Introducing a noninvasive, non-surgical, and reversible animal model for fecal incontinence using abobotulinumtoxinA in dogs

Mohammad Yasan Bangash1, Mir Sepehr Pedram1*, Valiollah Mehrabi2, Mohammad Mehdí Dehghan1, Korosh Mansoori3, Sarang Soroori1, Sanaz Bandifazl4, Forough Dadgar5, Mohammad Reza Mokhber Dezfooli6

1 Department of Surgery and Radiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; 2 Department of Pediatric Surgery, Children’s Medical Center, School of Medicine, Tehran University of Medical Science, Tehran, Iran; 3 Neuromusculoskeletal Research Center, Department of Physical Medicine and Rehabilitation, School of Medicine, Iran University of Medical Sciences, Tehran, Iran; 4 Department of Clinical Sciences, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran; 5 Department of Medicine, International University of the Health Sciences, Winnipeg, Canada; 6 Department of Internal Medicine, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

Abstract

The aim of this study was to introduce a new animal model of fecal incontinence (FI) by injecting abobotulinumtoxinA in the external anal sphincter (EAS) muscle of dogs which replaces models based on anal sphincter destructions that are invasive, mostly require surgical procedures, expensive, permanent, and painful to the animals. 4 healthy mongrel dogs were used in this study. First, they were received NaCl 0.09% (as control) injections in EAS muscle and effects were assessed by means of Electromyography (EMG) and clinically evaluated by sphincter pinch test and presence of leakage of feces for 2 weeks. Then, they received abobotulinumtoxinA in EAS muscle and reevaluated for 6 weeks to see short-term and medium-term effects of abobotulinumtoxinA injection. Saline had no significant changes in results obtained from EMG, however, there were significant decreases in amplitudes of action potentials after receiving abobotulinumtoxinA in comparison with no injection or saline injection in EAS muscle. Pinch tests were normal after saline injection assessment period; however, then started to be negative, ranging from two days after abobotulinumtoxinA injection to seven days after receiving abobotulinumtoxinA. Animals also had significant presentations of fecal incontinence (leakage of feces and cage contamination with feces) from the 1st week after receiving abobotulinumtoxinA until the 6th week after receiving abobotulinumtoxinA. AbobotulinumtoxinA caused paralysis in the EAS and produced FI conditions in dogs. This animal model was an appropriate substitute to the various invasive, expensive and also complicated procedures with an easy, feasible, noninvasive and non-painful single-stage abobotulinumtoxinA injection.

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Introduction

Fecal Incontinence (FI) is defined by the inability to control defecation or leakage of gas from the rectum for at least a month, in patients older than 4 years old which they were normal in the past.2,3 Complete pathophysiology of fecal incontinence is not fully understood. A study reported that 18.40% of adults can have some degrees of anal sphincter problems and the prevalence of FI could be something between 7.00 to 15.00% in communities.2,3 Ability to control defecation is something between 7.00 to 15.00% in communities.3

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*Correspondence:
Mir Sepher Pedram, DVM, DVS
Department of Surgery and Radiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
E-mail: mpedram@ut.ac.ir

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secrecy attached to this disorder can lead to a variety of mental, social, and physical health issues. Financial pressures over the patient and societies are other important issues that play a crucial role in patient management. For example, a study reported that 400 million US dollars are spent on adult diapers and about 1.50 to 7.00 billion US dollars are spent on care and treatment of the affected patient. Considering all of the mentioned aspects of FI, clinicians and researchers all around the world are seeking for the best treatment and cure for fecal incontinence. They led to a lot of options such as medical treatment, physiotherapy, and surgeries, however, none of these options introduced a perfect, pleasant and choice treatment for fecal incontinence. Still, there are a lot of researches conducting on the subject of FI and specially focusing on animal trials.

