Vocal fold injury models in rats: a literature review on techniques and methodology

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ABSTRACT
This study reviewed the current literature on technical aspects regarding controlled vocal fold injuries in the rat model. Data from PubMed, Embase, and Scopus database for English language literature was collected to identify methodological steps leading to a controlled surgical injury of the rat vocal fold. Inclusion criteria: full disclosure of anesthesia protocol, positioning of the rat for surgery, vocal fold visualization method, instrumentation for vocal fold injury, vocal fold injury type. Articles with partial contribution were evaluated and separately included due to the limited number of original methodologies. 724 articles were screened, and eleven articles were included in the analysis.

Anesthesia: ketamine hydrochloride and xylazine hydrochloride varied in dose from 45 mg/kg and 4.5 mg/kg to 100 mg/kg and 10 mg/kg. Visualization: The preferred method was the 1.9 mm, 25–30 degree endoscopes. The widest diameter endoscope used was 2.7 mm with a 0 or 30 degree angle of view. Instruments for lesion induction range from 18 to 31G needles, microscissors, micro forceps to potassium titanyl phosphate, and blue light lasers. Injury types: vocal fold stripping was the main injury type, followed by vocal fold scarring and charring. One article describes scaffold implantation with injury to the superior aspect of the vocal fold. Rats are good candidates for in vivo larynx and vocal folds research. A more standardized approach should be considered regarding the type of vocal fold injury to ease data comparison.

KEYWORDS: rat, vocal fold, review, injury, methodology.

INTRODUCTION
Voice quality after vocal fold injury, scarring, and subsequent surgery is still a significant concern for the clinician and patient. The vocal folds are uniquely layered structures that consist of stratified non-keratinized squamous epithelium, lamina propria (LP), and the thyroarytenoid muscles [1]. Primarily, the vibratory property of the vocal folds depends on the size and the extracellular matrix (EM) constituents of the LP [2–3]. Exposing the LP due to epithelial injury alters the composition of collagen, elastin, and glycosaminoglycan [4] in the EM and impairs vibratory function. These changes will clinically manifest as dysphonia [5].

Animal models have been widely used to document healing, scarring, and biomechanics of the vocal folds. The rat model is the most widely used, partially due to the same tri-layered vocal fold structure as humans and presents similar fibrous protein composition of the EM. Also, a great amount of research data is already available regarding chronic scarring of the vocal fold compared to the canine of the rabbit model [6–7]. A rat vocal fold injury model is difficult to replicate even throughout the study groups due to the lack of standardization and reproducibility of the vocal fold lesions themselves. Imaizumi et al. emphasized the importance of depth and length of a controlled injury in experimental studies by cross-referencing rat vocal fold injuries to human vocal fold pathology and excision extent [8]. Understanding vocal fold anatomy, histological layer succession, and the space provided by the rat pharyngeal lumen (Figure 1 and Figure 2) are key in performing controlled in vivo laryngeal surgery in concordance with the scope of the study. Although the rat model holds several advantages over other species, technical difficulty may arise when choosing the appropriate surgical methodology for the scope of the study.

This review article focuses on technical aspects of controlled vocal fold injury in rat models to synthesize and improve current knowledge on methodology and assist further experimental research regarding in vivo vocal fold healing in the rat model.
MATERIAL AND METHODS

A literature review was conducted in March 2021, following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines.

Literature search criteria

A primary electronic MEDLINE (PubMed), Embase, and Scopus database search for English language literature were carried out with advanced search criteria using MeSH terms: “rat” and "vocal fold". No publication period restriction was used. The scope was to identify all possible articles in these databases, discussing surgical injury to the rat vocal fold, regardless of the scope of the study.

Selection criteria

The objectives were to systematically identify the methodological steps that lead to a controlled surgical injury of the rat vocal fold. Based on the title and abstract, all identified articles were independently screened by two authors for inclusion.
Articles that did not indicate a vocal fold injury or healing process were excluded. The included articles were screened as full-text versions.

