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ORIGINAL ARTICLE

Observation and mechanism study of bladder wound healing after transurethral holmium laser resection of bladder tumor

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Abstract

This research aims to observe and compare the wound healing process of urethral bladder after transurethral holmium laser resection of bladder tumor (HoLRBT) and transurethral resection of bladder tumor (TURBT) and explore the possible mechanism of wound healing and bladder re-epithelialization after HoLRBT. An animal model of canine achieving HoLRBT and TURBT was established. Cystoscopy was performed at different time points (3 days and 1, 2, 3, and 4 weeks) after operation to observe the wound healing and re-epithelialization of bladder epithelium. Bladder mucosa specimens were obtained and histopathological changes of the bladder epithelium were observed under light microscope after HE staining. Immunochemistry was used to determine the cell expression ofCK5, CK14, EGF, EGFR; microRNA expressions of CK5, CK14, EGF, and EGFR were measured by qRT-PCR. The changes of urinary EGF concentration were detected by ELISA. The bladder epithelial wound was repaired and re-epithelialized at 1 week after HoLRBT. At the 4th week, the bladder wound was basically completed and re-epithelialized; repair of bladder epithelial wounds recapitulates the wounds with the proliferation and migration of residual epithelial cells under the wound and the bladder epithelium that proliferates alongside the wound surface to complete re-epithelialization. The process begins at 1 week after surgery and basically completes at 4 weeks after surgery. CK5 and CK14 positive cells were detected in the basal cells of the bladder epithelium after HoLRBT, and the expression of CK5 and CK14 mRNA in the basal cells of the bladder epithelium under hyperplasia was significantly higher than that of the normal bladder epithelial basal cells. Bladder epithelial wound repair of TURBT group was performed by the proliferative differentiation of the peri-bladder epithelium adjacent to the wound edge and crawled to the wound surface to complete the re-epithelialization process. The wound repair and re-epithelialization were significantly slower than HoLRBT group. The CK5 and CK14 positive cells can also be detected in the basal cells of marginal hyperplasia of basal margin, and the expression of CK5 and CK14 mRNA in the basal cells of the peri-bladder hyperplasia is obviously higher than that of the normal bladder epithelial basal cells. The expression of EGF in bladder regenerating epithelium gradually increased with time after HoLRBT. Bladder basal cells and bladder regenerating epithelium express high levels of EGFR after HoLRBT. The concentration of EGF in urine after HoLRBT and TURBT increased significantly after surgery, and peaked at 3 days after operation. The urinary EGF concentration in HoLRBT group was higher than that in TURBT group at 3 and 4 weeks after operation. The re-epithelialization process can be seen 1 week after the cystectomy with holmium laser cystectomy, and the epithelialization rate is faster than the traditional transection surgery. This is because the residual bladder epithelial stem cells and wound marginal epithelial cells are

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involved in the process of wound repair and re-epithelialization following HoLRBT. But only the marginal epithelial tissue participates in the re-epithelialization process after TURBT, so the repair rate of TURBT is slower. The repair of bladder epithelium after HoLRBT is related to the stimulation of tissue factor EGF. The regenerated bladder epithelium also participates in the wound repair process by means of autocrine of EGF.

Keywords Bladderepithelial stem cells · Wound repair · HoLRBT

Bladder cancer was the ninth most common malignancy worldwide [1]. On average, it is 3 to 4 times more common in men than in women [2]. Among the newly diagnosed bladder malignancies, non-muscle-invasive bladder cancer (NMIBC) accounts for 70%, of which Ta accounts for 70%, T1 accounts for 20%, and Tis accounts for 10%. At present, the standard treatment of non-muscle-invasive bladder cancer is transurethral resection of bladder tumors known as TURBT. Since Parson et al. [3] used laser technology in the urinary system for the first time, its advantages of safety and less bleeding are rapidly reflected and widely recognized. Laser technology has been popularized in the clinical practice of urology [4, 5]. Transurethral resection of the bladder tumor (HoLRBT) has been widely performed in the treatment of NMIBC. The prognosis and operation time of HoLRBT and TURBT are similar [6]. However, HoLRBT was superior to TURBT in terms of indwelling catheter time, hospital stay, bladder perforation, and obturator nerve reflex rate and incidence of postoperative bladder irritation [7, 8]. Although HoLRBT has obvious advantages compared with TURBT, it still cannot completely solve the problem of high recurrence and rapid progression of postoperative tumors. Postoperative bladder infusion chemotherapy can significantly reduce the recurrence of non-muscle invasive bladder cancer and is an important treatment after transurethral surgical procedure for NMIBC [9]. However, the side effect caused by chemotherapeutic agents such as frequent and urgent urination and urge urinary incontinence are still of high incidence. The complications of infusion chemotherapy seriously affect the quality of life and treatment of patients. Compliance has led to the occasional pause or termination of intravesical instillation chemotherapy, which has ultimately contributed to the recurrence of bladder cancer [9]. In order to reduce the side effects of infusion chemotherapy, it usually takes 2–4 weeks after TURBT to promote better repair and re-epithelization of bladder mucosa [10]. Therefore, the understanding of the process and mechanism of bladder re-epithelialization after HoLRBT and TURBT has important clinically applicable value and significance for achieving the fastest clinical wound healing and reducing the complications of bladder instillation. In recent years, it has been found that bladder epithelial stem cells are mainly present in CK5+/CK14+basal cells. These stem cells can self-regenerate and can differentiate and proliferate to form intermediate cells, thereby further generating superficial cells [11, 12]. In the study of re-epithelialization of the prostatic urethra after transurethral holmium laser surgery, it was found that the re-epithelialization of the prostatic urethra after surgery was due to residual prostate ducts and acinar epithelial cells under the wound [13]. The process of re-epithelialization of the prostate is also closely related to the stimulation of EGF [14]. However, the contrastive observation and mechanism study of the repair of bladder mucosal injury after HoLRBT and TURBT has been rarely reported. We hypothesize that the wound repair after HoLRBT is also related to the differentiation and proliferation of bladder stem cells in the basal cells of the urinary bladder, and has an important relationship with the secretion of tissue factor EGF. In this study, a canine transurethral holmium laser/electroresection bladder model was established to observe the healing process of cystoscopic wounds at various time points after operation and its mechanism was preliminary discussed.

