Effect of Direct Nicotinic Salts Laryngeal Exposure on the Physiologic Status of the Larynx

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Abstract: Background: The physiological effects of nicotine on the larynx are crucial for understanding its sensory impact. However, the lack of scientifically validated animal models to assess these effects has been a significant limitation in tobacco research. Objective: This study aimed to establish a novel animal model using advanced techniques to evaluate the direct physiological effects of nicotine and its salts on the larynx. Main Ideas: We utilized techniques such as oral buccal tube embedding, laryngeal electrode embedding, and real-time EMG signal recording. Mice were directly exposed to nicotine smoke, and changes in laryngeal EMG signals were dynamically monitored. The observations revealed a consistent increase in the amplitude of these signals with the duration of nicotine exposure, indicating a direct physiological effect of nicotine on the larynx. Furthermore, the effects of different nicotine salts on laryngeal physiology were objectively quantified by changes in the amplitude of electromyographic signals. Conclusion: Our study successfully established an animal model for assessing the physiological effects of direct nicotine exposure on the larynx. This model provides a valuable quantitative method for evaluating throat irritation and the sensory impact of nicotine salts, aiding in the development of safer and more satisfying tobacco products.

Keywords: Nicotine; Nicotinic Salts; Throat Hit Sensation; Sensory Analysis; Laryngeal Electromyographic Signals

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1 Introduction

In recent years, nicotine salt technology has gained widespread use in emerging tobacco products, generating protonated nicotine salts through organic acid-nicotine complexes, with nicotine predominantly existing in a protonated form [1]. Nicotine salt nebulizers mostly use benzoate, tartrate, lactate, acetylpropionate, malate, and citrate of nicotine as additives in nicotine salt atomizers [2, 3]. Some studies have indicated that incorporating nicotine salts can mitigate the throat irritation associated with the release of high levels of free nicotine, resulting in a milder and smoother sensory experience and increased physiological satisfaction [4-6]. However, a comprehensive understanding of the physiological effects of novel tobacco products, particularly the in vivo physiological throat irritation caused by different types and concentrations of nicotine salts, remains lacking. Therefore, evaluating the throat sensation induced by various tobacco products at the in vivo level holds significant importance for product optimization.

Throat hit, the sensation of irritation experienced in the throat when inhaling smoke, is a notable aspect for both new

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and long-time smokers [7, 8]. While new smokers may find it uncomfortable or itchy, long-time smokers typically associate it with the act of smoking. Throat hit primarily results from nicotine, with higher nicotine content correlating with a more pronounced throat hit [9, 10]. However, elevated nicotine levels can lead to symptoms such as dizziness and nausea. Despite the subjective nature of throat hit descriptions in the literature, no scientific definition exists from a biological standpoint [7]. A cross-sectional study among smokers suggests a connection between throat hit intensity, e-cigarette dependence, and perceived efficacy in reducing tobacco cravings [7]. Additionally, the propylene glycol (PG) to glycerol (VG) ratio in e-cigarette liquids may influence nicotine delivery and the user's throat hit experience [10, 11]. Menthol smokers tend to report a greater throat hit compared to non-menthol smokers [10, 12]. While subjective reports are prevalent in human studies, objective quantitative measures of throat hit are lacking in animal studies, highlighting the urgent need for an animal model to scientifically evaluate throat hit sensation.

Regarding the methodology for assessing laryngeal physiological function, laryngeal electromyography (LEMG) holds clinical value for evaluating the functional status of laryngeal muscles and nerves [13, 14]. In comparison to indirect techniques like ultrasound recording and Whole Body Plethysmography (WBP) [15, 16], which reflect physiological laryngeal status, LEMG provides a direct measure. Ultrasonic recording captures a mix of functional changes in the larynx, making it challenging to isolate the laryngeal sensation proportion [17, 18]. WBP primarily focuses on pressure changes related to respiration, with laryngeal responses being secondary and difficult to quantify [16]. Hence, both methods lack suitability as indices for evaluating the sensation of throat hit. This study innovatively employs LEMG to directly characterize the physiological effects of throat hit sensation, marking the first application of this technique to evaluate such effects in tobacco products globally.