We established five criteria as a part of a controlled vocal fold injury workflow for a methodology to be eligible. Criteria were as follows: full disclosure of anesthesia protocol; positioning of the rat for surgery; vocal fold visualization method; instrumentation for vocal fold injury; mentioning the type of vocal fold lesion created.

First, articles from the same author were screened to determine if the same methodological steps apply. If the methodology of the surgical injury was the same, the methodology discussed in the most recent article was excluded. Studies that cited and integrally used methods already published in the literature were eliminated. We also excluded the referenced article if the methodology for the vocal fold injury model was also cited from a previous article. The process was repeated until the original methodology was found. Furthermore, all articles discussing chronic vocal fold injury; laryngeal nerve injury, including other experimental animals of which the title and abstract screening did not pick up, were also excluded.

After including only the articles that discuss acute vocal fold injury in a rat model, a second screening was applied to include articles that mention all five methodological steps discussed above.

Finally, all original articles that met inclusion criteria were noted in an Excel spreadsheet and qualitatively analyzed. If discrepancies arose regarding any point of the screening process, the senior author stepped in as arbitrator.

Given the limited number of original methodologies found, a secondary manual search was conducted to include articles with a partial contribution. The partial contribution was defined as articles not mentioning all steps of the methodological process to be eligible for inclusion but adding value through original insight to the vocal fold injury process.

RESULTS

Study characteristics

The database search resulted in a total of 726 publications. MEDLINE n=266; Embase n=214; Scopus n=246. After removing duplicates, 326 articles were included for analysis. The selection process is illustrated in Figure 3. Fifty-seven articles were selected for full-text analysis, eleven of which were included for qualitative analysis. Seven articles that did not meet all inclusion criteria but partially contributed insight into the surgical methodological steps are synthesized in Table 1. Four articles that met all inclusion criteria as original methodologies are synthesized in Table 2.

Anesthesia and medication

Anesthesia protocols varied in terms of dose and means of administration. Induction anesthesia was not carried out in all instances. Ketamine hydrochloride and xylazine hydrochloride were used in all cases. When induction anesthesia was preferred, isoflurane [10, 14, 17, 18] or diethyl ether [13, 19] were used. Ketamine hydrochloride and xylazine hydrochloride varied in terms of dose from 45 mg/kg and 4.5 mg/kg [11–13] to 100 mg/kg and 10 mg/kg [10]. The preferred method of drug delivery was intraperitoneal. Intramuscular delivery was only used in two cases [7, 19]. The use of Atropine sulfate 0.005 mg/kg and 5% topical lidocaine was discussed by Tateya et al. [10]. Carprofen 5 mg/kg was used by Xu C. et al. [17].

Visualization of the rat larynx

Visualization of the rat larynx was carried out using different methods. Endoscopes were preferred over surgical microscopes or direct visualization. The endoscope was the preferred method [14, 15, 18]. The widest diameter endoscope used was 2.7 mm with a 30 degree angle of view [11]. The surgical microscope was used in one case [17].

In order to secure the endo-luminal view and pharyngeal patency, a series of custom-made laryngoscopes have been described. A 4 mm ear speculum was used by inserting it through the oral cavity to act as a suspension device [17]. Another method for securing the endoluminal patency was a custom-made spring wire device laryngoscope [16, 18]. Direct visualization of the vocal fold without optical instruments was described by Kanemaru S. et al. [19], using a sub-criconid incision to expose the subglottic region to visualize the vocal folds directly.

Instruments used for acute controlled injury

The controlled vocal fold injury instrumentation was divided into cold instruments and surgical lasers. Nine authors preferred cold instruments, and two authors used either potassium titanyl phosphate (KTP) or blue light (BL) laser as a means of injury. Cold instruments ranged from micro-scissors and micro forceps to different gauge needles. The smallest needle size was 32 G [19], and the biggest diameter needle was 18 G [17]. The two original methodologies using lasers as a means of controlled injury channeled the energy through an nr. 5 Frasier suction canula. The energy used for the KTP laser (532 nm) was at 10 W, 20 ms through a 0.4 mm fiber. For the BL laser (445 nm), the energy output was set at 2 W with 10 ms bursts.

