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Research Article

Effects of Qustul Hindi (*Saussurea lappa* L.) Root Extract on Sperm Morphology, Count, and Viability in Wistar Rats (*Rattus norvegicus* L.) Exposed to Cigarette Smoke

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ABSTRACT

Smoking-induced oxidative stress leads to Reactive Oxygen Species (ROS) production, reducing spermatozoa viability, morphology, and count. Antioxidants, such as those in Qustul Hindi (*Saussurea lappa*) root, which are consumed by many Indonesians, can counteract the effects of ROS. This study aims to ascertain how Qustul Hindi root extract affects the morphology, count, and viability of rat spermatozoa exposed to cigarette smoke. A true experimental posttest-only design with control groups was applied. Thirty healthy male Wistar rats (8–10 weeks, 150–200 g) were randomized into five groups: negative control (K1), positive control (K2, smoke exposure only), and three treatment groups (K3–K5) receiving 50, 100, and 200 mg/kg BW/day of Qustul Hindi root extract, respectively, alongside cigarette smoke exposure. Extracts were administered orally for 35 days. Outcomes were assessed using hemocytometer analysis for sperm count, giemsa-stained smears for morphology, and Eosin Y exclusion test for viability. Results show the lowest abnormal morphology was found in K1 (3.0±0.05 %), while the highest occurred in K3 (22.0±0.11 %). The highest sperm count was observed in K4 (32.69±6.43×10⁶/mL), and the lowest in K5 (11.87±6.76×10⁶/mL). Viability was highest in K4 (69.0±0.07%) and lowest in K5 (42.0±0.14 %). Statistical analysis revealed significant intergroup differences in morphology (p=0.013), count (p=0.000), and viability (p=0.000). As conclusion, administration of the extract at the dosages 50 and 100 mg/kg BW/day improved spermatozoa count and viability, while 200 mg/kg BW/day reduced both parameters. In terms of morphology, 50 and 200 mg/kg BW/day increased abnormalities, while 100 mg/kg BW/day reduced them.

Keywords: cigarette smoke; *Saussurea lappa*; spermatozoa count; spermatozoa morphology; spermatozoa viability

INTRODUCTION

Infertility is a significant reproductive health issue, affecting approximately 15 % of couples of childbearing age worldwide. Notably, around 50 % of these cases are attributed to male factors, primarily involving reduced spermatozoa quality and quantity (Agarwal et al., 2014). Among the key contributors to declining sperm quality are environmental exposures, particularly cigarette smoke. Cigarette smoke contains over 7,000 harmful chemical compounds capable of inducing oxidative stress, leading to DNA damage and structural alterations in sperm cells (Kumawat et al., 2018).

This issue warrants serious attention, especially given the high prevalence of smoking in the general population, including in Indonesia—a country where increasing tobacco use may exacerbate national infertility rates. According to the Indonesian Ministry

of Health, the prevalence of active smokers continues to rise and estimated 70 million active smokers with 7.4 % of them aged 10–18 years (Kemenkes RI, 2023). Alarmingly, the most rapid increase has occurred among children and adolescents. The Global Youth Tobacco Survey (GYTS) in 2019 revealed a rise in smoking prevalence among school-aged children (13–15 years), from 18.3 % in 2016 to 19.2 % in 2019 (WHO, 2019). Furthermore, SKI 2023 data showed that the highest proportion of smokers was found in the 15–19 age group (56.5 %), followed by the 10–14 age group (18.4 %).

The detrimental impact of cigarette smoke on sperm quality not only affects individual fertility but also poses a broader threat to population-level reproductive capacity. If this trend continues unchecked, countries with high smoking rates like Indonesia could face profound demographic and public health challenges related to declining fertility. Cigarette smoke exposure has been consistently associated with reduced sperm quality, which may contribute to decreased fertility at both individual and population levels. In countries with high smoking prevalence such as Indonesia, this pattern could have measurable implications for demographic trends and reproductive public health.

Alongside the growing global interest in natural-based therapies, Qustul Hindi (*Saussurea costus*) root extract has emerged as a promising candidate due to its well-documented antioxidant and anti-inflammatory properties, as well as its potential to mitigate cellular damage caused by oxidative stress (Ali et al., 2017). Despite its traditional medicinal use, scientific research investigating its specific effects on male reproductive parameters particularly under conditions of toxic environmental exposure such as cigarette smoke is still scarce and underdeveloped.

