Establishment of a Rabbit Model of Obstructive Sleep Apnea by Paralyzing the Genioglossus

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**IMPORTANCE** This study presents an innovative method for developing a neuromuscular model of obstructive sleep apnea (OSA).

**OBJECTIVE** To establish a new OSA animal model simulating real upper airway conditions during sleep.

**DESIGN AND SETTING** In vivo animal study at an academic tertiary referral center.

**SUBJECTS** A total of 27 New Zealand white male rabbits were used.

**INTERVENTION** Sleep was induced by intramuscular injection of 0.3 mL/kg of tiletamine hydrochloride plus zolazepam hydrochloride and 0.2 mL/kg of xylazine. Upper airway obstruction was induced by injecting botulinum toxin type A (2.5 U in 8 rabbits, 5.0 U in 10 rabbits, and 7.5 U in 1 rabbit) into the genioglossus. Eight rabbits were injected with normal saline as a control.

**MAIN OUTCOMES AND MEASURES** Drug-induced sleep was evaluated using a portable polysomnography device for electroencephalography, electrooculography, chin electromyography, nasal airflow, breathing efforts, and pulse oxymetry. Respiratory events (apneas or hypopneas) during sleep were evaluated using a sleep-screening tool.

**RESULTS** All the rabbits showed no apneas or hypopneas before injection of botulinum toxin type A. In the control rabbits injected with normal saline, apneas or hypopneas were not found. The respiratory events were observed in 5 of 8 rabbits injected with 2.5 U of botulinum toxin type A, whereas they were observed in 7 of 10 rabbits injected with 5.0 U of botulinum toxin type A. The median (interquartile range) apnea hypopnea index was 9.6 (5.3-14.8) per hour and 45.6 (21.5-70.5) per hour in the rabbits injected with 2.5 U and 5.0 U of botulinum toxin type A, respectively ($P = .03$).

**CONCLUSIONS AND RELEVANCE** An animal model of OSA could be developed by paralyzing the genioglossus in rabbits. This model may contribute to identifying the pathogenesis of upper airway obstruction in OSA and to developing new diagnostic or treatment devices targeting specific obstruction sites.

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Obstructive sleep apnea (OSA) is prevalent and an important risk factor for cardiovascular, metabolic, cognitive, and neurological diseases. However, its pathophysiologic mechanisms underlying the development of these complications are not completely understood. The pathogenesis of OSA also requires further investigation. In addition, to develop new treatment modalities, animal disease models are useful because there are many limitations in human studies.

Several kinds of animal models for OSA have been established during the last 2 decades. Animal models have some advantages in that experiments can be performed in controlled conditions, and the influences of confounders such as disease duration, chronologies of events, and genetic and environmental factors on the outcomes can be minimized. However, current OSA animal models have some limitations. The OSA animal models can be categorized into spontaneous or induced models. The historic natural spontaneous model of OSA is the English bulldog, which has an abnormal upper airway anatomy with a narrow oropharynx and enlarged soft palate. This model reproduces human features of OSA during sleep. Of the induced models, nonsurgical, noninvasive intermittent hypoxia models have been widely used to assess various consequences arising from oxygen desaturation. However, these hypoxic models do not replicate the obstruction of the upper airway per se in OSA. A couple of surgically invasive models have been designed to induce upper airway obstruction. However, most of the models used tracheotomy, and therefore they were more similar to intermittent hypoxic models rather than upper airway obstruction models.

In the present study, we developed a new noninvasive, physiologic animal model to mimic real pharyngeal conditions during sleep. We hypothesized that OSA could be induced if the genioglossus, which is a main dilator muscle of the upper airway, is paralyzed. Botulinum toxin was introduced for this purpose. It produces its paralytic effects by preventing vesicles containing acetylcholine from fusing with the cell membrane inside motor nerve terminals, and thus inhibits acetylcholine release at neuromuscular junctions. The present study demonstrates the establishment of a noninvasive neuromuscular model of upper airway obstruction.

Methods

Animals and Polysomnography During Drug-Induced Sleep
Twenty-seven 3-month-old New Zealand White male rabbits weighing 2.1 to 2.6 kg were used in this study. All the rabbits were acclimated for the first week and used for further experiments. Sleep was induced by intramuscular injection of 0.3 mL/kg of Zoletil (tiletamine hydrochloride plus zolazepam hydrochloride; Virbac) and 0.2 mL/kg of Rompun (xylazine; Bayer AG) at the same time.

