cell layer cells in scrotal skin, while the scar



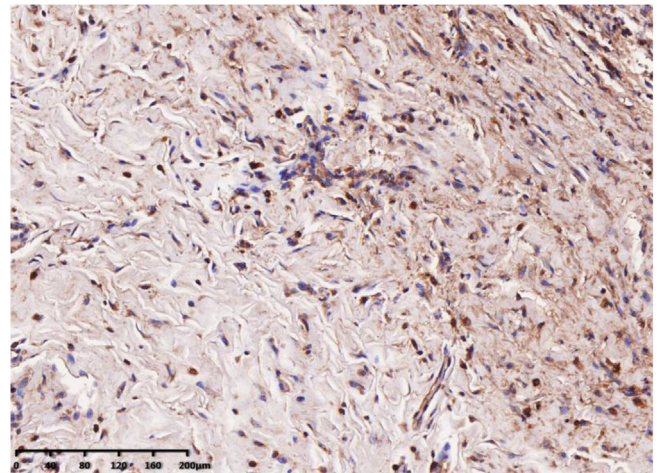
**Fig. 6F.** 4 W:TIMP-2 expression in mucosa, Immunohistochemistry ( $\times 100$ ).



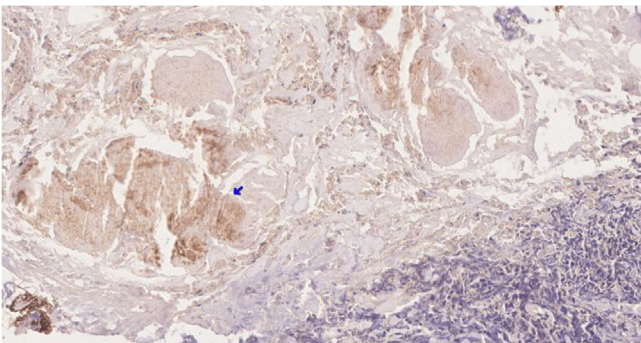
**Fig. 6I.** 8w:MMP-2 expression in mucosa, Immunohistochemistry ( $\times 100$ ).



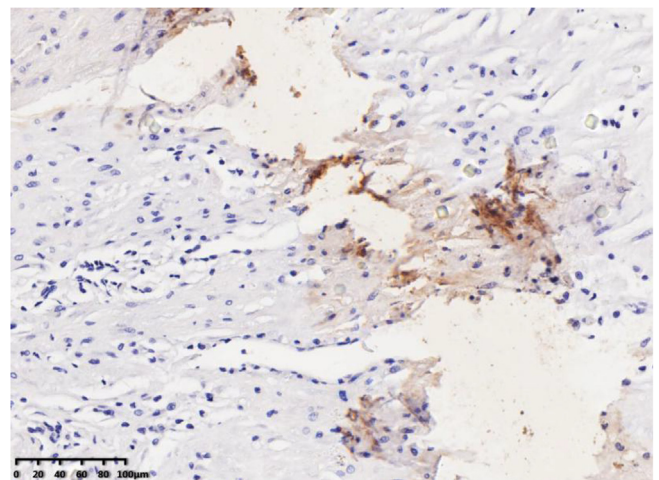
**Fig. 6G.** 4 W:MMP-2 expression in Scrotal skin, Immunohistochemistry ( $\times 100$ ).



**Fig. 6J.** 8w:TIMP-2 expression in mucosa, Immunohistochemistry ( $\times 200$ ).



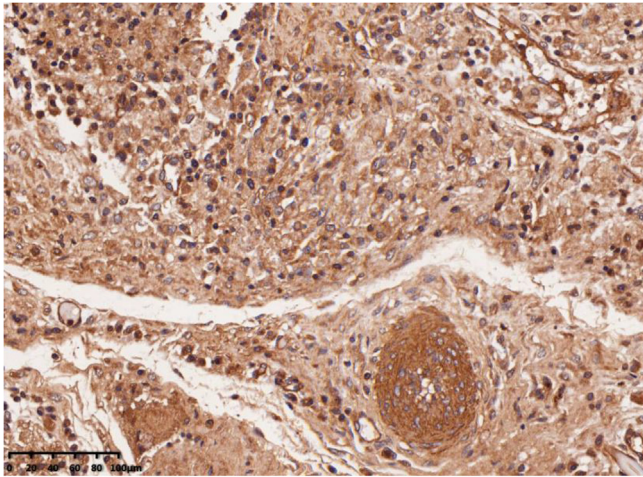
**Fig. 6H.** 4 W:TIMP-2 expression in Scrotal skin, Immunohistochemistry ( $\times 100$ ).



