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# Simultaneously administration of cabergoline and PMSG reduces the duration of estrus induction in anestrous bitches

**Mohammad Bolghanabadi[1], Hamideh Salari Sedigh[2]*, Pejman Mirshokraei[2], Masoud Rajabioun[2]**

*[1] DVM Graduate, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; [2] Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.*

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## Abstract

This study designed a protocol that would combine pregnant mare serum gonadotrophin (PMSG) and cabergoline (CAB) to induce estrus in bitches. Twenty clinically healthy adult and anestrous female dogs were randomly assigned into four groups. The first group was treated with 5.00 µg $kg^{-1}$ CAB until the onset of proestrus or for 25 days. The second group was treated with 20.00 IU $kg^{-1}$ PMSG for 5 days and 500 IU human chorionic gonadotropin (hCG) on the 5$^{th}$ day. The third group was treated with 5.00 µg $kg^{-1}$ CAB for 10 days in combination with 20.00 IU $kg^{-1}$ PMSG for 5 days and 500 IU hCG on the 10$^{th}$ day. The control group received 1.00 mL of normal saline. Ovarian changes were evaluated ultrasonographically, and the estrus cycle phase was examined by vaginal cytology. Respectively, three, three and four bitches showed clinical signs of proestrus in each treatment group. The intervals between treatment and proestrus for each group were 30.00 ± 3.05, 7.67 ± 1.20 and 13.00 ± 1.20 days, respectively. Two weeks after estrus, the progesterone mean was 14.51 ± 6.24, 19.96 ± 17.16 and 19.12 ± 9.26 ng $mL^{-1}$ for each group, respectively. In ultrasonography examination, the largest follicle was identified at 15.66 ± 1.33, 11.66 ± 2.40 and 8.75 ± 2.17 days after the onset of proestrus and the largest follicle's size was measured 6.50 ± 0.55, 4.83 ± 1.64 and 7.07 ± 1.49 mm for each group, respectively. Although the combined use of CAB and PMSG reduced the duration of treatment, alteration of the duration or PMSG dosage can be helpful to improve the results.



## Introduction

Estrus induction in bitches is an essential tool for breeding programs. It enhances the breeding management of a breeding colony, which would be advantageous in breeding establishments where a continuous supply of puppies would be required throughout the year (e.g., working dogs) to ensure future generations.[1] Also, synchronous estrus induction is necessary to control pregnancy and parturition timing during canine embryo transfer and research.[2,3] Also, in research programs, synchronous estrus induction is required to induce pregnancy and control the timing of birth.[4] The induction of estrus in clinical reproduction is an effective way of anestrus treatment, whether it is a primary or secondary condition.[5] It may also treat missed breeding opportunities and conception failure.[6]

Several protocols have been used in the last few decades to pharmacologically induce estrus in bitches.[7-10] These protocols comprise the use of dopamine agonists like cabergoline (CAB) and bromocryptine, gonadotropin hormone-releasing hormone (GnRH) agonists (deslorelin, fertirelin, buserelin, lutrelin and leuprolide), exogenous gonado-tropins (human chorionic gonadotropin [hCG]), follicle-stimulating hormone (FSH), luteinizing hormone (LH), equine chorionic gonadotropin (eCG), human menopausal gonadotropin and synthetic estrogens like diethylstilbesterol (DES).[11] There is a wide variation in the success of these methods concerning inducing estrus, determining the pregnancy rate following the induced estrus and maintaining the pregnancy after the induced estrus.[12] It should also be noted that some protocols are unsuitable for clinical veterinary practice due to their cost or labor intensity and some hormones are only available in some countries.[13]

In domestic and non-domestic felids, exogenous gonadotropin regimens combining eCG and hCG are frequently used in artificial insemination protocols.[14] In

***Correspondence:**

Hamideh Salari Sedigh. DVM, DVSc
Department of Clinical Science, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
**E-mail:** hssedigh@um.ac.ir

bitches, however, these protocols have caused a variety of results. When dopamine agonists are used to precipitate anestrus termination prematurely, the ovarian function will typically be stimulated due to the physiological estrus and spontaneous ovulation.[15] Based on comparisons of practical application and adverse effects of estrus and ovulation stimulation in bitches, satisfactory results could not be obtained with treatments of DES, GnRH, pregnant mare serum gonadotrophin (PMSG/eCG) and gonadotropin agonist administrations compared to CAB.[16-18]