Sleep staging during drug-induced sleep was performed using a portable polysomnography device (Embletta X100; Embla Systems) for 3 rabbits in an attended setting. The examinations included in the study were electroencephalography, electrocorticography, chin electromyography, nasal airflow or air pressure, breathing efforts, and pulse oxymetry (Figure 1). The sensors of Embletta X100 were applied onto the areas of rabbits equivalent to those of humans. For electroencephalography, only the central channel was used, and the electrodes were applied at C3/M2. Two electrodes for electrocorticography were applied at the lateral sides of both eyes and submental electromyography electrodes were applied at the submental muscle. Respiratory inductance plethysmography belts were applied on the thorax and abdomen to evaluate respiratory efforts and the pulse oximetry on the left leg. A real-time monitoring was maintained during the test. Sleep analyses were performed using a RemLogic v2.0 software (Embla Systems). All the experimental procedures were approved by the International Animal Care and Use Committee in Seoul National University Bundang Hospital.

Induction of Sleep-Related Breathing Disorder
To paralyze the genioglossus, a dilution of botulinum toxin type A (Allergan Inc) was prepared. A 100-U vial of botulinum toxin type A was dissolved in 4.0 mL of normal saline, 0.1 mL of which contains 2.5 U of botulinum toxin. After sleep was induced, 0.1 to 0.3 mL of diluted botulinum toxin type A was injected transorally into the genioglossus using an insulin syringe with a 30-gauge needle (Figure 2). Eight rabbits were injected with normal saline as controls, 8 rabbits with 2.5 U of botulinum toxin, 10 rabbits with 5.0 U of botulinum toxin, and 1 rabbit with 7.5 U of botulinum toxin.

Identification of Sleep-Related Breathing Disorder
The respiratory events associated with sleep-related breathing disorder were evaluated using ApneaLink (ResMed) in an attended setting. After induction of sleep, ApneaLink was applied and nasal airflow and oxygen desaturation were monitored (Figure 3). The recording continued for approximately 60 minutes in the supine position. On day 0, botulinum toxin was injected after baseline respiratory parameters were acquired using ApneaLink. At 1, 2, 3, 4, 6, and 8 weeks after in-
Injection, apnea hypopnea index (AHI) was measured. Body weight was measured every time before tests.

Apneas were defined as a 90% or greater drop in the peak nasal pressure compared with baseline. Hypopneas were defined as a 30% or greater drop in the peak nasal pressure of baseline associated with an oxygen desaturation of 4% or greater from baseline or a 50% or greater drop from baseline. Apneas or hypopneas were scored when they were 2-breath-or-longer events. First, AHI was automatically calculated using ApneaLink v7.0 software (ResMed) and then manually adjusted.

Evaluation of Genioglossal Activity
The muscle activity of the genioglossus was evaluated using needle electromyography (Oxford Instrument Medical Systems). Compound muscle action potentials of the hypoglossal nerve and motor unit action potentials were measured in a rabbit before and after injection of botulinum toxin.

Statistical Analysis
The average value of AHI is presented as median with interquartile ranges (IQRs). A Mann-Whitney test was used to compare the average AHI of rabbits injected with 2.5 U of botulinum toxin with that of those injected with 5.0 U of botulinum toxin. SPSS version 18.0 was used, and P < .05 was considered statistically significant.

Results
Demonstration of Sleep-Associated Parameters in Rabbits
During the drug-induced sleep, the electroencephalography was characterized by activities similar to low-amplitude mixed-frequency waves. Sleep spindlelike waves were frequently observed over the background of low-amplitude mixed-frequency activity (Figure 4). However, K-complexes or slow delta waves did not appear. Electrooculography showed no rapid eye movements, and there was no atonia on chin electromyography.

Induction of Apneas and Hypopneas
All the rabbits showed no apneas or hypopneas before injection of normal saline or botulinum toxin. When the botulinum toxin was injected, apneas or hypopneas were observed (Figure 5). However, apneas or hypopneas were not observed in the control rabbits.

Obstructive sleep apnea was induced in 5 (R1, R3, R4, R6, and R7) of 8 rabbits injected with 2.5 U of botulinum toxin type A. The AHI scores ranged from 2 to 114 (Table). Of 5 rabbits in which OSA was induced, 2 rabbits died of apneic events during drug-induced sleep. The other rabbits, which died before the study was finished, were found dead of unknown causes during regular checkups.