Injury type

Seven authors describing the use of cold instruments describe vocal fold stripping (with exposure of the thyroarytenoid muscle). Another injury type was described to facilitate the implantation of bioactive scaffolds. For this purpose, an 18 G modified spinal needle was used to create a 0.8 mm longitudinal incision at the superior surface on the posterior 1/3 of the rat vocal fold [17]. Grasping micro-forceps were used to induce focal lesion of the vocal fold by injuring the middle 1/3 of the vocal fold to create a model for membranous vocal fold scarring [14]. The two articles describing a thermal injury model using lasers describe charring and branching of the vocal folds as a mean injury [9, 16].

DISCUSSIONS

Experimental models permit the assessment of diverse bioactive agents on vocal fold wound healing that subsequently could translate into clinical application [20]. The rat model is well-established in investigating structural and functional modifications in all organ systems. Given the overall small size of rats and the 1 mm membranous vocal fold length, it is challenging to achieve consistency regarding vocal fold lesions throughout study groups [6, 21].
Records identified through PubMed, Embase and Scopus database search (n=726)

Records after duplicates removed (n=324)

Records screened for title and abstract (n=324)

Records excluded due to no indication of vocal fold injury in title or abstract (n=267)

Full text articles assessed for eligibility (n=57)

Full text articles excluded as having the same author and same methodology with different objectives (n=13)

Full text articles excluded due to not mentioning specific information on methodology regarding vocal fold injury (n=33)

Studies included in qualitative synthesis (n=11)

Full text articles with original methodology as defined in methods (n=4)

Full text articles with partial contribution to the literature as defined in methods (n=7)

Figure 3. PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) flowchart summarizing the search results and the application of eligibility criteria.

Table 1. The list of methodologies with partial contribution.

| Nr. | Author           | Year | Visualization | Lesion induction | Positioning of animal | Type of lesion  | Anesthesia                        |
|-----|------------------|------|---------------|------------------|-----------------------|-----------------|-----------------------------------|
| 1.  | Lin R. J et al.  | 2020 | 2.5 mm 0 degrees endoscope | 445 nm BL LASER at 2 W, 10 ms through nr. 5 Frasier cannula | Semi vertical position on custom operating platform | Superficial charring of the vocal fold | Not mentioned |
| 2.  | Kim C-S et al.   | 2018 | 2.7 mm rigid endoscope | Micro scissors | Semi vertical position on a custom made platform | Vocal fold stripping | Induction with isoflurane IP inj. of K: 100 mg/kg X: 10 mg/kg |
| 3.  | T. Morisaki et al. | 2018 | 2.7 mm 30 degrees endoscope | 25 G needle | Semi vertical position on a custom made platform | Vocal fold stripping | IP inj. of K: 45 mg/kg X: 4.5mg/kg |
Table 1. Continued.

| Nr. | Author          | Year | Visualization                  | Lesion induction                  | Positioning of animal                      | Type of lesion                      | Anesthesia                                                |
|-----|-----------------|------|---------------------------------|----------------------------------|--------------------------------------------|-------------------------------------|-----------------------------------------------------------|
| 4.  | R. Suzuki et al. [12] | 2017 | 30 degree rigid endoscope       | Micro scissors and microforceps   | Semi vertical position on a custom made platform | Vocal fold stripping                | IP inj. of K: 45 mg/kg X: 4.5 mg/kg                       |
| 5.  | M. Gugatschka et al. [13] | 2011 | Endoscope – no details mentioned | 90 degree bent scissor           | Vertical position on custom made platform   | Vocal fold scarring                 | Induction with diethyl ether IP inj. of K: 45 mg/kg X: 4.5 mg/kg topical anesthesia (5% lidocaine) |
| 6.  | B. H. Q. Johnson et al. [14] | 2010 | 1.9 mm diameter 30 degree endoscope | 27 gauge needle                  | Semi vertical position on custom made platform | Vocal fold stripping                | Induction with isoflurane IP inj. of K: 90 mg/kg X: 9 mg/kg IP inj. of atropine 40 µg/kg |
| 7.  | Ohno et al. [15] | 2009 | 1.9 mm 30 degree endoscope      | 22 gauge needle                  | Custom-made operating platform              | Vocal fold stripping                | IM inj. of K: 60 mg/kg X: 6 mg/kg                         |

BL – blue light; K – Ketamine; X – Xilazine; IP – intraperitoneal; IM – intramuscular.