Until now, there has not been a widely used practical, easy-to-apply and cost-effective protocol for the estrus induction in bitches. It would be beneficial if researchers could develop a method of combining PMSG and CAB to induce estrus in bitches effectively, predictably and safely.[19] This would be particularly promising in breeding colonies where it is vital to maintain a continuous supply of puppies. It is believed that simultaneous induction of folliculogenesis by using CAB along with PMSG will result in a successful protocol for triggering the onset of estrous cycle; this protocol is expected to have a high success rate.[20,21] Compared to the using dopamine agonists and estrogens alone, this method has fewer side effects.[22]

This study had the purpose of designing a protocol that would combine PMSG and CAB in an effective, predictable and safe way to induce estrus in bitches.

## Materials and Methods

**Animals and experimental design.** Twenty clinically healthy anestrus mixed-breed bitches were included. Animals were weighed 18.00 - 30.00 kg and aged 1-2 years. A balanced standard commercial dry food was fed once daily at an adequate rate to maintain body weight within individual indoor-outdoor runs. The bitches were provided with water *ad libitum* and exposed to the natural light. This study has received ethical review committee approval (Ethical Code: 29892). All bitches were clinically examined at the beginning of the study and the anestrus condition was confirmed based on vulvar observation and the absence of large intermediate or superficial intermediate cells in vaginal smears with a serum progesterone level of less than 1.00 ng $mL^{-1}$.[23,24] The start of proestrus was considered from the time of blood secretions from the vulva and the observation of intermediate and parabasal cells as well as red blood cells in the vaginal smear. Estrus onset was considered from observing hypertrophic cells with pyknotic nuclei in vaginal smear cytology or the first mating. The beginning of diestrus was determined from the reappearance of parabasal and intermediate cells in the vaginal smear.[23]

**Grouping and treatment.** Bitches were randomly assigned into four groups. The first group (CAB; n = 4) dogs were treated with oral 5.00 µg $kg^{-1}$ CAB (q 24 hr; Aburaihan Co., Tehran, Iran) until the onset of proestrus or for 25 days. The second group (PMSG; n = 6) dogs were treated with intramuscular 20.00 IU $kg^{-1}$ PMSG for 5 days (Hipra Co., Girona, Spain) and 500 IU hCG (BSV Co., Baesweiler, Germany) on the 5th day. The third group (CAB + PMSG; n = 6) dogs were treated with oral 5.00 µg $kg^{-1}$ CAB (q 24 hr) for 10 days in combination with intramuscular 20.00 IU $kg^{-1}$ PMSG from 5th day for 5 days and 500 IU hCG on the 10th day. The control group (n = 4) received 1.00 mL of normal saline (daily for 5 days) intramuscularly. During this study, ovarian alterations were evaluated by ultrasonography. Assessment of the estrus cycle was performed by vaginal cytology examination. After starting the treatment, the vulva of the dogs was checked twice a day to observe any changes.

**Vaginal smear.** A vaginal smear was taken from all dogs before the study initiation to determine the stage of the dogs' cycle. After starting the treatment, smears were taken from the treated dogs every 2 days. From that time, when the dogs showed the least signs of the beginning of proestrus, vaginal smears were taken daily to record the exact time of proestrus. In dogs responded to the treatment until the end of estrus, vaginal smear was taken. In the dogs not responding to the treatment, a vaginal smear was taken 20 days after the end of the treatment.

**Progesterone measurement.** Progesterone hormone was measured twice; the first was before the start of the treatment to ensure that the dogs were anestrus and the second was 2 weeks after the end of estrus to confirm ovulation and corpus luteum formation. To measure progesterone hormone, whole blood was taken from each dog and progesterone hormone level was measured using radioimmunoassay kits (IBL, Hamburg, Germany) according to the manufacturer's instructions.