Animal models for fecal incontinence are mainly based on anal sphincter destructions by blade or electrosurgery, caudal spinal or peripheral nerves (for example pudendal, pelvic, or cranial rectal nerve) damages, anal fistulas, anal sphincter complete resections, diabetic neuropathies, and a combination of nervous and muscular damages. We believe these methods are invasive, some of them require surgical procedures which can produce pain in the objects and could be expensive for the researcher, and can lead to irreversible damages which can leave the animal incontinent after the end of the study, and raises the likelihood of euthanasia in objects. Therefore, we decided to plan and evaluate a new animal model by means of injecting abobotulinumtoxinA into the external anal sphincter (EAS) muscle of dogs which is believed to be noninvasive, less expensive, easy to be applied and most importantly with no deteriorating or fetal damages to the animals.

Materials and Methods

Animals. Four healthy mongrel male dogs (Adult weight 25.00 - 27.00 kg; 16 weeks old) with no clinical signs of musculoskeletal disorders were used in this study. All of the objects were neurologically examined by two small animal surgeons and no sign of any neurological problem or lameness was evident. Complete hematological and biochemistry studies were performed for each dog and no abnormal finding was reported on blood samples. The dogs were housed individually in cages at the research kennel of Companion Animal Hospital of the University of Tehran with free access to water and were fed twice a day with commercial dry-foods. Antiparasitic therapy by bathing with lindane solution (Gilaranco, Rasht, Iran) and subcutaneous injection of ivermectin (1.00 mg kg⁻¹; Alfasan, Woerden, The Netherlands) were performed. Also they were treated with a single dose of oral praziquantel (50.00 mg per 10.00 kg of body weight; Bayer AG, Leverkusen, Germany) and also underwent two injections with four weeks intervals polyvalent (Bioveta, Prague, Czech Republic) and Biocan R rabies vaccines (Bioveta). All experimental procedures followed the Guidelines on Ethical Standards for experiment in animals and carried out according to a protocol approved U.S. National Institutes of Health “Guide for the Care and Use of Laboratory Animals” and approved by research committee of Faculty of Veterinary Medicine of University of Tehran (Approval No. 28885/6/17).

For the first 8 weeks, objects were housed under the care of two vets to get familiar and adapted to the new place and conditions. During that time animals were walked outdoor twice a day on an exact time (9:00 a.m. and 6:00 p.m.) in order to teach them defecate outside of the cages. Before they learn where to defecate, they were observed during the day and whenever they tried to take the position of defecation they were guided outside of the cage toward the yard to teach them not to defecate inside the cages. Finally, by the week 4, all of the animals were trained to defecate outside of the cage. Dogs were defecated once a day, mostly during the morning walking sessions. To test the habit the dogs kept three times indoor for the duration of 30 hr and they successfully did not defecate inside the cages.

Study design. In this study we tried to stimulate caudal rectal nerve branch of pudendal nerve—which innervates EAS- by means of bipolar needle electromyography or (EMG) placed over the anatomical position of pudendal nerve. Contraction force was recorded by means of a 0.50-cm electrode over the belly of muscle and reference electrode over the inactive region of the perineum (Fig. 1).

![Fig. 1. Perineal view in the EMG test in dogs. A: The point of insertion of bipolar needle-EMG over the anatomical position of the pudendal nerve (hatched line). B: The point of insertion of "Ground electrode". C: The position of placement of "Reference electrode" over the inactive region of the perineum, and D: The "Active electrode" positioned over the belly of external anal sphincter muscle.](image)
Induction of fecal incontinence using abobotulinumtoxinA. Our hypothesis for inducing fecal incontinence was to paralyze external anal sphincter muscle (EAS) by injecting the toxin. The evaluations were based on needle electromyography (EMG) of EAS and clinical signs of fecal incontinence in each subject after injection of abobotulinumtoxinA in comparison with NaCl 0.09% (IPPC Co., Tehran, Iran) injection (as control group). As it was mentioned dogs were housed and trained for 8 weeks not to defecate inside the cages. At the beginning of week 9, dogs were ready to receive saline and then abobotulinumtoxinA injections.