2 Methodology

1. Laboratory consumables, instruments, reagents

a) Instruments: single-channel smoking machine (HRH-SM120, Beijing Huironghe Science and Technology Co., Ltd.), mouse immobilizer (Melton Lab), R500 desktop anesthesia machine for small animals (Shenzhen RWD Science and Technology Co., Ltd.), home oxygen concentrator (8F-2A, Jiangsu Yuyue Medical Equipment Co., Ltd.), PowerLab electrophysiological recorder.

b) Reagents: 1,2 Propanediol (99.5%), Glycerol (>99%), Pure Nicotine (99.93%), Nicotinic Benzoate (99.5%), Nicotinic Tartrate (99.3%), Nicotinic Malate (99.8%), Nicotinic Lactate (99.9%), Nicotinic Acetyl Propionate (99.85%), Nicotinic Citrate (98.9%) (Shanghai Yunmei Biotechnology Co., Ltd.); Saline (0.9%) (Shanghai Medical Devices Wholesale Co., Ltd.).

C) Consumables: Hose Micro-Renathane® (inner tube, 30 mm long, 0.30 mm inner diameter, 0.64 mm outer diameter;.025" x 012" per ft.; Braintree Scientific, Inc.), self-coagulating tray powder and tray water (Heraeus Gussa Dental Co., Ltd.), 25 and 27 G disposable syringe needles (Ltd.), platinum iridium alloy wire (HONG KONG PLEXON Material #: 100-167), #0 disposable surgical sutures and #23 surgical blades (Shanghai Medical Equipment Wholesale Co., Ltd.).

2. E-cig smoking machine setup

Vaping machine: Vaping aerosol was produced using an ethylene carbonate (EC) atomizer coupled to a variable voltage EC battery. The specific EC battery had a 1300 milliampere-hour capacity with a nominal voltage range of 3.3–4.8 volts direct current. The e-cigarette puffing curve was trapezoidal, determining a vaping capacity of 55 mL, vaping time of 2 s, and vaping interval of 30 s.

3. Intraoral cheek fistula and laryngeal electrode surgery

Adult male C57BL/6 mice (8-16 weeks old) weighing 22-28 g were used for the experiment (Shanghai Jihui Laboratory Animal Breeding Co., Ltd.), and the mice were housed for at least one week in advance in an animal house with 12 hours of light and 12 hours of darkness alternately (lights on at 7 a.m. and off at 7 p.m.), with the temperature of the animal house at 22-26 ℃ and the humidity of the animal house at 40-60%, and all of them had free access to food and water. All mice had free access to food and water. All experimental procedures were approved by the Animal Ethics and Use Committee of Shanghai University of Science and Technology (Approval No. 20221020003) and were conducted in accordance with NIH guidelines.

Surgical procedure: mice were anesthetized with isoflurane; surgery was performed under continuous ventilation of an oxygen-isoflurane gas mixture. The eyes were coated with gentamycin eye cream, the skull surface of the head was fully exposed, and the needle of a 50-mL syringe was smoothed with sandpaper to serve as a guide syringe. The guiding syringe was inserted into the mouth and the needle was threaded through the incision on the cheek to the skull surface. The hose Micro-Renathane® was threaded