Table 2. List of original methodologies considering all inclusion criteria.

| Nr. | Author          | Year | Visualization                  | Lesion induction                  | Positioning of animal                      | Type of lesion                  | Anesthesia                                                |
|-----|-----------------|------|---------------------------------|----------------------------------|--------------------------------------------|--------------------------------|-----------------------------------------------------------|
| 1.  | Mallur P.S et al. [16] | 2009 | 2.5 mm 0 degrees endoscope; retraction on tongue; spring wire laryngoscope; retraction of the epiglottis with a blunt probe | 532 nm KTP LASER at 10 W 20 ms through a 0.4 mm fiber through size 5 Frasier suction tip | Supine in a horizontal position | Blanching of the vocal fold mucosa | IP inj. of K: 80 mg/kg X: 8 mg/kg                           |
| 2.  | Xu. C et al. [17] | 2009 | Surgical microscope and 4 mm ear speculum | A custom tool from an 18 G spinal needle | Custom made platform in semi-vertical supine position | 0.8 mm incision on the superior surface of the posterior one-third of the vocal fold of each vocal fold | Induction by inhalation of 4% isoflurane; K: 65 mg/kg X: 6.5 mg/kg Carprofen 5 mg/kg 100 µL 2% lidocaine on vocal folds |
| 3.  | Tateya T et al. [18] | 2005 | 1.9 mm 25 degrees endoscope; spring wire laryngoscope | 25 G needle and microscissors    | Semi vertical position on a custom operating platform | Vocal fold stripping            | Induction with isoflurane delivered at 0.8–1.5 L/minute; IP inj. of K: 90 mg/kg X: 9 mg/kg topical vocal fold anaesthesia with 5% lidocaine |
Table 2. Continued.

| Nr. | Author                  | Year | Visualization                                      | Lesion induction | Positioning of animal | Type of lesion        | Anesthesia                  |
|-----|-------------------------|------|---------------------------------------------------|------------------|-----------------------|------------------------|------------------------------|
| 4.  | Kanemaru S et al. [19] | 2005 | Direct visualisation through sub-cricoid incision | 32 G needle      | Supine in a horizontal position | Vocal fold stripping    | Induction with diethyl ether; IM inj. of K: 80 mg/kg X: 6 mg/kg |

KTP – potassium titanyl phosphate; K – Ketamine; X – Xilazine; IP – intraperitoneal; IM – intramuscular.

To the best of our knowledge, Imaizumi et al. [8] were the first to address the problem of consistency and comparability by describing and providing structure for the vocal fold injury model in rats. They emphasized the importance of the correlation between in vivo vocal fold injury in experimental medicine and the laryngeal classification proposed by Remacle and the European Laryngological [22]. The observations are essential for moving forward as they provide a classification for further studies, grouping vocal fold lesions in subepithelial, transmucosal, and transmuscular.

The literature regarding in-depth descriptions of the vocal fold injury process of the rat is scarce. Our review aimed to describe and synthesize various methodological steps for a controlled vocal fold injury in the rat model.

**Vocal fold injury types**

**Vocal fold stripping (transmucosal injury)**

To the best of our knowledge, vocal fold stripping in the rat model was first described by Tatsuya et al. [10] and subsequently gained the most traction among researchers (Tables 1 and 2). The definition of vocal fold stripping in the rat model would correspond to a transmucosal injury described later by Imaizumi et al. [8]. Earlier authors mention the exposure and visualization of the thyroarytenoid muscle, which would imply removing the deeper layers of the LP and not just the epithelium and superficial layer of the LP. This type of vocal fold injury would translate to clinical practice as a type II cordectomy (subligamental cordectomy). The vocal fold stripping model is a well-established first step to address the complex nature of EMC modifications, vocal fold scarring, and fibrosis using various solutions from tissue engineering to stem cells, growth factors, and antifibrotic agents [10–12, 15, 18, 19, 23, 24]. Instrumentation tends to be similar, using small gauge needles for the procedure. Needle size ranges from 22 to 26 G with auxiliary instruments helping with manipulation (micro forceps and scissors).