**Stage one: Saline administration and evaluations.** Day 1) All of the dogs were clinically and neurologically examined by two small animal surgeons initially to assure their general health. Positive anal sphincter pinch test was confirmed in all four dogs. Compound muscle action potential (CMAP) test using EMG unit was performed to confirm the normal activity of EAS muscle by stimulating caudal rectal nerve branch of pudendal nerve in each dog before any intervention and injection. A total volume of 1.50 mL NaCl 0.09% was injected in EAS muscle using Ambu® Neuroline Inoject EMG Needle (1.50”x 26-G); The needle had a wire which could conduct the electrical stimulations and was connected to EMG unit. We decided to inject 0.25 mL in 2, 4 (potential neuromuscular endplates), 8, and 10 (opposite to the endplates) o'clock of EAS muscle and 0.125 mL in 12, 3, 6, and 9 o'clock to cover all the remaining length of EAS muscle. Injection sites were confirmed by the maximum stimulation gained by the Ambu® Neuroline needle (Ambu A/S, Copenhagen, Denmark) just before injection (Fig. 2) CMAP test was performed after injection.

**Stage two: AbobotulinumtoxinA administration and evaluation.** Day 22) All of the objects were clinically and neurologically examined by two small animal surgeons. The objects were all healthy and normal. Positive anal sphincter pinch test was confirmed in all four dogs. Compound muscle action potential (CMAP) test using EMG unit were performed to confirm the normal activity of EAS muscle by stimulating the caudal rectal nerve in each dog before any intervention and injection of abobotulinumtoxinA. 250 - 300 IU of abobotulinumtoxinA (Dysport®; Ipsen Biopharmaceuticals Ltd., Wrexham, UK) in a total volume of 1.50 mL was injected in EAS of each dog. The dilution protocol and amount of abobotulinumtoxinA injection were on the basis of the description in the official prescribing information paper for Dysport® provided by IPSEN Biopharmaceuticals, Inc.; available on both drug package and Dysport® website.11 Total volume of 1.50 mL abobotulinumtoxinA was injected in EAS muscle using Ambu® Neuroline Inoject EMG Needle (1.50-inch length, 26-G); 0.25 mL in 2, 4 (potential neuromuscular endplates), 8, and 10 (opposite to the endplates) o'clock of EAS muscle and 0.125 mL in 12, 3, 6, and 9 o'clock to cover all the remaining length of EAS muscle (Fig. 2). Injection sites were confirmed by the maximum stimulation gained by the Ambu® needle just before injection. CMAP test was performed after injection. Day 30 and 37) Neurological examination, anal reflex and CMAP test were performed for each dog. From day 22 to day 37 all of the animals were walked outdoor twice a day at an exact time (9:00 a.m. and 6:00 p.m.). Leakage of feces from the rectum in each dog and defecation inside the cages were recorded if any. Although dogs after receiving saline were evaluated for 2 weeks, we decided to keep dogs for an extra 4 weeks after receiving abobotulinumtoxinA to evaluate the medium-term effects of abobotulinumtoxinA. Care of the dogs from day 38 to 65 was the same as the first 2 weeks after abobotulinumtoxinA injection and on day 65 final neurological examination, anal reflex and CMAP test were performed for each dog. It is important to mention that EAS pinch test was performed every day during the study and the presence of contraction of EAS was scored as 1 and the absence of contraction was considered as 0. These daily evaluations were done to observe the exact day of disappearance of (if happened)

![Fig. 2](image-url) Perineal view of dogs during injections of saline and abobotulinumtoxinA. A: The insertion point of Ambu® Neuroline Inject EMG needle as the cathode electrode over the belly of external anal sphincter. B: The point of insertion of anode electrode in midline, and C: The point of insertion of ground electrode.
EAS contraction, however, the days zero, one, seven, fourteen, twenty-two, thirty, thirty-seven, and sixty-five (in which we also performed EMGs) were considered as checkpoints for statistical analysis of data obtained from EAS pinch test.

**Statistical analysis.** Data obtained were analyzed by means of IBM SPSS Software (version 21.0; IBM Corp., Armonk, USA). The Average values of each group were expressed as mean ± sem. The repeated measurements ANOVA, t-test, Friedman and Wilcoxon were used for the analysis of parametric and non-parametric data. A p-value less than 0.05 was statistically significant.