**Ultrasonography.** Before starting the treatment, in all dogs, the left and right ovaries and the urogenital system were subjected to ultrasonography to check possible abnormalities and follicle situation using a real-time B-mode scanner (DP-6600; Mindray, Shenzhen, China) equipped by 7.50 and 10 linear transducers. In dogs responded to the treatment, ultrasonography was performed every 2 days at 14:00 to observe ovarian alterations. In each case, if a measurable follicle was observed, the first observation time of the follicle on the ovary, the size of the largest follicle and the observation time of the largest follicle were recorded. Ultrasonography was continued until the end of estrus. It was performed approximately 25 days after mating in dogs to detect possible pregnancy. In cases where the pregnancy was confirmed, ultrasonography was performed to check the condition of the fetuses. Finally, the number of embryos was determined and recorded.

**Statistical analysis.** First, a normality test was performed for each evaluated parameter. If the data were normal, an ANOVA test was used, and if the data were non-parametric, Kruskal-Wallis's one-way analysis was applied.

In all tests, a *p*-value less than 0.05 was considered significant. Statistical analysis was performed by SigmaStat software (version 4.0; Systat Software Inc., San Jose, USA).

## Results

**Treatments.** CAB group: The treatment in this group continued for 25 days; during this period, none of the dog showed signs of proestrus. Three dogs from a total of four treated dogs showed proestrus 30.00 ± 3.05 days after the start of treatment. In dogs of this group, proestrus and estrus lasted 10.33 ± 1.85 and 7.66 ± 2.40 days, respectively. Of these three dogs, after mating, two dogs became pregnant and five fetuses were confirmed totally. PMSG group: Three dogs out of the total of six treated dogs showed signs of proestrus 7.67 ± 1.20 days after the start of treatment. One of the three dogs showed mild proestrus signs. In dogs of this group, proestrus and estrus lasted 6.50 ± 0.50 and 6.50 ± 1.50 days, respectively. After mating, one of the dogs became pregnant and seven fetuses were confirmed. CAB + PMSG group: Four dogs out of the total of six treated dogs showed signs of proestrus 13.00 ± 1.20 days after the start of treatment. One of the dogs showed mild proestrus symptoms. Proestrus and estrus lasted 8.00 ± 3.05 and 7.00 ± 1.52 days, respectively. Only one of the dogs became pregnant after mating and three fetuses were confirmed (Table 1). Control group: None of the dogs showed signs of proestrus and subsequent estrus.

**Progesterone hormone.** The hormone levels in the anestrous period and 2 weeks after the end of estrus for different groups are presented in Table 2. No statistically significant difference was observed among the groups in both examination times.

**Smears.** Before the treatment, vaginal cytology showed parabasal and small intermediate cells. With the beginning of proestrus, the number of intermediate cells increased and red blood cells were observed in the slide. As the cycle progressed and approached estrus, intermediate cells progressed toward cornification. In such a way, their nucleus gradually disappeared and the cell became more elongated. Cornification of more than 80.00% of the cells was the beginning of the estrus stage. As the end of estrus approached, intermediary cells appeared in the smear. When more than 80.00% of the cells were intermediate and many neutrophils were observed, it was considered as the end of estrus and beginning of the diestrus phase.

**Ultrasonography.** Ultrasonography of the left and right ovaries and uterus was performed for all dogs before starting the treatment; the ovaries were inactive (4.90 - 7.20 mm height and 9.50 - 14.10 mm length) and no follicles could be detected by ultrasonography. With the onset of bleeding, ovarian ultrasonography was started to evaluate ovarian alterations. With the onset of proestrus, the size of the ovaries became larger and follicles with a diameter of less than 2.00 mm were often observed. The ovaries, which were initially small, round or elongated, became larger and had identifiable follicles when proestrus started and approached estrus. Follicles with a more than 10.00 mm diameter were observed in two dogs of the PMSG group. Also, in one dog in the PMSG group and one in CAB + PMSG group, where estrous symptoms did not continue, maximum follicular growth with a diameter of less than 3.00 mm was recorded. The average of the 1st day of follicle observation, the average observation time of the largest follicle and the average diameter of the largest follicle are presented in Table 3.