Cigarette smoke is a well-established reproductive toxicant that compromises sperm morphology, count, and viability through the generation of reactive oxygen species (ROS) and induction of oxidative stress. Considering the high prevalence of smoking and its impact on fertility, especially among young males, there is a critical need to explore protective interventions that are accessible, natural, and effective.

This study aimed to fill that gap by evaluating the effect of Qustul Hindi root extract on sperm quality (morphology, count, and viability) in Wistar male rats exposed to cigarette smoke, and to identify the optimal dose (50, 100, or 200 mg/kgBW/day) for maximum protective efficacy. Through this research, we aim not only to investigate the therapeutic potential of *Qustul Hindi* in mitigating smoking-induced reproductive damage, but also to contribute to the broader development of evidence-based, plant-derived interventions for male infertility.

METHODS

Research Design

This study used a true experimental research design to determine the effects of treatment on Wistar rats. Ethical approval for this study was obtained from the Animal Ethics Committee School Of Veterinary Medicine And Biomedical Science under approval number 060/KEH/SKE/III/2023. This study involved 30 healt hy and active male Wistar rats aged 8–10 weeks and weighing 150–200 grams. Rats from earlier trials and those with anatomical abnormalities were excluded. The rats were randomly selected and assigned to five groups.

This study used a posttest-only design with a control group. The sample was divided into a negative control group (K1), a positive control group (K2), and three treatment groups (K3, K4, K5). Groups K3, K4, and K5 were administered Qustul Hindi root extract at different doses. No pretest was conducted.

Preparation of Qustul Hindi

Qustul Hindi root extract was produced using a decoction method with water as the solvent, using a 70 % ratio of Qustul Hindi to water. The extract was filtered and evaporated, then thickened with corn starch, yielding 11 %. The thickened extract was dried and powdered into capsules. For administration, the Qustul Hindi root powder capsules were prepared according to the designated dose, diluted in 5 ml of water, and stirred until homogeneous.

Preparation of Animal Tests

A total of 30 male Wistar rats that met the inclusion criteria were selected for this study. The experimental animals were then acclimatized to temperature, drinking water, and standard 511 feed (feed consisting of corn, bran, oil cake, soybeans, wheat flakes, canola, meat, and bone meal with a maximum water composition of 13 %, protein 21-23 %, fat minimum 5 %, fiber maximum 5 %, ash maximum 7 %, calcium minimum 0.9 %, and phosphate minimum 0.6 %) for seven days at standard temperature (±20-28 °C) and humidity ±50–60 %.

The experimental animals were placed in containers with sawdust bases, with one rat per container. During the second to sixth week, the research intervention period took place. Groups II, III, IV, and V were exposed to cigarette smoke for 35 days at 09.00 WIB. Each group received exposure equivalent to two cigarettes per session, based on the consideration that the average Indonesian population consumes approximately 13 cigarettes per person per day (moderate level), and a 2013 study demonstrated

that exposure to two cigarettes could reduce three sperm quality parameters.

The cigarettes used in this study were kretek filter cigarettes. Cigarette smoke exposure was conducted in a glass chamber with a hole at the top, using a smoking pump connected to a hose attached to the base of a lit cigarette. Cigarette smoke and Qustul Hindi extract were administered using standardized procedures. All rats were randomly assigned to groups using simple randomization. Each rat received standard feed 511 and 1 % Na-CMC as the vehicle throughout the study period. Cigarette smoke exposure was delivered using kretek filter cigarettes, administered through a suction pump connected to a lit cigarette placed at the inlet of a closed glass exposure chamber (volume: 60 × 40 × 40 cm). Smoke was circulated for 10 minutes per session, corresponding to two cigarettes, and allowed to diffuse uniformly before ventilation. Exposure was performed once daily at 09.00 WIB for 35 days.

Qustul Hindi extract was administered via an oral stomach tube to ensure accurate dosing. The treatment allocation was as follows:

K1 (Negative control): No smoke exposure; received standard feed only.

K2 (Positive control): Exposed to cigarette smoke daily; received standard feed only.

K3: Exposed to cigarette smoke; received Qustul Hindi extract 50 mg/kgBW/day.

K4: Exposed to cigarette smoke; received Qustul Hindi extract 100 mg/kgBW/day.

K5: Exposed to cigarette smoke; received Qustul Hindi extract 200 mg/kgBW/day.