Materials and methods

Canines

Thirty male beagle dogs, aged 2, weighing 13–17 kg, were randomly distributed into two group: a TURBT group and a HoRBT group. The dogs were adaptively fed with tap water and two meals per day for 2 weeks at the Animal Experiment Center of Shanghai General Hospital, Jiaotong University, Shanghai. Approval for the animal studies was obtained from the Medical Science Ethics Committee of Shanghai General Hospital.

Modeling of two-micron laser resection

All operations were performed using the same two-micron continuous wave Tm:YAG laser system (RevoLix; Lisa Laser Products, Katlenburg, Germany). The laser wavelength was 2.013 μm and the energy was transmitted at 40 W of power output through a flexible 550-μm-diameter fiber. After general anesthesia had been achieved with 10% chloral hydrate (0.003 ml g^{-1}), the canine was placed in the supine position on the operating table. The lower abdomen was entered through a medial and longitudinal incision, and the anterior wall of the bladder was freed. A purse suture was performed in the anterior wall the bladder, an incision was made within the purse to allow the placement of a 26F continuous-

flow resectoscope, and then the suture was fastened. Under saline irrigation, a resectoscope was placed into the bladder sidewall. The TURBT and HoLRBT procedure is the same as is applied in patients as described previously [15]. Using holmium laser and cutting rings injure the bladder mucosa. After the procedure was completed, a suprapubic tube was sutured in place for follow-up observation and biopsy of the bladder wound. Then, the abdominal wall was closed around the suprapubic tube and the tube was clamped. No transurethral catheter was required.

Histopathologic examination

Each group was randomly divided into five groups, and each group has three canines. The canines were sacrificed, and wound specimens from the bladder sidewall harvested and fixed in 4% formalin at 3 days and 1, 2, 3, and 4 weeks after laser treatment. After embedding in paraffin, 5-μm slides were examined histologically by hematoxylin and eosin (H and E) staining.

Immunohistochemistry staining

Immunohistochemical staining was performed as described earlier [16]. Briefly, the sections were treated with blocking buffer (Dako Denmark A/S, Glostrup, Denmark) for 30 min at room temperature (RT) and thereafter incubated with anti-cytokeratin14 (CK14)antibody (1:300 dilution in Tris–NaCl buffer; Abcam, Cambridge, UK), anti-CK5 antibody (1:250 dilution in Tris–NaCl buffer; Epitomics Inc., Burlingame, CA, USA), anti-epidermal growth factor (EGF) antibody (1:500 dilution in Tris–NaCl Abcam, Cambridge, UK), and anti-epidermal growth factor receptor (EGFR) antibody (1:250 Abcam, Cambridge, UK) overnight at 4 °C. Following a thorough rinse in Tris–NaCl buffer, the sections were incubated with secondary antibodies; example, biotinylated goat anti-mouse IgG or biotinylated goat anti-rabbit IgG (diluted 1:200 in Tris–NaCl buffer) for 60 min at RT. Sections were subsequently incubated with avidin-biotinylated enzyme complex and DAB and then dehydrated with increasing concentrations of ethanol, cleared withxylene, and mounted in permount. Negative controls for these immunohistochemical procedures were incubated with nonimmune serum instead of the primary antibodies, which resulted in no detectable staining.

ELISA

Morning urine samples were collected from each dog through the suprapubic tube on days 3, 5, 7, 10, 14, 21, 28, and before the surgery. The urine specimens were centrifuged at 1000×*g* for 5 min and the supernatant was used for analysis. Urinary inflammatory cytokine quantification was assessed by ELISA using commercial kits for canine EGF (RayBiotech, Inc., Norcross GA, USA).

Real-time polymerase chain reaction

The mRNA levels of CK14, CK5, EGF, and EGFR in the regions of proliferating bladder epithelial cells and unproliferating residual bladder epithelial cells far away from the wound at the bladder wound from 3 days to 4 weeks after the surgery were detected by real-time polymerase chain reaction (PCR). Real-time PCR was performed as described earlier [16]. In brief, we selected the regions of proliferating bladder epithelial cells and unproliferating residual bladder epithelial cells far away from the wound according to H and E staining from 3 days to 4 weeks, respectively. Total RNA was extracted from these two regions with the RecoverAll Total Nucleic Acid Isolation Optimized for formalin-fixed paraffin-embedded samples kit (Ambion Inc., Austin, TX, USA). The RNA preparations were treated with three units of RQ RNase-free DNase (Promega) for 10 min at 37 °C in order to remove any traces of genomic DNA present. The purity and quantity of RNA were determined with UV spectrophotometer with A260/A280 ratio > 1.9. Total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase and Oligo-dT primers. The forward and reverse primers for selected genes were designed using Primer Express software (Biosystems, Foster City, CA, USA) and listed in Table 1. The real-time quantitative reverse transcription-PCR was performed by using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems) and analyzed with GeneAmp 7300 SDS software, in which the SYBR green Master Mix (Applied Biosystems, Foster City, CA, USA) was used. The transcript levels were estimated by using the formula $2^{-\Delta CT}$, where ΔCT represents the difference in cycle time (CT) values between target and housekeeping assays. The relative differences in expression between these two regions were expressed using CT values, in which the difference in CT between the genes of interest was first normalized with 18s, and then calculated as relative increases by setting the regions of unproliferating residual bladder epithelial cells as 100% in comparison.

Table 1 PCR primers used in the study

mRNA	*Sequences*
CK5	5’ -GTTCTTTGAGGCGGAGCTGT- 3’ 5’ -TAGAGGCGTTGTGGTTCGTT- 3’
CK14	5’ -GCTGACGACTTCCGTACCAA- 3’ 5’ -TGCTCCTCCTTTACTTGCGA- 3’
EGF	5’ -GCAGTATCTTCTCACCATCAGCAC- 3’ 5’ -AAAACCAGAGCCCCAAACAA- 3’
EGFR	5’ -CTATGACCCTACCACCTACGA- 3’ 5’ -AAACTCACCGATTCCTATTCC- 3’
18s	5’ -CGCCGCTAGAGGTGAAATTCT- 3’ 5’ -CATTCTTGGCAAATGCTTTCG- 3’

PCR polymerase chain reaction, *CK5* cytokeratin 5, *CK14* cytokeratin 14, *EGF* epidermal growth factor, *EGFR* epidermal growth factor receptor

Statistical analysis

The means were compared by the two-tailed Student's *t* test using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) in order to determine if there were statistically differences.