**Table 1.** Treatments outcome. Data are presented as Mean ± SD.

| Groups | Treatment duration (Day) | No. of dogs responded to treatment (%) | Interval until the onset of proestrus (Day) | Length of proestrus (Day) | Length of estrus (Day) |
|---|---|---|---|---|---|
| **CAB (n = 4)** | 25 | 3 (75.00) | 30.00 ± 3.05 | 10.33 ± 1.85 | 7.66 ± 2.40 |
| **PMSG (n = 6)** | 5 | 3 (50.00) | 7.67 ± 1.20 | 6.50 ± 0.50 | 6.50 ± 1.50 |
| **CAB + PMSG (n = 6)** | 10 | 4 (66.66) | 13.00 ± 1.20 | 8.00 ± 3.05 | 7.00 ± 1.52 |

CAB: Cabergoline; and PMSG: Pregnant mare serum gonadotrophin.

**Table 2.** Progesterone hormone levels in all experimental groups. Data are presented as Mean ± SD.

| Groups | Hormone levels before starting the treatment (ng mL$^{-1}$) | Hormone levels two weeks after estrus (ng mL$^{-1}$) |
|---|---|---|
| **CAB** | 0.50 ± 0.12 | 14.51 ± 6.24 |
| **PMSG** | 0.34 ± 0.12 | 19.96 ± 17.16 |
| **CAB + PMSG** | 0.57 ± 0.16 | 19.12 ± 9.26 |
| **Control** | 0.16 ± 0.13 | - |

CAB: Cabergoline; and PMSG: Pregnant mare serum gonadotrophin.

**Table 3.** Ovarian follicles alteration in treatment groups. Data are presented as Mean ± SD (Minimum - Maximum).

| Groups | The first day of follicle observation | Follicle diameter (mm) | Day of observation of the largest follicle | Diameter of the largest follicle (mm) |
|---|---|---|---|---|
| **CAB** | 5.00 ± 2.30 (1 - 9) | 4.06 ± 0.63 (3.00 - 5.20) | 15.66 ± 1.33 (13 - 17) | 6.50 ± 0.55 (5.40 - 7.00) |
| **PMSG** | 5.00 ± 1.00 (3 - 6) | 2.93 ± 0.58 (2.00 - 4.00) | 11.66 ± 2.40 (7 - 15) | 4.83 ± 1.64 (5.80 - 8.00) |
| **CAB + PMSG** | 3.25 ± 1.10 (1 - 6) | 3.87 ± 0.37 (3.00 - 4.80) | 8.75 ± 2.17 (5 - 15) | 7.07 ± 1.49 (5.00 - 11.50) |

CAB: Cabergoline; and PMSG: Pregnant mare serum gonadotrophin.

## Discussion

There are various protocols for inducing estrus in dogs; however, the success rate of each in estrus induction is different.[25-27] Dopamine agonists and gonadotropins have been more successful in aforementioned protocols.[7] Using dopamine agonists to induce estrus requires a long time drug administration.[28] The results of using gonadotropins to induce estrus have been successful, however, the pregnancy rate following their use is low due to the side effects.[29]

Alkaloids derived from ergots, such as bromocriptine, CAB or metergoline, bind to dopamine receptors and act as dopamine agonists.[16] Despite this, neither metergoline nor bromocriptine acts as a selective dopamine agonist. In addition, to act as an anti-serotonergic substance, metergoline has a dopaminergic effect and shortens inter-estrous intervals only at high doses.[30] Veterinary use of bromocriptine is not approved, as it can cause anorexia and emesis in the early stages of treatment.[31] The effects of CAB on the central nervous system are less than those caused by bromocriptine because it has a high level of specificity for D2 receptors and a long-lasting effect on pituitary lactotropic cells.[32] Fertile estrus has been induced successfully with CAB in bitches; but, such drugs are not readily available for veterinary use in some countries.[33] The CAB can also be purchased over the counter to treat hyperprolactinemia in women.[34] However, its use for the induction of estrus in heavy-breed dogs may be more cost-effective.

In 2003, Rota *et al*. reported that 10 of 12 litters (83.33%) treated with CAB at a dose of 5.00 µg $kg^{-1}$ body weight daily until the onset of proestrus or for 30 days, enter proestrus 23.50 ± 2.30 days after starting the treatment.[33] In the present study, the treatment interval until the start of proestrus lasted 30.00 ± 3.05 days. All the dogs responded to the treatment in the Rota *et al.* report mated, became pregnant and gave birth with an average of 5.20 ± 0.50 puppies, being similar to the results of this study.