**Results**

Neurological examination and anal reflex test results. All of the animals were neurologically examined during the study and no sign of any neurological impairment or disease were observed before and after interventions. Gait and weight bearings were all normal, no sign of any muscle atrophy and lameness were present on both hindlimbs after the injections. No sign of scoliosis was evident in the caudal lumbar and sacral vertebral region after saline and abobotulinumtoxinA injection. The tail was not paralyzed in any of the animals after saline or abobotulinumtoxinA injection. Anal reflex and contraction of EAS were assessed by pinching EAS of each animal every day. Pinch tests were positive in all of the objects before and after saline injections, however, after injection of abobotulinumtoxinA, pinch tests started to be negative, ranging from two days after injection (two dogs) to 7 days after injection (First dog on 5th day and the last one on the 7th day after injection). Saline injection showed no significant difference in EAS pinch test before, 1 week and 2 weeks after saline injection. Statistical analysis of data obtained showed that the EAS pinch test was negative and significantly decreased (p < 0.05) from the first week after abobotulinumtoxinA injection in comparison with no injection and saline injection. EAS pinch test negative results continued to be the same until 6 weeks after abobotulinumtoxinA injection (p < 0.05). Finally, a comparison of amplitudes between abobotulinumtoxinA and saline (as control injection) showed that there was a significant decrease after receiving abobotulinumtoxinA compared to saline group. This decrease in amplitude was statistically confirmed in both point-to-point periods comparisons [(1 week after saline injection (1,294.04 ± 131.00 µV) vs. 1 week after abobotulinumtoxinA injection (339.86 ± 103.11 µV; p < 0.05); 2 weeks after saline injection (1,221.92 ± 205.98 µV) vs. 2 weeks after abobotulinumtoxinA injection (391.05 ± 44.41 µV; p < 0.05)] and analysis of whole period of study, which means effects during the whole period after saline and abobotulinumtoxinA injections (Final mean amplitude was 145.25 ± 45.96 µV; p < 0.05). (Fig. 3).

Electromyography results. Compound muscle action potential (CMAP) Test using needle EMG device was performed and amplitudes of action potentials of EAS muscle were recorded by a computer. Amplitudes were considered as parameters showing the impact of contractibility of EAS muscle. Amplitudes were assessed as follow: Before receiving any treatment (Day 0), after receiving saline as control treatment (Day 1, week 1, and week 2 after treatment) and after receiving abobotulinumtoxinA (Day 1, week 1, week 2, and week 6 after treatment). As it is shown in Table 1 there were two amplitude data for each dog in each session which were representing the results after stimulation of the left and right caudal rectal nerve branches of pudendal nerves innervating left and right side of EAS muscle, respectively. Statistical analysis showed that there was no significant decrease in amplitude of EAS in a two-week period after treatment with saline (amplitudes were 1,062.03 ± 91.50 µV before receiving any treatment, 1,684.47 ± 186.43 µV one day after saline injection, 1,294.04 ± 131.00 µV one week after saline injection and 1,221.92 ± 205.98 µV two weeks after saline injection; p > 0.05). In contrast, there were highly significant decreases in amplitude after treatment with abobotulinumtoxinA-both in 2-week period after injection and 6-weeks period after injection-in comparison with no treatment (amplitudes were 1,062.03 ± 91.50 µV before receiving any treatment, 876.47 ± 102.66 µV one day after abobotulinumtoxinA injection, 339.86 ± 103.11 µV one week after abobotulinumtoxinA injection, 391.05 ± 44.41 µV two weeks after abobotulinumtoxinA injection, and 145.25 ± 45.96 µV four weeks after abobotulinumtoxinA injection; p < 0.05). Finally,a comparison of amplitudes between abobotulinumtoxinA and saline (as control injection) showed that there was a significant decrease after receiving abobotulinumtoxinA compared to saline group. This decrease in amplitude was statistically confirmed in both point-to-point periods comparisons [(1 week after saline injection (1,294.04 ± 131.00 µV) vs. 1 week after abobotulinumtoxinA injection (339.86 ± 103.11 µV; p < 0.05); 2 weeks after saline injection (1,221.92 ± 205.98 µV) vs. 2 weeks after abobotulinumtoxinA injection (391.05 ± 44.41 µV; p < 0.05)] and analysis of whole period of study, which means effects during the whole period after saline and abobotulinumtoxinA injections (Final mean amplitude was 145.25 ± 45.96 µV; p < 0.05). (Fig. 3).