Results

Cystoscopy changes in re-epithelialization in canine bladder urethra after resection of the bladder

Three days after surgery, the wounds were pale and mainly composed of coagulative necrosis in both groups. There was a small amount of blood leakage. No epithelium is covered on the wound surface. After 1 week in the laser group, the wound was ruddy and there was a small amount of bleeding sites. A few areas are scattered over thin layers of regenerated epithelium. The transection group still saw a large number of pale necrotic tissues covering the wound surface and no epithelial covering of the wound surface. From 2 weeks to 4 weeks after the surgery, the area and thickness of the regenerated epithelium among the wound in laser group had obviously increased. The wounds and normal bladder epithelium are clearly differentiated. In transaction group, the wound healing process is similar to laser group, but the healing is much slower, and the wounds and normal bladder epithelium are not clearly differentiated (Fig. 1).

Histopathology changes in re-epithelialization in canine bladder urethra after resection of the bladder

Three days after laser operation, cavitation, a zone of coagulation necrosis and acute inflammatory exudate on the wound surface was evident. There was no epithelium coverage on the wound surface. Under the necrotic tissue, residual bladder epithelial cell proliferation was seen. Inflammation cells infiltrate and mildly hyperplasia of small blood vessels. Compared with the laser group, wounds of in the electrotomy group had a larger range. There were more coagulative necrosis and inflammatory exudate adhesions. There was no epithelium coverage in the wound, and there was no residual bladder epithelial cell proliferation under the necrotic tissue.

One week after operation, the necrotic tissue and inflammatory exudate were still seen in the wounds of the laser group. Under the necrotic tissue, there was a significant proliferation of bladder epithelial cells with some squamous metaplasia. The nucleus was large, the nucleoli were obvious, and chromatin increased. Granulose tissue hyperplasia was seen; most epithelial wounds were not covered by epithelium, and a few wounds had flaky patches and reconstructed epithelium covered with small lamellae. This shows that the neo-epithelial neoplasia is consistent with the proliferating bladder epithelium under the wound edge. Peripheral urinary tract also has marginal proliferation and crawling. The coagulation necrosis and inflammatory exudate adhesion were still seen in the electrotomy group. There was no epithelium coverage on the wound surface. There was no residual bladder epithelial cell proliferation under the necrotic tissue. The urinary tract around the wound edge showed a marked hyperplasia.

After 2 weeks, more wounds in the laser group were covered by regenerated neo-epithelial neoplasia, and 2–3 layers of neonatal epithelium were not obvious. There was still a small amount of coagulative necrosis and inflammatory exudate adhesions in the electrotomy group. The peri-urinary tract around the wound edge gradually proliferated, crawled, and covered the wound.

Three weeks after operation, the regenerated epithelium of the laser group basically covered the wound, and the epithelium thickened to 5–6 layers. The cells were arranged in a polar array. The umbrella cells appeared on the surface, and the granulation tissue under the epithelium became mature. The fibrous tissue was slightly proliferated and the inflammatory

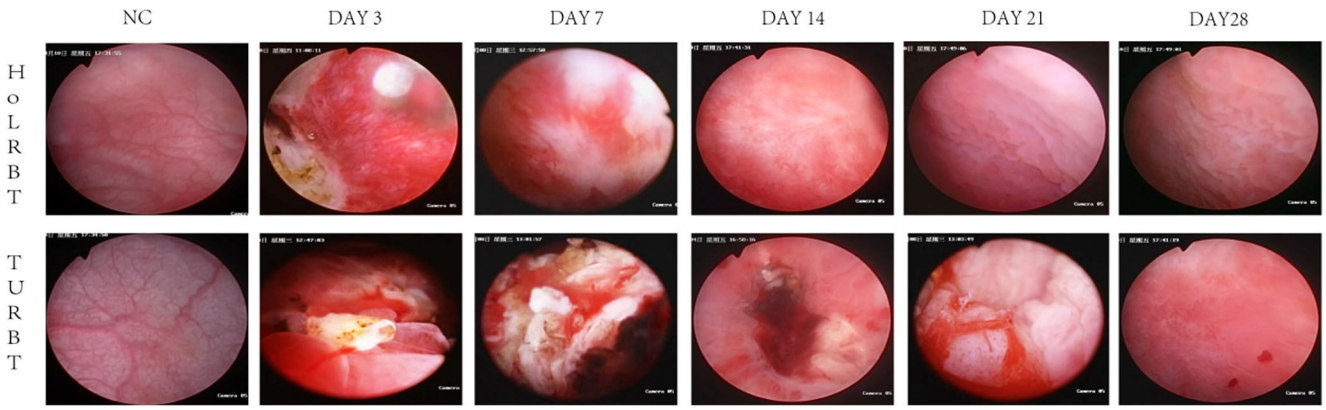

Fig. 1 Cystoscope observations of the bladder wounds. Compared with normal bladder epithelium (normal control, NC), 1 week after the surgery, there were no distinct differences between the wounds of the two groups. At each time point that followed, the wound healing of bladder of the laser group was much better than that of the transection dogs

cells were decreased. In the transection group, the wounds on both sides of the wound surface were basically covered with regenerative epithelium, and the polarity was not obvious.

At 4 weeks postoperatively, HE staining of the laser group was similar to that at 3 weeks postoperatively, and the proliferation of fibrous tissue under epithelial tissue was slightly higher than that at 3 weeks after surgery. In the electrotomy group, the wounds were basically covered by the regenerative epithelium, and the cells were in a polar arrangement, showing umbrella cells. The surgical wound healing process in the TURBT group was similar to that in the laser group after surgery, but the duration of inflammation was longer than that of the laser group. The time of appearance of the regenerating epithelium and the speed of covering the wound were slower than that of the laser group (Fig. 2).

Immunohistochemical results of CK5 and CK14 in bladder wounds after surgery

Three days after operation, a small amount of CK5- and CK14-positive epithelial cells were seen in the basal cells and neo-epithelial neoplastic cells under the coagulation necrosis in the laser group. No coagulation-negative cells were found in the transection group.