Gobello *et al*. reported hair color changes in seven dogs received CAB for more than 14 days from the 2nd week of administration.[35] In the present study, hair color change was not observed in any dog during and after treatment.

Spattini *et al*. found that eight out of 10 dogs (80.00%) treated with CAB at a dose of 5.00 µg $kg^{-1}$ of body weight (beagles) and 3 dogs out of six dogs (50.00%) treated with CAB at a dose of 5.00 µg $kg^{-1}$ of body weight (greyhounds) enter proestrus for 4 weeks.[17] Three out of four treated dogs (75.00%) responded to the treatment and entered proestrus. The time from the start of treatment to the beginning of proestrus was 13.30 ± 1.90 days, the length of proestrus was 10.60 ± 0.50 days and the length of estrus was 8.90 ± 0.90 days for the beagles, and the time from the start of treatment to the start of proestrus was 20.30 ± 1.70 days, proestrus length was 11.70 ± 0.50 days and estrus length were 8.60 ± 0.40 days in greyhounds. The observed difference in the average interval between treatment and the beginning of proestrus can probably be due to the difference in the geographical area or the breed of studied dogs. They also reported ovarian enlargement and follicular growth in nine out of 10 dogs (90.00%) in the beagles and five out of six dogs (83.33%) in the greyhounds between the 2nd and 3rd weeks of treatment. Still, only eight beagles and three greyhounds entered proestrus. Follicular growth was imaged in 75.00% of the treated dogs by ultrasonography.[17]

Ajitkumar and Praseeda investigated the induction of estrus in 20 anestrous dogs of different breeds and ages using CAB at a dose of 5.00 µg $kg^{-1}$ body weight for 20 days.[36] They reported that 16 out of 20 dogs (80.00%) entered proestrus within 13.44 ± 3.12 days after the start of treatment. Proestrus and estrus lasted 10.11 ± 0.68 and 8.00 ± 0.29 days, respectively. Out of 16 litters, 14 litters (87.50%) became pregnant. In the present study, the treatment time until the onset of proestrus was 30.00 ± 3.05 days; this difference could be due to the difference in the breed and age of the dogs according to the above-said study.

In the present study, it was not possible to detect the anestrus stage of dogs. The results of this study confirmed that using CAB with the usual dose to induce the estrous cycle in dogs was associated with success.

In 1991, England and Allen have examined the induction of estrus by administering PMSG at a dose of 20.00 U $kg^{-1}$ of body weight for 5 days, followed by the administration of 500 U of hCG on the 5th day with a natural estrous cycle in 6 dogs.[37] Five litters of 6 treated litters entered proestrus in 6.00 ± 1.70 days. Proestrus lasted 7.40 ± 1.50 days and estrus lasted 12.00 ± 1.90 days. They also reported no significant difference between the natural estrous cycle and induced estrus during proestrus and estrus. The length of estrus in the present study was higher than the reported amount; but, there was no significant difference.

Weilenmann *et al*. studied the induction of estrus in 14 anestrous dogs using PMSG at a dose of 20.00 U $kg^{-1}$ of body weight daily for 5 days along with the administration of 500 U of hCG on the 5th day.[38] They reported that all dogs entered proestrus between 4 and 6 days after the start of treatment. All dogs entered estrus and mated 9 to 15 days after the start of treatment and 6 dogs out of 14 dogs (42.85%) became pregnant.

Horoz *et al*. have also reported that administering 500 U of PMSG twice with an interval of 6 days does not affect the estrus induction in dogs.[27]

Nak *et al*. probed the induction of estrus in primary and secondary estrous dogs using PMSG at a dose of 20.00 U $kg^{-1}$ of body weight.[39] In the 1st group (primary anestrus), nine dogs out of 10 treated dogs (90.00 %) and in the 2nd

group (secondary anestrus), eight dogs out of nine treated dogs (88.88%) entered proestrus. The treatment interval until proestrus onset was 5.80 ± 0.90 days for the 1st group and 8.40 ± 0.40 days for the 2nd group. In both groups, all the dogs responded to the treatment mated; in each group, only one became pregnant (11.10% in the 1st group and 12.50% in the 2nd group). Pregnancy in one dog of the 1st group and one dog of the 2nd group did not continue due to the embryo absorption on day 25. In this study, three litters of six treated litters entered proestrus with an average of 7.67 ± 1.20 days after the start of treatment. Proestrus stopped in one of the dogs and did not continue. Two other dogs entered estrus and mated; one became pregnant (16.66%), being consistent with the above study results.