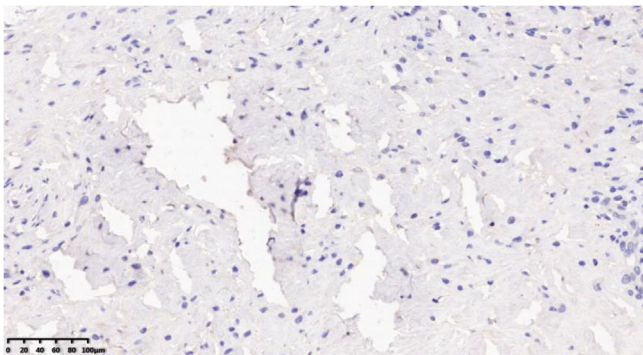
**Fig. 6K.** 8w:MMP-2 expression in Scrotal skin, Immunohistochemistry ( $\times 200$ ).

tissues were mostly located in the cytoplasm of epidermal cells and vascular endothelial cells, and a small amount were concentrated in the cytoplasm of fibroblasts, which manifested as clay bank stained particles (Fig. 6K); TIMP-2 expression was located in the cytoplasm of basal cell layer cells in scrotal skin, while the scar tissues were mostly located in the cytoplasm of epidermal cells and vascular endothelial cells, and a small amount were concentrated in the cytoplasm of fibroblasts (Fig. 6L). At 16 W, MMP-2 expression in the mucosa was mostly located in the cytoplasm of vascular endothelial cells and fibroblasts at the junction between scar and the normal mucosal tissues, and a small amount was concentrated in the cytoplasm of epidermal cells, which manifested as the clay bank stained particles (Fig. 6M); the TIMP-2 expression was mostly located in the cytoplasm of vascular endothelial cells and fibroblasts at the junction between scar and the normal mucosal tissues, and a small amount was concentrated in the cytoplasm of epidermal cells, which manifested as the clay bank stained particles (Fig. 6N). At 16 W, MMP-2 expression in scrotal skin was

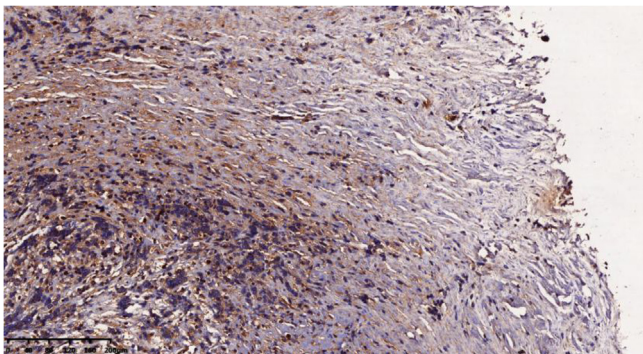
mostly located in the cytoplasm of vascular endothelial cells at the junction between scar and epidermis, and a small amount was located in the cytoplasm of fibroblasts, which manifested as the clay bank stained particles (Fig. 6O); TIMP-2 expression was mostly located in the cytoplasm of vascular endothelial cells and fibroblasts at the junction between scar and epidermis, and a small amount was located in the cytoplasm of fibroblasts at the scar site,