One week after laser irradiation, a large number of CK5- and CK14-positive hyperplasia epithelial cells were found in the basal cells and neoplastic epithelial cells under the coagulative necrosis in the cystic tissue of the laser group. In the transection group, no positive staining cells were found under the wound surface, but there were a few positive cells stained with CK5 and CK14 in the bladder epithelium near the wound edge.

At 2 weeks after operation, proliferating basal cells and neo-epithelial cells in the laser group still showed a large number of CK5- and CK14-positive hyperplastic epithelial cells and had basically covered the wound surface. However, in the TURBT group, no positive cells were stained, and CK5- and CK14-positive cells were seen around the wound surface and were covered by wounds.

At 3–4 weeks, the positive expression of CK5 and CK14 in hyperplasia of epithelial cells and regenerated epithelial cells was decreased in laser group postoperatively. The transection epithelium basically covered the wound in the transection group, showing positive staining of CK5 and CK14 in the regenerating epithelium (Fig. 3).

Changes of CK5 and CK14 mRNA levels in bladder wound tissue

In laser group, the levels of CK5 and CK14 mRNA in proliferating cells were higher than those in bladder epithelium away from the wound from 3 days to 4 weeks after operation. The differences were statistically significant. One week after surgery, the mRNA levels of CK5 and CK14 peaked and gradually decreased from 2 weeks after surgery. There was also high expression of CK5 and CK14 mRNA in the wounds of the electrotomy group. Compared with the normal bladder epithelial tissue away from the wound, the mRNA expression of the CK5 and CK14 was statistically significant from the second week. At the same time, the mRNA levels of CK5 and CK14 were lower than the laser group at the same time, and the difference was statistically significant. The peak appeared at 2 weeks after operation (Fig. 4).

Immunohistochemical results of EGF and EGFR in bladder wounds after laser surgery

At 3 days after operation, EGF and EGFR were positively expressed in inflammatory exudates and necrotic tissues of the bladder epithelium, neutrophils, and mononuclear macrophages. There was no expression of EGF in bladder epithelial cells adjacent to the necrotic tissue of the wound. However,

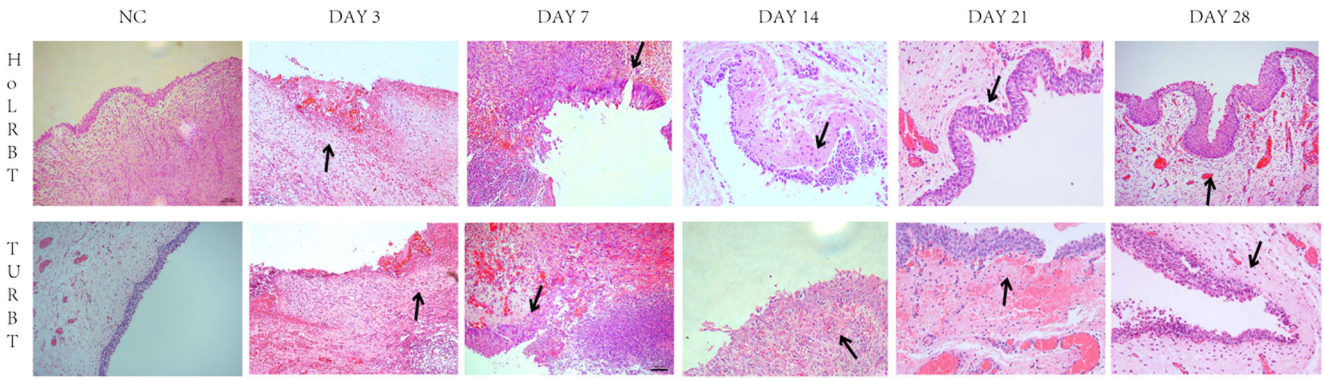

Fig. 2 Changes of regenerated epithelium on the wound surface of the three groups at each time point (200×). Compared with normal bladder tissue (NC), residual vesical epithelial cell proliferation was seen in necrotic tissue on the 3rd day after laser irradiation (day 3), and the epithelium under the wound surface and the wound surface could be seen and re-epithelialized after one week (day 7–28). In the electrosurgical group, epithelial hyperplasia was observed on the wound surface 1 week after surgery (day 7), and the epithelialization rate was slower than that of the laser group