Stornellia *et al*. administered the eCG with a dose of 50.00 U $kg^{-1}$ of body weight intramuscularly and 500 hCG U seven days later to 16 dogs of different breeds.[19] They reported that all dogs entered the cycle 14.4 ± 3.40 days after drug administration. Fifteen out of 16 dogs (93.75%) mated and 12.75% became pregnant and gave birth to 3.62 ± 0.41 puppies. No side effects were observed due to the administrations of eCG and hCG. The level of progesterone hormone before the start of treatment was 0.71 ± 0.31 ng $mL^{-1}$, and its level on the day of the onset of diestrus was 22.85 ± 4.27 ng $mL^{-1}$, being similar to the present study. In our study, the PMSG group showed progesterone levels similar to this study.

Hase *et al*. assessed the relationship between the determination of ovulation day using ultrasound and the level of LH and progesterone.[40] They reported that ovarian follicles were visible by ultrasonography 3 - 8 days (5.10 ± 0.50) after the start of vaginal bleeding. The size of the follicles at the time of observation was 0.30 - 0.50 cm (0.40 ± 0.02). Ovarian follicles reached their largest size within 6 - 12 days (9.10 ± 0.60) after the beginning of bleeding from the vulva. In this study, the ovarian follicles in the CAB group reached their largest size in 16.66 ± 1.76 days, in the PMSG group in 9.00 ± 2.08 days and in the CAB + PMSG group in 9.33 ± 2.96 days after the start of proestrus. The largest size of the follicle observed by ultrasound was 0.50 - 0.80 cm (0.58 ± 0.03).

England *et al*. have analyzed follicular growth, ovulation and conception rate in the natural estrous cycle of dogs.[41] They reported that ultrasonography could detect follicles at the end of anestrus, 60 - 100 days before the sudden surge of LH hormone before ovulation, and their sizes increase as they approach the beginning of the cycle. Furthermore, their sizes reach more than four mm approximately two days before the LH hormone elevation.

Large follicles can be detected 10 days before the sudden increase of LH hormone. They also evaluated follicular growth in dogs in which the estrous cycle was induced using CAB and PMSG. They reported follicular growth in dogs in which the estrous cycle was induced using CAB. It was induced at a dose of 5.00 μg $kg^{-1}$ of body weight until the beginning of proestrus. It was similar to the follicular growth in the normal estrous cycle. The only difference was that more small follicles remained in the luteal phase in the induced cycle using CAB compared to the normal estrous cycle. In dogs in which the estrous cycle was induced using eCG with a dose of 20.00 U $kg^{-1}$ of body weight for 5 days and administration of 500 U of hCG on the 5th day, there was a large number of small follicles before ovulation in ovaries being in the luteal phase.

Using a high dose and a long treatment period of PMSG, in addition to being economically unaffordable, can cause excessive ovarian stimulation.[42] Also, using PMSG to induce estrus is associated with several complications.[43] On the other hand, oral CAB is difficult to induce estrus in dogs due to its low dose and requires a long treatment period.[44,45]

In this study, the combined protocol of using CAB and PMSG was proposed and implemented for the first time to eliminate the duration of CAB use and poor results caused by PMSG. This protocol made ovarian follicles visible, reaching their largest size in a shorter time than the other two groups. In addition, dogs treated with this protocol had larger follicles on the ovaries than the other two groups; although, this difference was not statistically significant. This issue can indicate that the administration of CAB at the beginning caused ovarian sensitivity to gonadotropins and the administration of PMSG after CAB accelerated follicular growth.

In the present study, in the CAB + PMSG group, the speed and amount of follicle growth were much higher than the CAB group. The low pregnancy rate may be attributed to the low quality of the oocytes. Therefore, reducing the amount or duration of PMSG consumption is recommended.

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## Conflict of interest

The authors declare no conflict of interest.

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