through the guiding syringe out of the mouth, and the two incisions were glued with tissue glue. Especially the incision on the cheek needed to be tightly glued between the inner tube, the subcutaneous muscle, and the incision to prevent the hose from retracting back under the skin inside the mouth and interfere with the smooth delivery of the fumes. A 5 cm length of platinum-iridium electrode with insulation was used with about 2 mm of insulation removed from both ends. The incision was made along the midline of the mouse pharynx to expose the mouse pharynx, and the pharyngeal muscles were fully exposed by bluntly separating the subcutaneous tissue. The pharyngeal muscles were fully exposed, and the pharyngeal-cranial subcutaneous channel was established with a guiding syringe. After passing the platinum-iridium electrode through the subcutaneous channel, the broken end of the insulating layer was removed and inserted into the muscle layer of the throat, and the electrode was sufficiently fixed to the subcutaneous tissue with a suture, and the pharyngeal incision was sutured. The orally embedded tube guided to the skull surface with the platinum-iridium electrode was fixed to the skull surface with dental cement (Figure 1). After the cement had set, the mice were routinely injected with penicillin intramuscularly to prevent infection. The mice were housed individually and tested for signaling after postoperative recovery.

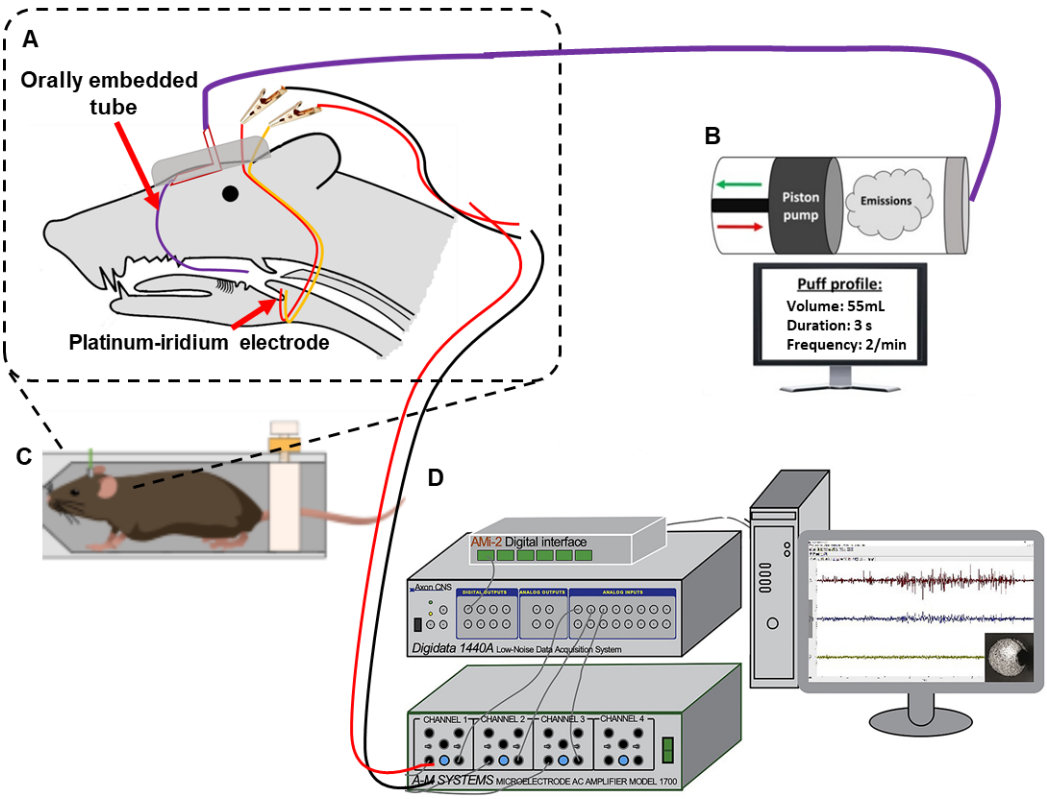

Figure 1 Schematic diagram of experimental procedure

(A and C) Experimental diagram diagram of intraoral cheek fistula and laryngeal electrode surgery. (B) Schematic diagram of smoking machine. (D) Schematic diagram of electrical signal acquisition.