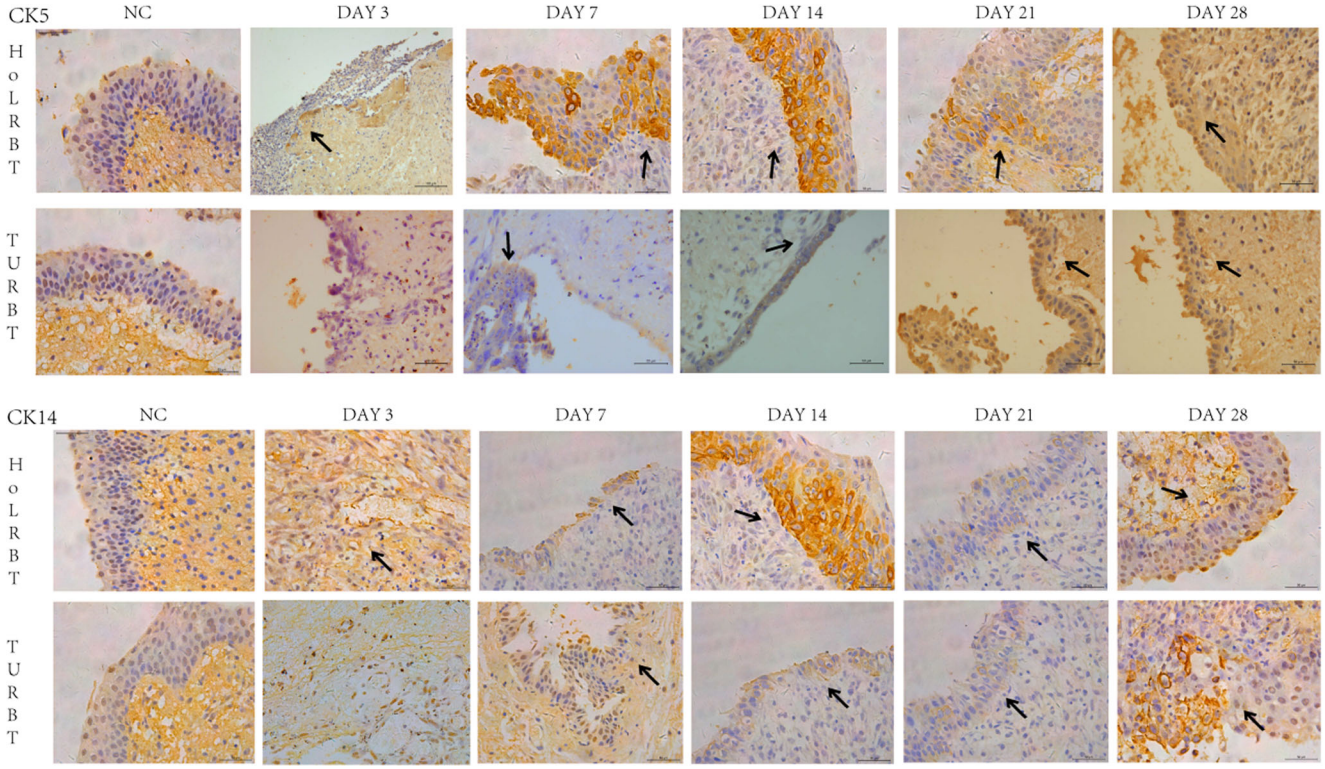

Fig. 3 The expression of CK5 and CK14 in canine bladder wound by immunohistochemical staining. Epithelial cells stained with CK5 and CK14 were seen under the coagulation necrosis of the bladder wound at 3 days after operation. In the laser group, epithelial cells stained positive for CK5 and CK14 were observed under the coagulation necrosis of the bladder wound at 3 days after operation. CK5 and CK14-positive epithelial cells were seen below the wound in 1–4 weeks after operation. In the transection group, CK5- and CK14-positive epithelial cells were also seen from the second week after surgery, and continued expression was observed four weeks after surgery

the expression of EGFR was positive and stronger than that of the basal cells in the non-proliferative bladder epithelium of the wound.

At 1 week after surgery, neutrophils and mononuclear macrophages showed positive expression of EGF and EGFR, and there was no expression of EGF in bladder epithelial cells adjacent to the wound necrotic tissue, but the expression of EGFR was positive. The intensity of EGFR expression was slightly stronger than that at 3 days after surgery. A small number of wounds showed weak expression of EGF and EGFR in regenerated epithelium.

At 2 weeks after operation, EGF and EGFR were positively expressed in neutrophils, mononuclear macrophages, and fibroblasts. The epithelial cells adjacent to the wounds did not express EGF and positively expressed EGFR. The intensity of expression was similar to that of postoperative 1 week. The positive expression of EGF and EGFR in regenerated epithelial wounds was stronger than that in the first week after surgery.

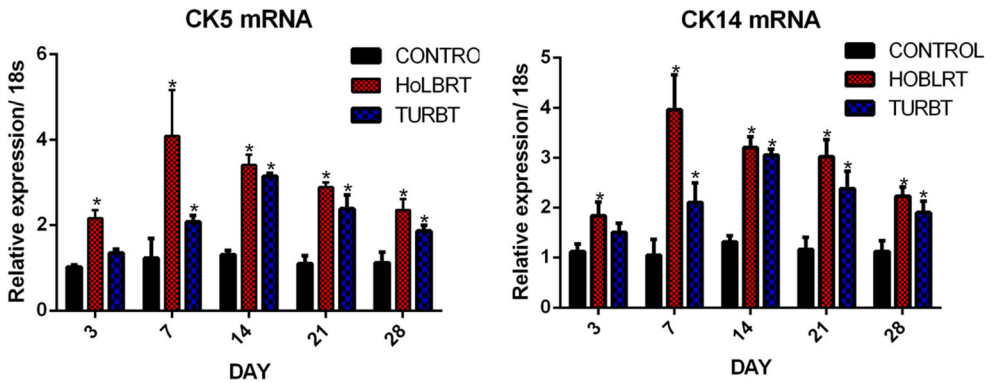

Fig. 4 CK5 and CK14 mRNA levels in bladder wound tissue after the surgery. The CK5 and CK14 mRNA levels of proliferating cell stem cells in the laser group were higher than those in the bladder epithelial tissue away from the wound from 3 days to 4 weeks after operation ($P < 0.05$). The peak value was reached 1 week after operation. The CK5 and CK14 mRNA levels of proliferating cell stem cells in the transection group were higher than those in the bladder epithelium away from the wound surface from the first week ($P < 0.05$) (*P<0.05 vs control)

At 3 and 4 weeks after surgery, the number of inflammatory cells decreased, and a small amount of neutrophils, mononuclear macrophages, and fibroblasts showed weak expression of EGF and EGFR. A small amount of EGFR in bladder epithelial cells underwent weak expression. The intensity of expression was basically the same as that of the non-proliferating bladder epithelial cells. No EGF expression was found. Positive expression of EGF and EGFR in regenerated epithelial wounds compared with expression increased 2 weeks after operation (Fig. 5).

Changes of EGF and EGFR mRNA levels in bladder wound tissue after laser surgery

At 1, 2, and 3 weeks after operation, the expression of EGF mRNA in the regenerated epithelial cells in the laser group gradually increased and peaked at 3 weeks after surgery and slight decline in the fourth week. At each time point, the expression of EGF mRNA in wounded regenerated epithelial cells was higher than that in bladder epithelial cells that were not proliferated. The difference was statistically significant. At 3 days, 1 week, and 2 weeks after surgery, the expression of EGFR mRNA in the basal cells of the necrotic tissue adjacent to the wound increased, peaked 1 week after surgery, and gradually decreased afterwards, and the difference of EGFR mRNA expression between the proliferating bladder basal cells and the basal cells away from the wounds without proliferating bladder epithelium was statistically significant. At 2, 3, and 4 weeks after operation, the expression of EGFR mRNA in the regenerated epithelium of the wound increased, peaked at 3 weeks after operation, and decreased slightly during the fourth week. At each time point, there was a statistically significant difference in the expression of EGFR mRNA in the regenerated epithelium of wounds compared with those in the non-proliferative bladder epithelial cells (Fig. 6).