In the 7 (R10, R12, R14, R15, R16, R17, and R18) of 10 rabbits injected with 5 U of botulinum toxin type A, OSA was induced. The AHI scores ranged from 4 to 242 (Table). Of the 7 in which OSA was induced, 4 rabbits (R10, R14, R15, and R17) died of apneic events during drug-induced sleep. The other 3 rabbits (R9, R11, and R13) in this group that showed no OSA during the first 4 weeks were killed after 4 weeks because of a very low chance of delayed development of sleep apneas. The median (IQR) AHI was 9.6 (5.3-14.8) per hour and 45.6 (21.5-70.5) per hour in the rabbits injected with 2.5 U and 5.0 U of botulinum toxin type A, respectively (P = .03). The rabbits injected with 7.5 U of botulinum toxin type A died 1 day after injection owing to severe apneic events during drug-induced sleep.
Identification of Paralysis of the Genioglossus

In the hypoglossal nerve conduction, we identified remarkable differences of the compound muscle action potential responses of the genioglossus before and after botulinum toxin injection. The amplitude and area of the compound muscle action potential were higher before botulinum toxin injection (40.5 mV and 24.7 mV × milliseconds [mVms], respectively) (Figure 6A) than those after injection (3.7 mV and 2.0 mVms, respectively) (Figure 6B). The electromyographic activity recorded in the genioglossus showed normal configuration of motor unit action potential and firing frequencies (5-10 Hz) before injection (Figure 6C). However, the insertional activity into the muscle remarkably decreased, and motor unit action potentials were sparsely detected after injection (Figure 6D).
In the present study, we described a new rabbit model of OSA. Our hypothesis for developing a new animal model of OSA was that paralysis of the tongue-protruding muscle will cause the retroglossal airway space to be collapsed and apneas or hypopneas to be induced. The genioglossus is already known to be a major tongue protrusor or upper airway dilator. In contrast to humans, rabbits are obligate nasal breathers due to their epiglottis positioned rostrally to the soft palate.12 Several studies in animals and humans have shown that stimulation of the hypoglossal nerve or direct stimulation of the genioglossus could widen the upper airway space in the retroglossal area and improve sleep-related breathing disorder.13,14 Thus, in spite of anatomical differences of the upper airway, we assumed that collapse of the airway around the tongue base by genioglossus paralysis would cause OSA in rabbits. In contrast to the previous studies focusing on treatment of upper airway obstruction by electrically stimulating the hypoglossal nerve or tongue muscles, we tried to paralyze the tongue muscle and obstruct the upper airway for development of an OSA animal model.

Our study exhibited that the genioglossus could be easily accessible and transoral injection of botulinum toxin was effective in paralyzing the muscle and collapsing the upper airway. Most of the apneas or hypopneas were associated with occurrence of oxygen desaturation. Although all of the 3 different amounts of botulinum toxin were able to induce apneas or hypopneas, the increased severity of OSA was associated with an increased amount of toxin.

Our study also showed that rabbits were appropriate as an OSA model. Because the rabbits were medium-sized animals, they could be easily handled,13,15 and sleep-monitoring devices used for humans could be applied. The size of their nostrils and legs was suitable for application of the nasal pressure cannula and pulse oximetry sensor, respectively.

The amplitude and area of the compound muscle action potential were higher before botulinum toxin injection (A) than after injection (B). Before injection, the electromyographic activity of the genioglossus was normal in configuration of motor unit action potential (C). However, the insertional activity remarkably decreased, and motor unit action potentials were sparsely detected after injection (D).