**Vocal fold scarring (subepithelial lesion) and superficial thermal injury**

Superficial lesions of rat vocal fold were described using cold instruments and lasers. Cold instruments used for vocal fold scarring are twenty-seven gauge needles and 90° micro scissors. The instruments were used to scar the membranous portion of the vocal folds [13–14].

This form of injury would correspond to the subepithelial injury model [8] or, in clinical terms, to a type I cordectomy. These methods could be used to study reepithelization and modifications of the superficial layer of the LP without disturbing the vocal ligament.

Superficial epithelial blanching and charring of the vocal fold were achieved with the help of KTP and blue light (BL) laser. Mallur PS et al. described using the KTP on the rat vocal fold epithelium to replicate potential modifications in the clinical setting. Consistent blanching was achieved with a 10 W 20 ms pulse. Higher power led to eschar formation. No modification of the underlying cellular architecture was noted other than cellular infiltration. The energy of the 532 nm KTP laser was delivered with a 0.4 mm fiber introduced through a number 5 Frasier tip suction handpiece [16].

Compared to the KTP, the BL laser is a novel technique for laryngeal surgery. The epithelial effects and injury depth were assessed by Lin RJ et al. [9]. They evaluated the effects of the BL laser on the vocal fold epithelium and the LP. The energy was delivered at a 2 W and 10 ms pulse to simulate the same clinical appearance and effects of the procedure as described before (blanching and charring). They noted no significant destruction of vocal fold cellular architecture but significant postsurgical submucosal inflammation (day 1). Long-term comparison (90 days) between KTP and BL laser showed significantly higher fibrosis in the KTP group.

**Surgical model for implantable materials in the rat vocal fold**

Engineered synthetic scaffold implantation shows promise in vocal fold reconstruction and regeneration [25]. As far as we are aware, Xu C et al. [17] article is the only one that gives surgical insight for scaffold implantation in the rat model. The authors describe the implantation of an acellular xenogenic extracellular matrix scaffold through a 0.8 mm incision on the superior surface of the posterior one-third of the vocal fold. For this purpose, a custom-made 18 G spinal needle was used under the guidance of an operating microscope.

**Visualization**

Endoscopes are the most common means of visualization. Endoscope size ranged from 1.9 mm to 2.7 mm. Endoscope angle was 25–30 degrees in most cases [11, 12, 14, 15, 18] and 0 degrees only in 2 cases [9, 16]. Proper visualization is essential, and the 25–30s degree, small diameter endoscopes provide the best angle and room for manipulation. Even though optimal visibility is provided, care should be taken regarding time exposure due to possible thermal injury of the surrounding tissues. It is recommended to use minimal light intensity that provides good visibility and avoid touching the mucosa. Also, limit the continuous exposure to less than 10 minutes [26, 27]. Xu C et al. [17] used a surgical microscope to aid scaffold implantation. This procedure differs from other types of vocal fold injuries by positioning the incision in the posterior and superior aspects of the vocal folds. In this case, the pharyngeal lumen patency was secured using an ear speculum. Other devices were mentioned to aid the visualization of the vocal folds and were described as custom-made.
spring wire laryngoscopes [16, 18]. These devices were fabricat-
ed from a 1 mm steel wire and were shaped to a coil at the mid-
dle, providing counter tension to the tongue base and posterior
pharyngeal wall. We have learned so far that the most suitable
instruments for visualizing the true vocal folds that lie deep to the
epiglottis would be smaller diameter endoscopes (1.9–2.4 mm)
with a 25–30 degree angle. Procedures regarding scaffold im-
plantations, vocal fold injections, injuries to the posterior 1/3
of the vocal folds, or arytenoid surgery would best benefit from
microscopic guidance. Historically, Kanemaru et al. were one of
the first to use rats in laryngeal studies. They describe a sub-cric-
coid incision to visualize the true vocal folds directly and provoke
injury using a 34 G needle. Nowadays, with optical devices, this
technique is no longer useful. In most cases, the positioning of
the rat for surgery is in posterior decubitus and a near-vertical
position. A series of custom-made operating platforms have been
described and illustrated [12, 13, 16, 18].