4. Recording of electromyographic signals in the larynx under vaping exposure.

After the animals had been operated for 1 week, the end of the platinum-iridium electrode on the head was connected to the EMG signal recorder to test the EMG signal. In order to exclude the influence of the animals on the EMG signals during their activities, similarly, the animals were placed in a mouse immobilizer for three consecutive days before the smoke exposure test, from 9-11 a.m. every day for three days, and were given air exposure to acclimatize the animals to the whole course of the experiment and to reduce the influence of stress and exercise on the EMG signals. On the fourth day, the laryngeal EMG signals of mice were observed before and after nicotine exposure. The physiological status of the larynx was characterized by the magnitude of the changes in the EMG signal, which was used to reflect the strength of the effect of nicotine and nicotinic acid salts on the laryngeal sensa-

tion of the mice under smoke exposure.

5. Analysis of the laryngeal EMG signals.

The recorded data were exported as mat files for further analysis in Matlab software, first preprocessed by steps such as baseline calibration smoothing the data and marking the stimulus onset time. The baseline standard deviation of more than twice the baseline standard deviation was used as the calculation criterion for the identification of EMG signal events, and the plot function of Matlab software was applied to plot the amplitude of signal changes before and after stimulation in single mice. Numerical data were expressed as mean ±SEM. Off-line data analysis was performed using software GraphPad Prism 6 (GraphPad Software, USA). Statistical significance was determined by ANOVA followed by Bonferroni post-tests for multiple comparisons among more than two groups. n refers to the number of mice. Every group of mice in each experiment was from at least 3 animals. For all results, $p < 0.05$ was accepted as being statistically significant.

3 Results

3.1 Evaluation of the General State of the Animal

To evaluate the effect of surgery on the basal physiologic status of the animals, we set up a sham surgery group and weighed the basal body weights of all animals on the day before surgery, as well as their general mobility under absenteeism, and there were no differences in basal body weights as well as 5-minute absenteeism between the two groups on the day before surgery (n = 10) (Figure 2). Weight was measured daily after surgery, and no weight loss was noted in the surgical group (n = 10). On the day of smoke exposure, there was no difference in 5-minute absentee activity tested (n = 10). These results indicate that surgery had essentially no effect on the basal physiologic status of the animals (Figure 2).

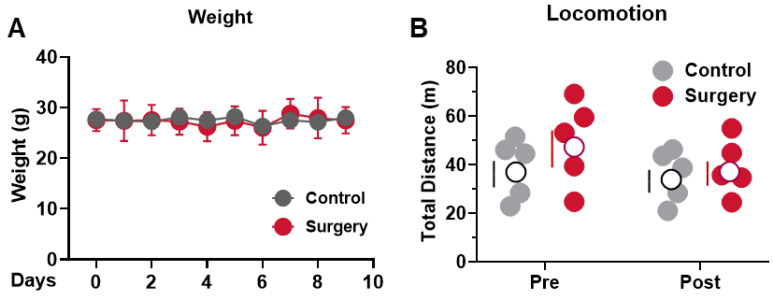

Figure 2 Effect of surgery on body weight and activity in mice

(A) There was no effect on the body weight of mice observed for 10 consecutive days after surgery (n = 10, two-way ANOVA, P > 0.05). (B) No difference in locomotion between pre- and postoperative mice (n = 10, two-way ANOVA, P > 0.05).

3.2 Effects of Nicotine or Nicotinic Salts on Electromyographic Signals in the Larynx

We tested mice exposed to different types of e-cigarette nicotine or nicotinic acid salts (nicotine, nicotinic acid benzoate, nicotinic acid lactate, nicotinic acid malate, nicotinic acid acetylpropionate, nicotinic acid tartrate, nicotinic acid citrate) during the exposure time period in which the laryngeal EMG signals were subjected to an increase in event and amplitude, and each group was set up with four gradient concentrations (0.06, 0.12, 0.25 and 0.37 mol/L) (Table 1) (Figure 3). Further, by comparing the magnitude of increase of different nicotine salts, we found that the magnitude of increase of all nicotine salts was smaller than the magnitude of increase of pure nicotine (Figure 3), which was consistent with the previous studies that the throat-hitting sensation of pure nicotine was greater than the effect of nicotine salts, and indicated that it was feasible to characterize the strength of throat- hitting sensation by the magnitude of electromyographic signals of the larynx. Further we found by fitting the concentration and magnitude curves of the effects of different nicotine and nicotinic salts that the different substances all showed a nonlinear fit and exhibited different fitting characteristics (Figure 4). These results indicate that all can cause changes in the physiological state of the mouse larynx, but this change has no correlation with the concentration, may be related to the higher concentration of nicotine used in our experiments, the lowest concentration is already a higher dose, due to the ceiling