**Fig. 3.** schematic change in voltage of amplitudes of EMG tests before treatment and time points after treatment with saline and abobotulinumtoxinA (Bot).

* indicate significant decrease in amplitudes (p < 0.05).
Clinical signs of incontinence. The animals were kept for 8 weeks before the beginning of injections, they were trained not to defecate inside the cages. Four weeks before saline treatment it was confirmed that they usually defecate once in a day, especially in morning walking sessions. Meanwhile, contamination of the cages with feces was recorded twice a day and all of the animals kept their cages clean in the final 4 weeks before the beginning of injections. From the beginning of the injections, cleanliness or contamination of cages with feces was considered as a clinical sign of incontinence and feces leakage from the animals. After the injection of saline to external anal sphincter muscle, all of the animals kept their cages clean and no changes in habit of defecation were observed. For the first 3 weeks after saline injection, there were no changes in habit of defecation and animals were all continent. The ability to hold the feces was checked once after the first week of injection by keeping the animals 30 hr inside the cages and they successfully did not defecate inside the cages. However, after receiving abobotulinumtoxinA, behaviors were changed dramatically. A small amount of leakage of feces were observed in two animals just 2 days after receiving abobotulinumtoxinA. The other two started to leak feces from days 4 and 5 after receiving abobotulinumtoxinA. By the end if the first week after injections, all of the animals were clinically fecal incontinent. Cages were all contaminated with feces during the day, which obliged us to clean their cages three times a day. For statistical analysis of clinical fecal incontinence, contamination of cages and leakage of feces from each animal were scored as 1, and cleanliness of cages scored as 0 every day. Results showed that animals had significant presentations of fecal incontinence from the 1st week after receiving abobotulinumtoxinA \((p < 0.05)\) until the 6th week after receiving abobotulinumtoxinA in comparison with EAS muscle saline treatment period \((p < 0.05)\).

Discussion

Animal studies have an important role in researches aiming to improve human health.\(^\text{12}\) Although there are many controversies about whether the animal studies are appropriate predictors of human reactions to exposures or not, we cannot ignore this fact that animal model-based studies are still forming a great amount of health care studies.\(^\text{12,13}\) There are a lot of important regulations for animal experiments all around the world and one of the most valid ethical guidelines is presented by Institutional Animal Care and Use Committee (IACUC). The IACUC declares that each protocol must include: A reduction in number of animals to be used, elimination or minimizing the pain and discomfort for the animal, a description of efforts used to find alternative method to painful procedures and a description of searches used to declare that the experiment does not duplicate previous researches.\(^\text{14}\) Based on these important guidelines the authors of the present study tried to find an alternative and noninvasive fecal incontinent animal model, which can replace previous models producing a lot of pain and discomfort to the animals, and many of them require surgical interventions that produce irreversible damages to the animal and requires euthanasia at the end of the study consequently.

In the present study, we decided to find an alternative way to previous animal models of fecal incontinence by focusing on the pathophysiology and way of establishment of FI in the animals. As we figured out, all of the previous FI animal models were focusing on EAS muscle in order to eliminate this muscle or reduce and/or eliminate the contraction function of this muscle.\(^\text{10}\) In order to achieve these targets some of the researchers tried to damage the EAS muscle by cutting EAS (single or multiple cuts), partial excision (25.00, 50.00, or 75.00% of EAS), or complete removal of the EAS.\(^\text{15-21}\) Dogs, Rodents, and rabbits were the animal models used in this kind of study models.\(^\text{10}\) Two studies tried to damage EAS by creating anal fistulas in pigs and rabbits. The aim of these two studies were the study of Fistula-In-Ano disease. They tried to make fistulas by passing stones through catheters, however, both of these animal models of fistula were led to damage to EAS muscle and induced FI.\(^\text{22,23}\) Some other researchers tried to focus on the elimination of function of EAS instead of removing it. Therefore, they tried to induce a neuropathic FI by damaging pudendal, pelvic or caudal rectal nerves. They adopted multiple methods like using vaginal or