Anesthesia and medication

There is no standardized anesthesia protocol for laryngeal
surgery in rats. Ketamine and Xylazine were used with or with-
out induction anesthesia in all instances. The ketamine dose
ranged from 45 mg/kg to 100 mg/kg and the Xylazine dose
ranged from 4.3 mg/kg to 10 mg/kg. We would argue that the
higher dose ranges would be necessary in most cases because the
vocal fold injury process itself is just the first step of the method-
ology in many experiments. Applying diverse bioactive agents or
scaffold implantation would lengthen the time of the procedure.

Nevertheless, to the best of our knowledge, there is only one
study to support the optimal anesthesia dose regarding exper-
iments limited to the rat larynx [20]. According to Suzuki Y et al.,
a 67.5 mg/kg ketamine and a 6.75 mg/kg xylazine dose would
be too low, and epiglottal movement would still be present. An
optimal minimal intraperitoneal dose of 90.0 mg/kg ketamine
hydrochloride and 9.0 mg/kg xylazine hydrochloride would provide
sufficient anesthesia for the placement of a suspension laryngoscope.

Additional medication was prescribed in a few cases. Atro-
pine sulfate in a dose of 0.005 mg/kg can be administered to
reduce sputum secretion. A 2–5% topical lidocaine can be deliv-
ered to the vocal folds to provide additional local anesthesia [17, 18].
The use of 5 mg/kg Carprofen was discussed in one case
where the probability of laryngeal edema was higher given the extent
of the lesion [17].

Clinical interest and limitations of current injury models

All articles contributed to the methodology and technique of
performing vocal fold lesions in the rat model following a particu-
lar interest. The studies also give significant insight regarding cell
messaging, extracellular matrix composition, and understanding
of the healing cascade and the injured vocal fold scarring. Un-
derstanding the basic physiology and cell biology of healing and
scarring gives the opportunity to further study bioactive agents
that can serve as adjuvant treatment options in clinical practice
[29]. Bioactive agents could reduce the need for prolonged voice
rest or prevent vocal fold scarring after trauma or phonosurgical
intervention.

Although precise and replicable vocal fold injury models
(suprabasal epithelial injury and vocal fold stripping) have been
described up to date, there is a lack in the literature regarding a
subepithelial surgical model as described by Imaizumi et al. [8].
Only two methodologies describe this type of injury [13, 14], and
the injuries provoked were only focal lesions of the vocal fold. We
believe that a more elaborate subepithelial injury model would be
better replicate a type I cordectomy in humans, thus serving as a
more practical starting point in bridging the gap between exper-
imental vocal fold models and clinical practice. We believe that
the described injury models are appropriate to analyze cellular
modifications and observe scarring of the vocal fold, but a more
adapted surgical model should be used to address specific prob-
lems of the superficial lamina propria.

CONCLUSIONS

Rats are good candidates for in vivo larynx and vocal folds
research. Although the small size may impede consistent vocal
fold injury, a vast amount of research data is already available.
We believe that a more standardized approach should be consid-
ered regarding the type of vocal fold injury. Transmucosal
injuries (vocal fold stripping) are most widely used as a means
of injury. A great variety of instruments can be used to achieve
the desired length and depth of the lesion. Angled endoscopes
permit the best visualization of the true vocal folds, but a surgical
microscope can be used in cases where exposure of the posterior
glottis is sufficient. Anesthesia doses vary greatly, but higher doses
are required to limit the reflex movement of the epiglottis.

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Authorship
PLU contributed to conceptualization, methodology, data
collection, analysis, writing the original draft, and editing the
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and writing of the original draft and manuscript. AAM contrib-
uted to editing the manuscript. MC contributed to conceptualization, methodology, and editing of the manuscript.

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