effect of the response, even if the concentration is further increased the effect does not increase further. We realized the physiological effects of the larynx under direct exposure to oral smoke, and established an objective and quantifiable method of evaluating the strength of the laryngeal sensation in an animal model.

Table 1 Changes of LEMG signal in mice exposed to e-cigarette group

	0.06 (mol/L)	**0.12 (mol/L)**	**0.25 (mol/L)**	**0.37 (mol/L)**
Nicotine	3.69±0.75	4.38±0.61	5.41±0.42	5.49±0.48
Nicotine benzoate	0.66±1.01	1.77±0.81	1.09±0.14	1.86±0.07
Nicotine lactate	0.94±0.23	1.20±0.08	1.46±0.31	0.80±0.17
Nicotine malate	0.74±0.31	1.50±0.26	1.49±0.26	1.46±0.33
Nicotine laevulinic	1.07±0.08	1.82±0.17	1.41±0.32	1.39±0.24
Nicotine ditartrate	0.13±0.06	0.69±0.17	1.15±0.28	0.87±0.16
Nicotine citrate	0.53±0.09	1.48±0.26	1.29±0.16	2.05±0.19

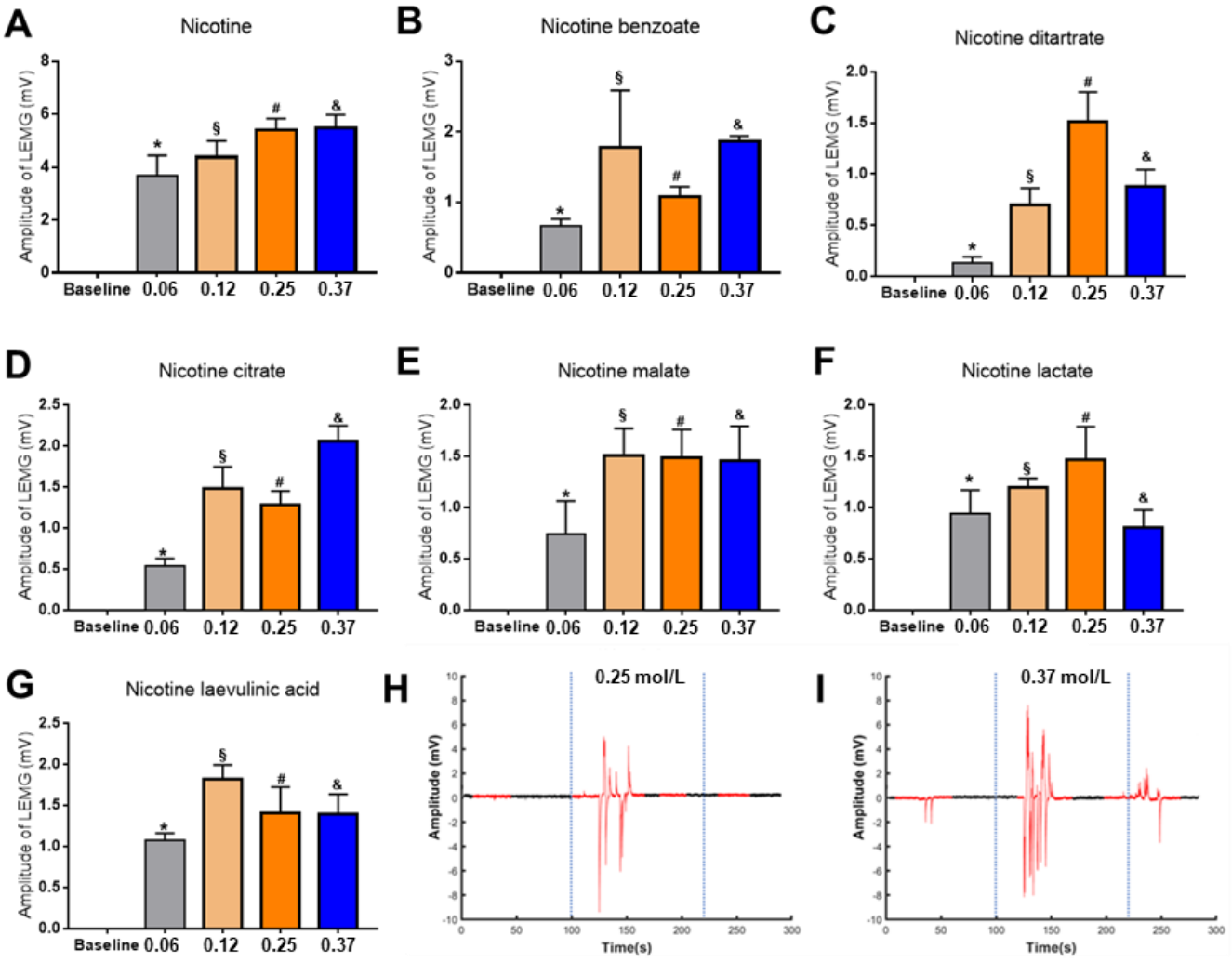

Figure 3 Effect of nicotine salts on LEMG amplitude

(A) Increase in LEMG amplitude by different concentrations of nicotine (n = 5, one-way ANOVA, $P < 0.05$). (B) Increase in LEMG amplitude by different concentrations of nicotine benzoate (n = 5, one-way ANOVA, $P < 0.05$). (C) Increase in LEMG amplitude by different concentrations of nicotine ditartrate (n = 5, one-way ANOVA, $P < 0.05$). (D) Increase in LEMG amplitude by different concentrations of nicotine citrate (n = 5, one-way ANOVA, $P < 0.05$). (E) Increase in LEMG amplitude by different concentrations of nicotine malate (n = 5, one-way ANOVA, $P < 0.05$). (F) Increase in LEMG amplitude by different concentrations of nicotine lactate (n = 5, one-way ANOVA, $P < 0.05$). (G) Increase in LEMG amplitude by different concentrations of nicotine laevulinic acid (n = 5, one-way ANOVA, $P < 0.05$). (H) Representative LEMG at a concentration of nicotine 0.25 mol/L. (I) Representative LEMG at a concentration of nicotine 0.37 mol/L.