**Fig. 6L.** 8w:TIMP-2 expression in Scrotal skin, Immunohistochemistry ( $\times 200$ ).

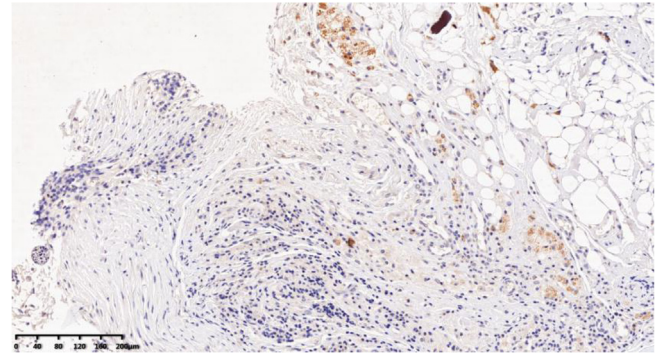


**Fig. 6M.** 16w:MMP-2 expression in mucosa, Immunohistochemistry ( $\times 100$ ).

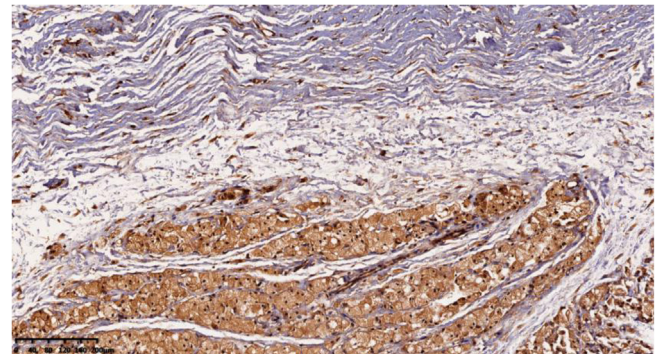


**Fig. 6N.** 16w:TIMP-2 expression in mucosa, Immunohistochemistry ( $\times 100$ ).

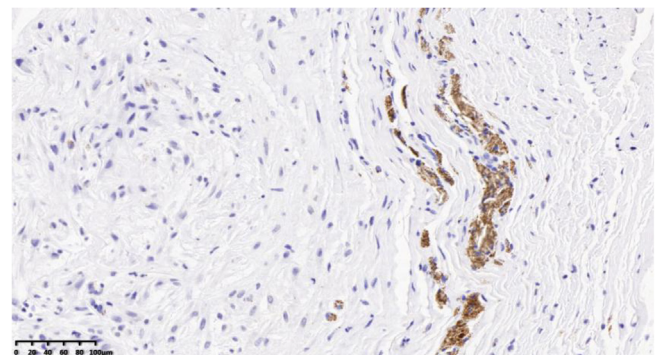
which mainly manifested as the clay bank stained particles (Fig. 6P). At 24 W, MMP-2 expression in mucosa was mostly located in the cytoplasm of fibroblasts at the junction between scar and normal mucosal tissues, and a small amount was concentrated in the cytoplasm of vascular endothelial cells, which manifested as the clay bank stained particles (Fig. 6Q); TIMP-2 expression was mostly located in the cytoplasm of vascular endothelial cells and fibroblasts at the junction between scar and normal mucosal tissues, which manifested as the clay bank stained particles, besides, positive expression could be seen in the scar central region, which mainly manifested as clay bank particles (Fig. 6R). At 24 W, MMP-2 expression in scrotal skin was mostly located in the cytoplasm of



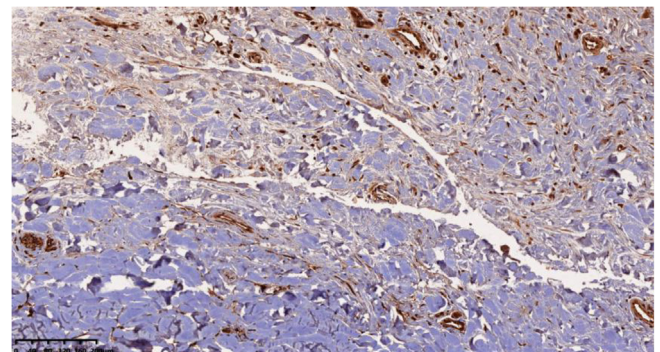
**Fig. 6O.** 16w: MMP-2 expression in Scrotal skin, Immunohistochemistry ( $\times 100$ ).