Change of EGF concentration in urine

The peak concentration of EGF detected in the urine at each time point in the laser group and the electrotomy group appeared on the third day after operation and then decreased gradually, and high expression of EGF was still seen 4 weeks after operation. It can be seen from the 14th day after surgery that the decreasing trend of EGF concentration was slowed down, and the concentration of EGF at each time point was significantly different from the preoperative one. The concentration of EGF in urine was higher in the laser group on the 21st and 28th days after operation than that in the TUR group. The difference was statistically significant ($p < 0.05$) (Fig. 7).

Discussion

The curative treatment of NMIBC is transurethral resection of bladder tumor (TURBT). TURBT is also an important method for diagnosing NMIBC. However, many previous studies have shown that transurethral holmium laser resection of the urinary bladder (HoLBRT) was superior to TURBT with regard to the parameters obturator nerve reflex, bladder perforation, catheterization time, hospitalization time, and 24-month recurrence rate. Moreover, HoLBRT can offer a more accurate result of the tumor's pathological stage and grade [6, 17]. The study of prostate holmium laser surgery showed that the reason for the smaller damage of prostate laser surgery than that of prostate resection is the difference in the way of epithelial repair and the difference in the speed and mechanism of re-epithelialization [13]. So we speculate that this is the same in bladder surgery.

Re-epithelization of the wound is one of the key steps in the wound repair process. An important criterion for the body to complete the repair process is whether the wound is complete and then re-epithelialized to restore the integrity of the wound. Conceptually, re-epithelization can be the result of three

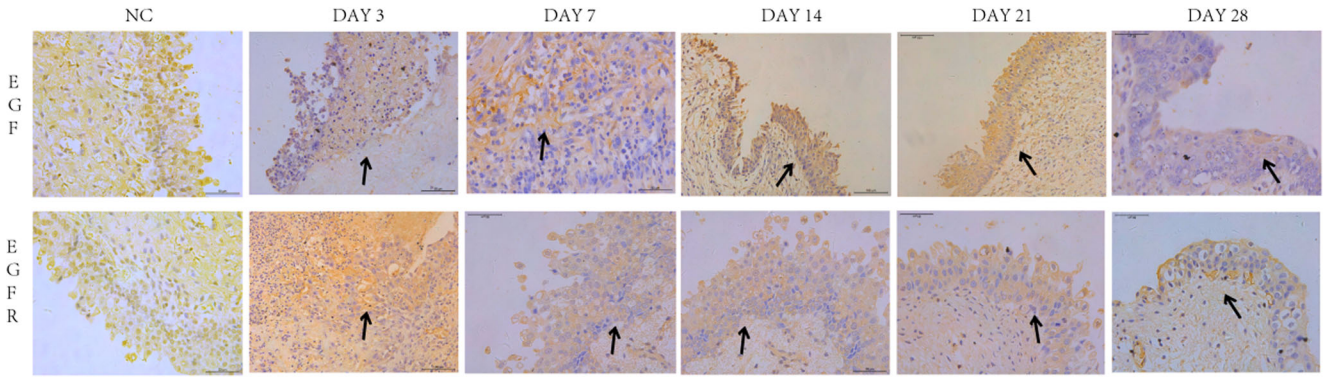

Fig. 5 EGF and EGFR expression in bladder wounds after laser surgery by immunohistochemistry staining. The expression of EGF and EGFR in inflammatory exudate and necrotic tissue in neutrophils was detected on the 3rd day after operation, while the basal cells in hyperplasia on the wound surface expressed high levels of EGFR and did not express EGF; at 1 week to 4 weeks, EGFR was highly expressed in epithelial basal cells of the wound, while neonatal bladder epithelium expressed high levels of EGF and EGFR

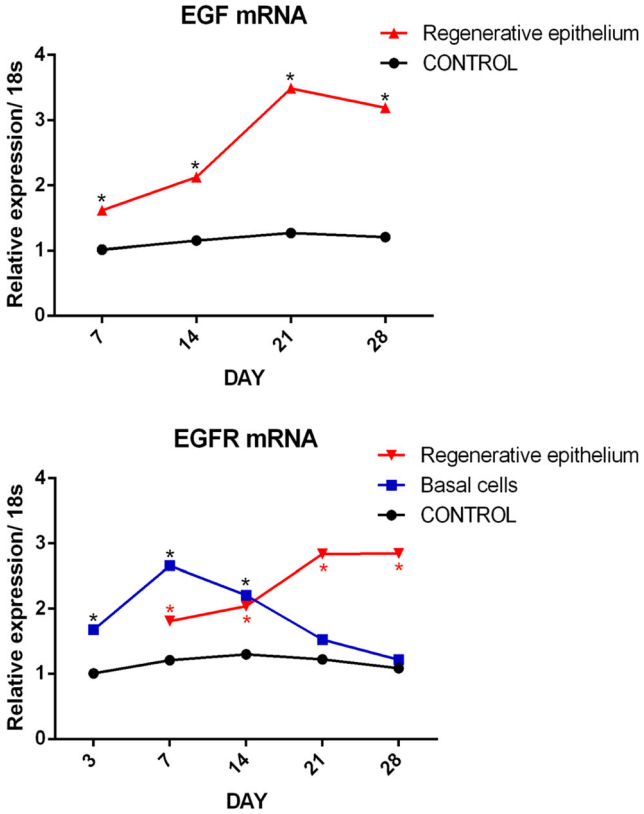

Fig. 6 Changes of EGF and EGFR mRNA levels in bladder wound tissue after laser surgery

overlapping functions of cells: cell proliferation, migration, and differentiation. The study of skin wound repair showed that the wound surface of the skin wounded by epidermal keratinocyte proliferation, migration, covering the wound and then differentiated to complete the process of wound re-epithelialization [18].