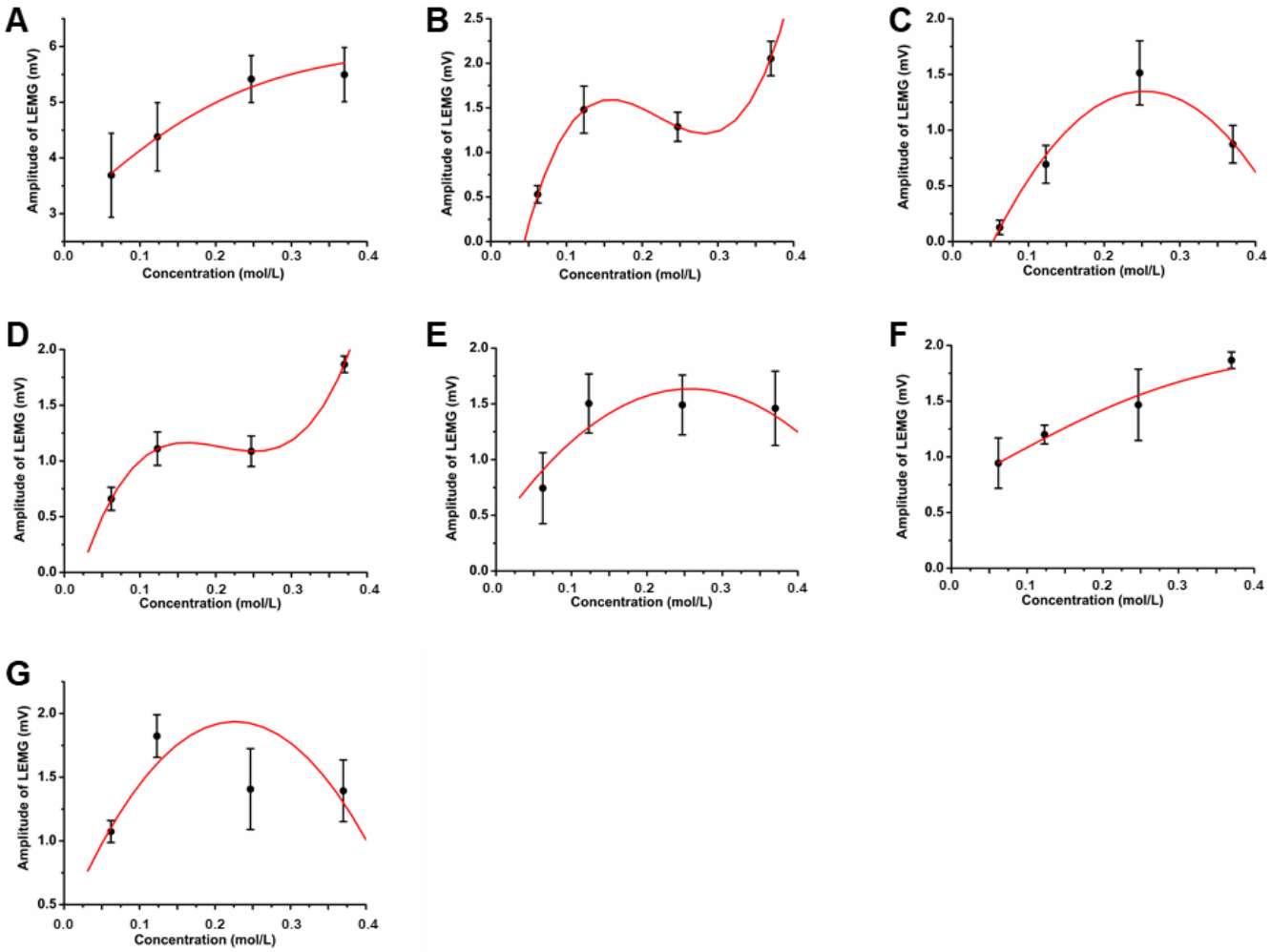

Figure 4 Dose-response curve of LEMG signal changes and nicotine salts

(A) Exponential fit concentration effect curves for nicotine. (B) Exponential fit concentration effect curves for nicotine citrate. (C) Exponential fit concentration effect curves for nicotine ditartrate. (D) Exponential fit concentration effect curves for nicotine benzoate. (E) Exponential fit concentration effect curves for nicotine malate. (F) Exponential fit concentration effect curves for nicotine lactate. (G) Exponential fit concentration effect curves for nicotine laevulinic acid.

4 Discussion

In this experimental study, we devised a method to assess the intensity of throat sensation elicited by nicotine by closely monitoring changes in the electromyographic signals (EMG) of the larynx in mice. This involved the direct exposure of the oral cavity to smoke using an orally embedded tube, employing the mouse as a conventional model animal. The alteration in the amplitude of laryngeal EMG signals served as an indicator of the physiological state of the mouse larynx, with the peak increase in EMG signals during direct oral smoke exposure representing a quantifiable measure of the physiological throat sensation induced by nicotine. Our study demonstrates that both nicotine and nicotinic salts induce changes in the physiological state of the mouse larynx, as evidenced by alterations in laryngeal EMG signals.