**Fig. 6P.** 16w:TIMP-2 expression in Scrotal skin, Immunohistochemistry ( $\times 200$ ).



**Fig. 6Q.** 24 W:MMP-2 expression in mucosa, Immunohistochemistry ( $\times 100$ ).



**Fig. 6R.** 24 W:TIMP-2 expression in mucosa, Immunohistochemistry ( $\times 200$ ).

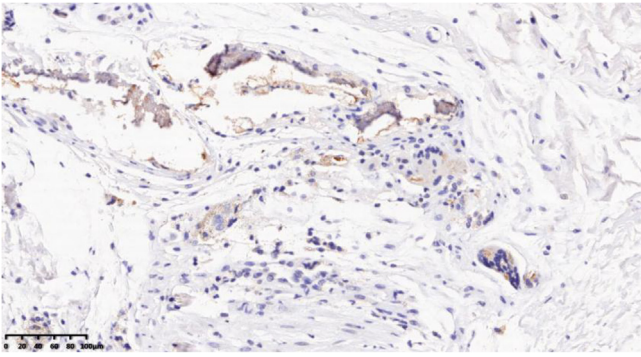


Fig. 6S. 24 W:MMP-2 expression in Scrotal skin, Immunohistochemistry ( $\times 200$ ).

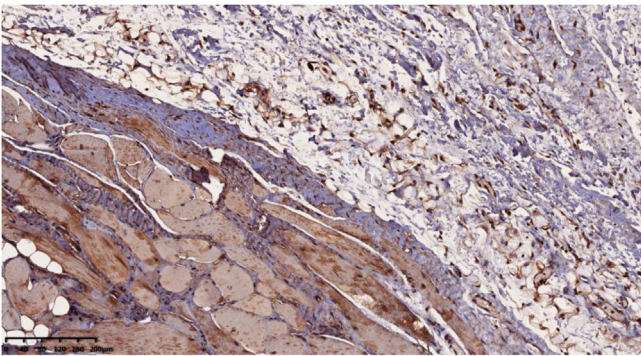


Fig. 6T. 24 W:TIMP-2 expression in Scrotal skin, Immunohistochemistry ( $\times 200$ ).

vascular endothelial cells at the junction between scar and epidermis, and a small amount was located in the cytoplasm of fibroblasts at the scar site, which manifested as the clay bank stained particles (Fig. 6S); TIMP-2 expression was mostly located in the cytoplasm of vascular endothelial cells at the junction between scar and epidermis, and a small amount was located in the cytoplasm of fibroblasts at the scar site, which mainly manifested as the clay bank stained particles (Fig. 6T).

## 6. Discussion

Transplantation of free tissue or local flap has been applied for years to reconstruct part of urethra, and the options of materials include oral mucosa, tunica vaginalis of testis, bladder mucosa or scrotal flap (Pons et al., 2005; Baskin and Duckett, 2010; Li et al., 1995). However, some materials have been gradually abandoned due to the severe postoperative complications, while oral mucosa for urethral reconstruction has been extensively applied in hypospadias repair and urethral defect reconstruction since its first proposal by Humby in 1941 (Barbagli et al., 2017; Djordjevic et al., 2008), which can be ascribed to its favorable compliance, high transplant survival rate and low scar contracture rate. It has been discovered in numerous studies that, oral mucosa allows for less scar or even no scar healing, which is based on the moist microenvironment in the oral cavity, as well as the high concentrations of epidermal growth factor and saliva protease (Roh et al., 2017; Evans, 2017). For free mucosa transplanted in the changed matrix microenvironment, Mokhless et al. (2007) had observed the histopathological changes at 6 months before and after oral mucosa transplantation for urethral reconstruction, and discovered that the mucosal epithelium was subjected to mild keratinization, the epithelial papilla within the lamina propria was extended, and the submucosa was rich in capillaries, which facilitated vascular-