We established a canine transurethral cystospasm laser and electrosurgical model to observe the process of wound repair and re-epithelialization. The results showed that the laser-damaged bladder epithelium was mainly inflammatory in three

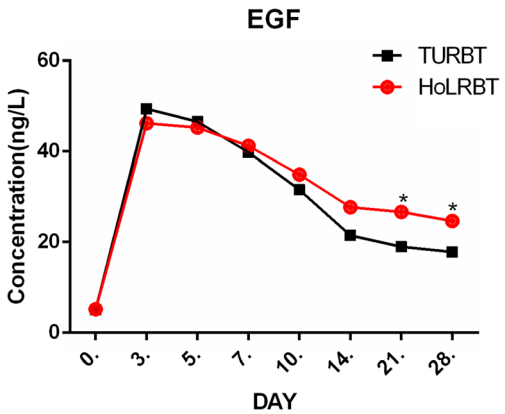

Fig. 7 EGF levels in the urine from each dog were analyzed by ELISA at each time point. *$p < 0.05$

days after operation, and the wound was re-epithelialized from the beginning of 1 week. HE staining showed that there was a significant proliferation of vesical epithelial cells in the necrotic tissue with some squamous metaplasia, and a few of the wounds had been covered with patchy regenerated epithelium, showing that the neo-epithelial neoplasia continued with the proliferative bladder epithelial cells under the wound edge. Peripheral urinary tract also has marginal proliferation and crawling. After 2 to 4 weeks of surgery, the wound was gradually covered by regenerative epithelium and gradually thickened, and by 4 weeks after surgery, the bladder epithelium had basically completed the re-epithelialization process. In the transection group, there were no hyperplastic vesical epithelial cells after necrotic tissue. The wounds were mainly covered by inflammatory necrosis tissue. Two weeks after surgery, the uninjured urothelium near the wound edge gradually re-epithelized by proliferating, crawling, and covering the damaged bladder surface. Through comparison between the two groups, we found that the rate of re-epithelialization in the transection group was significantly slower than that of the laser group. This may be due to the fact that the laser has a smaller lesion depth than the excision, and has less damage to the basal cells of the bladder epithelium under the wound surface so that basal cells can rapidly differentiate and proliferate after epithelial damage and together with the marginal epithelium complete re-epithelialization. However, in the TURBT group, the wound was larger, and the tissue under the wound surface could not effectively differentiate and proliferate and participate in the process of wound repair and re-epithelialization. Therefore, normal vesical epithelial cells beside the wound edge can be proliferated and differentiated and cover the wound surface, which is the reason why the laser group has obvious advantages over the TURBT group in the repair rate and re-epithelialization degree.

In the process of wound healing, basal cells play a crucial role in the re-epithelialization process. Potten CS et al. think that there are three kinds of basal cells in the basal layer of epidermis, namely stem cells, transit amplifying cells (TA cells), and committed cells. The epidermal stem cells account for about 10% of the total number of basal cells [19]. Stem cells still maintain a high self-renewal capacity in adulthood, and they are ultimately responsible for the maintenance of epidermal tissue and repair during trauma [20].

Wound healing is a complex, multi-step process. A critical and important feature of a healed wound is the restoration of an intact epidermal barrier through wound re-epithelialization. Re-epithelialization can be conceptually viewed as the result of three overlapping cell functions: proliferation, migration, and differentiation [18]. It has been reported that prostatic basal cell layer may have stem cells with differentiation potential that maintain the development, maturation, and function of the prostate [21]. They maintain the development, maturation, and function of the prostate and are associated with the development of prostate cancer and benign prostatic

hyperplasia [22]. The most plausible explanation for this powerful regenerative capacity of bladder epithelium is the presence of stem cells with self-renewal and proliferation capabilities in bladder epithelium. Existing studies suggest that urothelial stem cells are mainly found in basal cell CK5+/CK14+ cells. These stem cells can rapidly regenerate after injury to the bladder epithelium, and differentiate and proliferate to form intermediate and superficial cells, which helps to complete the re-epithelialization of bladder epithelium, and participate in the wound repair process [11, 12]. In this study, it was also observed that the proliferative cells under the wound surface are characterized by large nucleus, obvious nucleoli, and increased chromatin, which are similar to the morphological features of dermal stem cells [23], and these cells expressed CK5 and CK14 from the third day after operation, suggesting that they may be differentiated by the microenvironment of bladder epithelial stem cells. The proliferation of residual bladder epithelial cells was observed under HE staining, and immunohistochemistry showed high expression of CK5 and CK14, which may be the differentiation of basal stem cells expressing CK5 and CK14. The CK5 and CK14 mRNA levels were higher in the bladder epithelial tissue away from the wound from 3 days to 4 weeks after operation, which also confirms this conclusion. It can be seen that the mRNA levels of CK5 and CK14 peaked 1 week after surgery and gradually decreased from 2 weeks after surgery. These all suggest that the proliferation and differentiation of stem cells derived from residual basal cells under the wound after laser surgery quickly seals the wound surface and further re-epithelialization. In the transection group, the basal cells of the vesical epithelial cells near the wound surface also had the expression of CK5 and CK14, but they were mainly expressed in the urinary bladder epithelium on both sides of the wound edge and appeared later than the laser group. At the same time, the mRNA levels of CK5 and CK14 were lower than that of the laser group at the same time, and the peak appeared at 2 weeks after operation. Therefore, we speculate that the reason for the faster re-epithelialization of the laser group compared with the TURBT group is that the holmium laser cystectomy has a smaller lesion depth than the cystectomy and the superficial coagulation layer of the wound. It has less damage to basal cells of bladder epithelium, so stem cells in the remaining basal cells of bladder epithelial cells can rapidly differentiate, proliferate and migrate under the action of inflammatory factors and chemokines, and quickly complete the process of wound repair. In the TURBT group, due to the large depth of injury, the stem cells in the wound surface of the bladder are more damaged due to the large depth of injury. Therefore, the process of wound repair consists of proliferation and migration of the epithelium beside the wound edge, covering the wound and then diverging to complete the process of wound re-epithelialization. The re-epithelialization time of the TURBT group was longer than that of the laser group and the appearance of cells of CK5 and CK14 and the expression time of mRNA were later, and the expression level of mRNA was also smaller than that of the laser group.