Our investigation into the effects of nicotine or nicotinic salts on laryngeal EMG signals revealed intriguing insights into the concentration-response relationships of these compounds. By subjecting mice to various concentrations of e-cigarette nicotine or nicotinic acid salts, we observed distinct patterns in the concentration and magnitude curves of the EMG signals. Firstly, our findings indicated that all tested substances exhibited nonlinear concentration-response relationships. This suggests that the physiological effects on the larynx are not strictly proportional to the concentration of nicotine or nicotinic salts. Such nonlinear relationships could stem from complex interac-

tions between these compounds and the receptors or signaling pathways in the laryngeal tissue. Interestingly, we observed variations in the magnitude of these effects between different types of nicotine salts. Specifically, pure nicotine elicited a more pronounced increase in EMG signal amplitude compared to other nicotinic salts. This finding is consistent with previous reports emphasizing the importance of nicotine content in determining the strength of throat-hitting sensation [10, 19-21]. The reduced magnitude of EMG signal increase with nicotine salts may reflect differences in the bioavailability or pharmacokinetics of these compounds, highlighting the importance of considering formulation-specific effects in tobacco product development.

Nicotine salt technology, increasingly prevalent in emerging tobacco products, offers a promising avenue for reducing irritation to mucous membranes in the oral cavity and throat [22]. Compared to free nicotine, nicotine salts provide a smoother sensory experience, enhancing physiological satisfaction for consumers [4, 23]. Our results suggest that while nicotine salts generally induce heightened laryngeal EMG signals, their effect may be moderated compared to pure nicotine. This highlights the potential of nicotine salts to mitigate the harshness associated with traditional nicotine delivery methods [5, 24].

Despite these advancements, the specific physiological effects of different types of nicotine salts on throat irritation remain unclear [25, 26]. Moreover, there is a notable absence of an objective and quantitative animal experimental model for evaluating throat irritation. In our study, we addressed this gap by leveraging our laboratory's technological strengths to establish a more operationally efficient method for recording laryngeal EMG signals. To align with the practical requirements of directly exposing the larynx to smoke, we integrated the well-established technology of oral tube embedding in neuroscience into our smoke delivery approach. This approach is more in line with the real-world application of assessing the throat hit sensation of tobacco products.

Moving forward, it is imperative to validate whether our method can distinguish effects at lower concentrations and whether the physiological signals comprehensively and objectively reflect the throat sensation akin to human smoking behavior [27, 28]. This ongoing research will contribute to a deeper understanding of the nuanced physiological responses to various nicotine formulations, ultimately aiding in the development of safer and more satisfying tobacco alternatives [29, 30].

5 Conclusion

Our study lays the groundwork for evaluating the effects of nicotine and nicotinic salts on throat sensation using a novel animal model approach. By elucidating the physiological mechanisms underlying throat irritation, we pave the way for informed decisions in the development of tobacco products that prioritize consumer satisfaction and safety. By elucidating the complex relationships between compound concentration, magnitude of physiological response, and formulation-specific characteristics, we advance our understanding of how these compounds influence throat sensation. This knowledge is essential for informing the design and optimization of tobacco products that offer enhanced sensory experiences while minimizing potential adverse effects on consumer health.

Author Contributions

Xiaonan Li, Lihuan Qiu, Lehua Lu. designed, carried out and analyzed stereotaxic surgery experiments. Xiaonan Li, Lihuan Qiu. carried out EMG signal recording. Xiaonan Li, Huaquan Sheng, Xu Jiang, Peicai Cui., Guangchao Liu. analyzed the date of EMG signal. Xiaonan Li, Ming Chen, Yihan Gao. supervised and assisted in experimental planning and wrote the manuscript. All authors read and approved the final manuscript.

Availability of Data and Materials

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

Competing Interests

The authors report no biomedical financial interests or potential conflicts of interest.

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