ization survival after transplantation. Filipas et al. (1999) had transplanted the swine free flap and oral mucosa to the bladder, and compared the influence of the two after long-term exposure in the urine environment; their results suggested that, mucosa had better transplantation advantages than the free flap in terms of postoperative scar contracture and transplant survival rate; besides, that research had avoided the influence of exposure to urine. This may be because that oral mucosa for urethral reconstruction is generally carried out in two-stage surgery in clinic; at the first-stage, the mucosal reeplie is transplanted to the ventral penis, the ectopic urethral orifice at the root of penis is used as the major urination orifice within half a year after surgery, while the free mucosal flap in the distal ventral penis is less or not exposed to urine environment, which can better simulate the scar hypertrophy and elastic tensile changes after the first-stage surgery. Existing studies have shed light on the basic understanding of the histological changes before and after mucosal transplantation, but few studies have investigated the great tensile changes in urethral length applied in penis reconstruction. In this study, we had carried out histological observation to examine the influence of long-term scar hypertrophy on the elastic tension of the transplanted mucosa with changed matrix environment, which could provide favorable experimental foundation for the clinical application of mucosa in repair the urethral defect.

During the wound healing process, matrix metalloproteinases (MMPs) mainly hydrolyze the extracellular matrix (ECM), while the tissue inhibitor of metalloproteinases (TIMPs) can form the complex with MMPs to suppress its expression (Wang et al., 2017; Yang et al., 2017). The expression of these two is at a dynamic balance, which can stabilize the intracellular environment and structure; however, the imbalanced expression ratio of these two will result in ECM deposition, decomposition and remodeling abnormality (such as collagen), thus leading to the formation of pathological scar. It has been suggested in numerous studies that, MMPs and TIMPs are highly expressed at the early and middle stages of scar formation, and the two are still at a dynamic balance; however, TIMPs expression is higher than MMPs at the late stage, which can suppress ECM degradation and give rise to abnormal collagen deposition, thus rendering scar formation (Li et al., 2012; Imaizumi et al., 2010; Liu et al., 2001). In our study, the MMP-2 and TIMP-2 protein expression in the transplanted tissues was localized and quantitatively analyzed, and it was discovered that the MMP-2 and TIMP-2 were lowly expressed in normal oral mucosa and scrotal skin, either in experimental group or in control group; in addition, the MMP-2 and TIMP-2 expression levels in both groups at 2 W and 4 W time points were remarkably up-regulated compared with those at  $T_0$  time point; besides, the AOD values between MMP-2 and TIMP-2 showed no statistical significance, revealing that scar formation at the early and middle stages was related to the high expression levels of MMP-2 and TIMP-2. Additionally, the TIMP-2 expression in both group at 8–24 W time period was higher than that of MMP-2, but the TIMP-2/MMP-2 ratio in scrotal skin at  $T_{8W}$  time point was higher than that in oral mucosa, and those in oral mucosa at 16 W and 24 W time points were elevated, which showed no significant difference compared with those in scrotal skin. Such findings could well explain our observation that scar tissue in scrotal skin occurred earlier than that in oral mucosa; moreover, at 16 W time point, scar tissues were also observed in the transplanted oral mucosa, but the free transplanted tissue and the recipient site were at the revascularization and cell reconnection processes between tissues, which might be slightly different from the expression time of MMP-2 and TIMP-2 in traditional wound healing. We had carried out HE staining to observe the mucosa and scrotal skin tissues after 16 weeks of transplantation, and found that the collagen fibers in submucosa were mostly arranged in a wavy manner along the wound surface direction; whereas the submucosal collagen fibers were irregularly