### Table. Apnea Hypopnea Index in Rabbits Before and After Injection of Botulinum Toxin A

<table>
<thead>
<tr>
<th>Rabbits</th>
<th>Apnea Hypopnea Index (per Hour) at Weeks After Injection of Botulinum Toxin Type A</th>
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<tbody>
<tr>
<td></td>
<td>W0</td>
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<tr>
<td>2.5-U injection</td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>0</td>
</tr>
<tr>
<td>R2</td>
<td>0</td>
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<tr>
<td>R3</td>
<td>0</td>
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<td>R4</td>
<td>0</td>
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<td>R5</td>
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<td>R6</td>
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<td>R7</td>
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<td>R8</td>
<td>0</td>
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<tr>
<td>5.0-U injection</td>
<td></td>
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<td>R9</td>
<td>0</td>
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<td>R10</td>
<td>0</td>
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<tr>
<td>R11</td>
<td>0</td>
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<td>R12</td>
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<td>R17</td>
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<td>R18</td>
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Abbreviations: NA, not applicable because of death; R, rabbit; W, week.
To our knowledge, this is the first OSA model based on a neuromuscular mechanism. The causes of OSA can be simplified into the imbalance between mechanical loads imposed by bony structures and soft tissues and neuromuscular responses to airway obstruction. Between mechanical loads and neuromuscular responses, most of previous animal models of OSA focused on simulation of mechanical loads imposed on upper airway using tracheostomy tube or hypoxic air delivery through a hypoxic chamber. However, there has been some criticisms in the respect that it cannot exactly mimic real upper airway conditions during sleep. In our model, we could develop functional narrowing of upper airway, causing desaturation during drug-induced supine sleep by injection of botulinum toxin to the genioglossus.

Our study also has some limitations. First, sleep in our study was not natural but induced by drugs. Zoletil and Rompun were used to induce sleep in our experiment. Because zolazepam, a component for Zoletil, is one of benzodiazepines that has activities for sedation, hypnosis, antianxiety, and muscle relaxation, it can induce and aggravate OSA. However, there were few apneas or hypopneas in the control group even though they were also anesthetized. Thus, the drugs were less likely to induce sleep respiratory events. Besides, during drug-induced sleep, sleep spindles were found over a background of low-amplitude, mixed-frequency activity, but it is difficult to identify whether it is associated with the drugs or non-rapid eye movement sleep.

Second, OSA could be observed only during drug-induced sleep in a supine position, even if rabbits sleep naturally mostly in the prone position. However, given that control rabbits showed few sleep apneas even in a supine position, supine position is less likely to be the only cause of sleep apneas observed in the rabbits injected with botulinum toxin. In addition, we could not observe respiratory events in prone or decubitus position during natural and drug-induced sleep.

Third, sleep staging was performed using surface electrodes instead of electrodes implanted on the brain cortex, and respiratory events were measured using devices developed for humans. There have been a few studies showing the electroencephalographic findings of natural sleep of rabbits or the effects of benzodiazepines on electroencephalographic findings in rabbits. These in reports, electrodes were invasively implanted on the brain cortex to monitor natural sleep. However, because we tried to prove the hypothesis that the neuromuscular mechanism may be involved in the pathogenesis of sleep apnea rather than to investigate full-sleep status during drug-induced sleep, we used noninvasive surface electrodes to identify the sleep status. Our study showed that human devices could be applied to evaluate sleep staging and respiratory events in rabbits. Because rabbits weigh 2 to 3 kg, we assumed that they may be almost equivalent to human newborns in terms of body weight.

Fourth, there were several rabbits that did not respond to botulinum toxin, and there were some fluctuations in the severity of respiratory events in rabbits. Nonresponsiveness might be attributed to technical errors such as denaturation of the toxin or incorrect injection. The day-to-day variation is difficult to explain and might be caused by a short duration of recording and potential differences in depth of sleep despite a regular dose of anesthetics.

Lastly, elevation of AHI due to induced paralysis of genioglossus in rabbits may not correspond to that of OSA in humans because of the difference in normal upper airway anatomy between rabbits and humans as previously discussed. Our animal models may not reproduce all the phenomena occurring in human OSA. However, in the respect that retraction of the tongue base causes OSA, our OSA animal model may play a role in part as a animal model for human OSA. Further studies including cine computed tomography or magnetic resonance imaging, which can show the upper airway change in real time during OSA events, will help this animal model. The present animal model is also likely to be used for the development of devices to treat human OSA and for studies to identify its pathogenesis and complications.

In conclusion, in the present study a new rabbit model of OSA based on a neuromuscular mechanism was developed by paralyzing the major upper airway dilator, the genioglossus, with botulinum toxin. In the future studies, the long-term effect of genioglossus paralysis should be investigated because botulinum toxin may act temporarily. Also, the upper airway space should be monitored using radiographic methods to identify the obstruction sites. This novel OSA model may contribute to identifying the pathogenesis of upper airway obstruction in OSA and to developing new diagnostic or treatment devices targeting specific obstruction sites.

**ARTICLE INFORMATION**

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