Trauma repair is a complex process. The interaction between the wound microenvironment and repairing cells is very important. This requires the synergistic interaction between multiple cellular components, cytokines and extracellular matrix [24]. Growth factors not only promote animal growth and development but also play an important role in wound healing and wound healing. When the body is traumatized, the level of growth factors in the wound microenvironment changes rapidly. Each growth factor binds to a specific receptor on the target cell and initiates the tissue repair process through multiple pathways. These include the activation of rest cells that are in a resting state, the proliferation and migration of cells, the increase of extracellular matrix biosynthesis, and the regulation of granulation tissue formation and re-epithelialization processes [25]. Many literatures have confirmed that various growth factors play an important role in skin wound repair, including epidermal growth factor (EGF) closely related to re-epithelialization [26]. EGF is widely distributed in humans and animals and promotes the growth of many types of cells. EGF binds to its specific receptor EGFR and forms an EGF-EGFR complex, which functions through a series of complicated processes such as activation of tyrosine phospholipase by G protein. Studies have shown that after skin damage, platelets, inflammatory cells, and damaged keratinocytes that are locally accumulated on the wound surface can release EGF to stimulate epithelial cell proliferation and accelerate wound healing and repair [27]. The glycosaminoglycans in the extracellular matrix of bladder epithelial cells, including heparan sulfate, inhibit the out-diffusion of growth factors, form pools of factor concentrations, and release these factors, including EGF. EGF can be autocrinely produced by damaged bladder epithelial cells and activate the transcription factor SOX9 to promote the proliferation and repair of bladder epithelial cells [28, 29].

We analyzed the concentration of EGF in the urine as the progress of postoperative time. It was shown that the concentration of EGF in the urine was significantly elevated after operation, peaked at 3 days after surgery, and decreased slowly at 2 weeks postoperatively. In the laser group, the concentration of EGF was higher compared with the electrotomy group 3 weeks and 4 weeks after surgery, which suggests that in the process of re-epithelialization after HoLRBT, the tissue factor EGF and its receptor EGFR play an important role in the wound healing. We studied the distribution and expression changes of EGF, EGFR, and their relationship with repair time in bladder wounds of thulium laser dogs. The results of immunohistochemistry showed that EGF and EGFR were expressed in neutrophils and macrophages of the inflammatory exudate and necrotic tissue of the thulium laser cyst wound 3 days after the operation, while the proliferative bladder basal cells under the wound surface expressed high levels of EGFR and no

expression of EGF, and 1 week to 4 weeks postoperatively, neutrophils and mononuclear macrophage cells express EGF and EGFR in the inflammatory exudate and necrotic tissue, while the proliferative epithelial basal cells under the wound surface express EGFR highly and the newborn bladder epithelium expresses EGF and EGFR. The biological effects of EGF need to be achieved by acting on specific receptors distributed on repair cells. The expression of EGF-specific receptor EGFR is enhanced in the proliferative basal cells of the bladder epithelium under the wound. The mRNA expression of EGF and EGFR in the thulium laser showed that the peak of EGFR expression in the bladder basal cells under the wound after surgery appeared at 2 weeks after operation and then gradually decreased, which remained higher than normal bladder epithelial tissue until 4 weeks after surgery. Over time, the mRNA expression of EGF and EGFR in the regenerating epithelium gradually increased and remained highly expressed after 4 weeks. Neutrophils, macrophages, and fibroblasts are major inflammatory and repair cells during wound repair. In the early and mid-trauma period (3 days and 1 week after surgery), exuded macrophages and proliferating fibroblasts secrete EGF [30]. We speculate that in the early and mid-reparation stage, platelets and inflammatory cells aggregated locally around wounds secrete EGF, and the proliferative basal cells under the wounds also show EGFR positive expression and peak at 1 week after surgery, and these cells are precisely bladder epithelial stem cells with CK5+ and CK14+. Exogenous EGF combined with EGFR of proliferating bladder epithelial stem cells promoted the proliferation and differentiation of bladder epithelium. In the later period of repair (3 weeks and 4 weeks after operation), the wound was basically covered with regenerative epithelium, the inflammation was gradually controlled while the inflammatory cells and fibroblasts were reduced. The main changes of wound healing were that the regenerating epithelium differentiated into epithelial and intermediate epithelium of the bladder. The expression of EGFR in bladder epithelial basal cells decreased to non-proliferative level, while the expression of EGF and EGFR peaked in the regenerated epithelium of the wound, which may be the main source of EGF. The regenerative epithelium promotes its own hyperplasia and differentiation into the urothelium through an autocrine approach. The thickness of regenerated epithelial and re-epithelialization degree in the laser group were better than those in the electrotomy group at 3 and 4 weeks postoperatively, which also explained the phenomenon that the concentration of EGF in the urine of the laser group was higher than that of the electrotomy group postoperatively 3 and 4 weeks. Therefore, our results show that there is EGF and its receptor distribution and expression changes in the bladder epithelial wound of the thulium laser cystectomy, the changes of which are closely related to the time of repair. This is due to the accumulation of inflammatory cells, platelets and their secretion of EGF after wound formation. The EGF that stimulates the EGFR of CK5+ and CK14+ bladder epithelial stem cells remaining in the basal portion of the bladder under the wound surface causes proliferation and differentiation of the bladder stem cells and further completes wound repair and re-epithelialization.

In summary, thulium laser bladder surgery has the characteristics of small injury scope and shallow depth of injury. It has less damage to bladder stem cells under the wound and can activate bladder epithelial stem cells at the early stage of injury to achieve rapid healing of wounds, reducing re-epithelialization time, improving wound healing quality, reducing the operative complications and speeding up the recovery of patients and other good results. At the same time, the rapid re-epithelization process after thulium laser surgery also minimizes the damage of the bladder perfusion to the wound surface, which can effectively reduce the complications of postoperative perfusion of bladder tumors, and can play an important role in clinical practice. Also, we find that EGF and its receptor EGFR are important for the repair of bladder epithelium after thulium laser surgery. Also, bladder wound regenerating epithelium is also an significant source of EGF in the later stage of wound re-epithelialization. In this study, the establishment of a canine transurethral bladder thulium laser cystectomy model was used to explore the differences in the histological repair of wounds during 2 weeks after the laser and electrotomy excision, and the mechanism of re-epithelialization of vesical wounds after thulium laser was explored preliminarily. Future research directions will focus on the effect of EGF concentration and the regulation of androgen or other factors affecting wound repair on the repair of wounds as well as the effect of drugs used in intravesical instillation on bladder re-epithelialization. Similarly, how to reduce the negative impact of infusion drugs on the wound is also the important direction of future research. We hope to provide a reference for better treatment of bladder tumors and promotion of thulium laser bladder surgery through the observation and mechanism study of the wound healing after thulium laser surgery.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Approval for the animal studies was obtained from the Medical Science Ethics Committee of Shanghai First People's Hospital.

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