arranged in a staggered way, which was consistent with the histological research results by Zhe Yang et al. (Mungadi and Ugboko, 2009) on the autologous transplantation of rabbit mucosa, and it could well explain the low contracture rate of mucosa after surgery. Besides, the longitudinal mechanical elastic tensile detection suggested that the elastic modulus of oral mucosa was smaller than that of scrotal skin. Oral mucosa had stronger flexibility and poorer resistance to deformation; as a result, the oral mucosa was more likely to be deformed than scrotal skin when the same tensile force was applied; at this moment, the maximum tensile length of oral mucosa at the time of rupture was markedly greater than that of scrotal skin when the maximum longitudinal tension was applied to break the oral mucosa and scrotal skin. In the experimental group, the longitudinal tensile length per mm before 8 weeks of transplantation was  $0.96 \pm 0.31$  mm, while that in control group was  $0.82 \pm 0.49$  mm, and there was no significant difference in the maximum tensile displacement between the two groups, which indicated that the longitudinal elastic tensile capacity of mucosa was close to that of scrotal skin before transplantation. However, when compared the data in both groups before and after 8 weeks of transplantation, the maximum tensile displacement per mm of oral mucosa before and after 8 weeks of transplantation was not markedly declined, which showed stable biomechanical property; while the maximum tensile displacement per mm of scrotal skin after 8 weeks of transplantation was evidently decreased than that before. In addition, the maximum longitudinal tension required to break the scrotal skin before 8 weeks of transplantation was smaller than that after transplantation, HE staining had revealed the earlier occurrence of scar tissues in scrotal skin than in oral mucosa at 8 W time point after transplantation, and the change in the oral mucosal length after transplantation was smaller than that in scrotal skin, suggesting that the long-term scar hypertrophy in the scrotal skin after transplantation was more obvious than that in oral mucosa. The more obvious scar hypertrophy was associated with the greater resistance to deformation, as well as the longitudinal tension required for deformation. At the same time, the scar hypertrophy had reduced the tissue elastic displacement, which had reduced the elastic tensile capacity of the transplanted material. For oral mucosa, which had less scar formation characteristics and stable tensile stress property, could maintain the original elastic tensile capacity after transplantation, even though it was transplanted to the microenvironment different from that of the oral cavity, which was more suitable to be transplanted into the reconstructed organ with great change in volume before and after surgery.

Our experimental results from animal modeling to the final mechanical analysis could provide objective foundation for the long-term scar contracture and mechanical tensile changes of the transplanted mucosal tissues in clinical application. Nonetheless, the mechanical changes of mucosa exposed to urine environment for a long time should be further investigated. Moreover, the tissue scar healing mechanism is complicated, which has involved various types of growth factors and cytokines (Olsen et al., 2018; Mandapalli et al., 2017; Dwivedi et al., 2017). On this account, this study was not sufficient enough to represent the scar healing role of all transplanted tissues. However, the location and quantitative analysis of MMP-2 and TIMP-2 expression in the transplanted tissues can provide a new thinking for the healing mechanism of transplanted tissues with less or no scar in the future.

## 7. Conclusions

The maximum elastic tensile displacement and change per mm of the transplanted tissue before and after surgery can be known from the elastic mechanical detection of the transplanted oral mucosa and scrotal skin. At the same time, the long-term scar

hypertrophy can result in the declined elastic tensile capacity of the transplanted tissues. Compared with skin, mucosa has a smaller scar contracture rate and stable biomechanical properties, which is more suitable for the reconstruction of the urethral defect. In normal oral mucosa and scrotal skin, MMP-2 and TIMP-2 are lowly expressed; after two weeks of transplantation, the expression quantities of these two are remarkably enhanced, revealing that scar formation is correlated with the high expression of MMP-2 and TIMP-2. From the 8<sup>th</sup>–24<sup>th</sup> weeks, the AOD values of TIMP-2 in oral mucosa and scrotal skin are remarkably higher than those of MMP-2, and the TIMP-2/MMP-2 ratio in scrotal skin at the 8<sup>th</sup> week of transplantation is higher than that in oral mucosa, which can well explain the earlier scar formation in scrotal skin than in oral mucosa; besides, such findings indicate that the different expression levels between TIMP-2 and MMP-2 are the important cause leading to scar formation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

QL wrote the manuscript. QL and WW analyzed IHC and HE result. YL and ZY designed the study and performed the experiment. NM and WW contributed to observation indexes analysis. The final version was read and adopted by all the authors. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The study was approved by the Ethics Committee of Plastic Surgery Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College.

## Consent for publication

Not applicable.

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