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| Table 1. Amplitudes (\(\mu\)V) of action potentials of EAS muscle after stimulation pudendal nerve. |
|------------------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Case | Day 0 | 1 day | 1 week | 2 weeks | 1 day | 1 week | 2 weeks | 6 weeks |
|------|-------|-------|--------|--------|-------|--------|--------|-------|
| 1    | R\(^1\) | 928.40 | 1,492.70 | 1,435.50 | 1,107.50 | 842.60 | 616.30 | 210.60 | 32.21 |
| 2    | L\(^2\) | 1,365.30 | 2,512.30 | 1,320.20 | 1,121.30 | 1,076.70 | 834.80 | 405.70 | 436.90 |
| 3    | R\(^1\) | 1,014.30 | 1,560.30 | 958.36 | 624.10 | 1,084.50 | 436.90 | 475.90 | 124.80 |
| 4    | L\(^1\) | 1,490.30 | 803.60 | 1,560.40 | 2,496.10 | 1,310.10 | 425.30 | 569.50 | 202.80 |

\(^1\text{Right and left rectal nerve branch of pudendal nerve, respectively.}\)

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intrapelvic balloons to induce pressure damage to pelvic floor muscles and their innervation, crushing or cutting the pudendal nerve or caudal rectal branch and finally inducing diabetic neuropathies leading to fecal incontinence.10

In the present study, we used abobotulinumtoxinA to remove the function of EAS. AbobotulinumtoxinA is one of the most reliable and common therapeutic forms of botulinum toxins mainly recognized with the trade name Dysport®. Botulinum toxins are protein complexes produced by anaerobic gram-positive Bacilli called 

_Clostridium botulinum_.24 In 1950s Burgen and Vernon discovered that botulinum toxins presynaptically blocked the release of acetylcholine from motor nerve endplates and that was the main cause of toxification and death of many patients eating foods containing this toxin.25 Nowadays scientists have realized that these toxins degrade N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins in neuronal endings. This could prevent the attachment of vesicles containing acetylcholine and prevents the normal function of neuromuscular end-plates.24,25 In the 1970s Alan Scott introduced botulinum neurotoxin A as a therapeutic agent. He used botulinum toxin to weaken muscles for correction of strabismus and blepharospasm.26 Therefore today, therapeutic use of botulinum toxins has a wide range from cosmetic uses for facial rejuvenation, treatment of muscular disorders like muscle stiffness or spasms, movement diseases such as cervical dystonia and torticollis, treatment of patients suffering urge urinary incontinence and every now and then researchers add a new treatment option by means of botulinum toxin to mentioned list.27

Considering the pathophysiology of the botulinum toxins, the fact that abobotulinumtoxinA can prevent EAS muscle actions, as the main target structure of almost all of the animal models of Fecal Incontinence, was our main hypothesis for this study. Previous studies on human patients showed that the duration of muscle relaxation after the injection of abobotulinumtoxinA could range from 2 to 6 months based on the site, the amount and the use of injection (cosmetics and muscle spasm treatment).20,21 However, similar studies in veterinary practice are sparse and there is no study focusing of the duration of EAS muscle relaxation after the injection of abobotulinumtoxinA in veterinary medicine. We believed that the period of muscle relaxation of EAS after abobotulinumtoxinA injection could be similar to the human patients, however, to completely confirm this period, long-term studies (at least 3-6 months) should be carried out. We selected dogs for this study because we had the option of manipulation of the defecation habit of the animals and used this behavior as a clinical parameter for assessing the effectiveness of abobotulinumtoxinA in fecal incontinence.

Our results showed that abobotulinumtoxinA could cause paralysis to the EAS and produced FI condition in dog models. We believed this animal modeling was very helpful for the researchers in the field of FI, especially those who aimed to eliminate the function of EAS muscle since it could provide both clinical signs of the disease and electromyographic data which could be statistically analyzed before and after manipulations during studies. And, finally it replaced the various invasive, expensive and also complicated procedures with an easy, feasible, noninvasive and non-painful single-stage abobotulinumtoxinA injection.

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**Conflicts of Interest**

The authors declare no conflict of interest.

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