Sperm Analysis

Sperm count, morphology, and viability were evaluated according to standard protocols. Sperm count was measured using a hemocytometer following WHO laboratory manual procedures (WHO, 2010). Sperm morphology was assessed using eosin–nigrosin staining; abnormal morphology included head defects, midpiece defects, and tail abnormalities based on WHO criteria. Sperm viability was determined using eosin staining, classifying live sperm as unstained.

Preparation of Animal Test Prior to Sperm Analysis

After 35 days of treatment, rats were anesthetized using ketamine (80 mg/kgBW) and xylazine (10 mg/kgBW) administered intraperitoneally and allowed to reach surgical anesthesia (5–10 minutes). Euthanasia was performed by cervical dislocation. A midline incision was made, and the cauda epididymis was removed for sperm analysis. Epididymal spermatozoa were processed for analysis of sperm morphology, count, and viability using epididymal fluid obtained from the cauda epididymis.

Sperm Count Analysis

The cauda epididymis was minced in 1 ml phosphate-buffered saline (PBS) and allowed to disperse for 5 minutes. The sperm suspension was homogenized by gentle manual shaking. A 0.005 ml aliquot was drawn and diluted using a hemocytometer pipette to the 1.01 mark. A drop was placed at the cover glass edge, filling the Improved Neubauer chamber via capillary action. Spermatozoa were counted under 400× magnification.

Sperm Morphology Assessment

Morphology was evaluated using Giemsa staining. A smear of sperm suspension was air-dried and fixed with methanol for 10 minutes, then stained with 5 % Giemsa for 15–20 minutes. Abnormal morphology was classified according to WHO criteria (head, midpiece, and tail defects).

Sperm Viability Assessment

Viability was assessed using eosin–nigrosin staining. A drop of suspension was mixed with equal parts 1 % eosin Y for 30 seconds, followed by 10 % nigrosin. A smear was made and examined at 400×. Live sperm appeared unstained, while dead sperm stained red or pink. Assessment of spermatozoa morphology is done by taking a spermatozoa suspension dripped on an object glass to make a smear preparation then dried in air. The preparation is fixed using methanol then stained using giemsa and washed using distilled water. Next, observe the preparation under a microscope at 400x magnification to see the morphology of 100-150 mouse spermatozoa then calculate the percentage of abnormal spermatozoa. Viability observation based on live and dead spermatozoa conducted on 200 spermatozoa cells under a light microscope with 400x magnification. Live spermatozoa will not be stained by Eosin Y.

Statistical Analysis

Data were analyzed using SPSS version 25. Normality was assessed using the Shapiro–Wilk test. For normally distributed data, differences between groups were evaluated using one-way ANOVA, followed by the LSD (Least Significant Difference) post-hoc test to determine pairwise differences. For non-normally distributed data, the Kruskal–Wallis test was used. A p-value < 0.05 was considered statistically significant.

Table 1: Percentage Of Spermatozoa Morphology, Count, And Viability Across Treatment Group

Sample	Percentage of Spermatozoa Abnormal Morphology (%)	Mean±SD (%)	Count of spermatozoa (x10^6/mL)	Mean±SD (%)	Percentage of Spermatozoa Viability (%)	Mean±SD (%)
Negative Control (K1)	0 0 0 10 10 0	3.0±0.05	29.75 16 16.5 31 22.5 21.9	22.95±6.36	64 62 72 62 66 74	67.0±0.05
Positive Control (K2)	29.4 5.7 18 11.7 19.5 18.2	17.08±0.07	11.55 17.9 24.5 15.9 18 14.5	17.05±0.07	60 68 60 58 64 68	63.0±0.04
Treatment 1 (K3)	44 20 22.9 14.1 18.9 13.2	22.0±0.11	29.25 28 25.5 21.3 21.25 18	23.88±4.39	66 70 70 66 60 53	64.0±0.07
Treatment 2 (K4)	14.8 1.5 14.6 16.4 3.2 28	13.08±0.09	28 27.5 32.25 29.25 44.65 34.5	32.69±6.43	64 62 66 74 81 66	69.0±0.07
Treatment 3 (K5)	4 13.6 15.4 24.7 29.4 24.6	19.0±0.09	23.75 7 10.5 5.5 9 15.5	11.87±6.76	66 38 36 23 44 42	42.0±0.14

RESULTS AND DISCUSSIONS

Results

A total of 25 male Wistar rats were divided into five groups (n = 5 per group). The measured outcomes included spermatozoa morphology, count, and viability after exposure to cigarette smoke and administration of Saussurea lappa root extract.

Spermatozoa Morphology

The improvement in sperm morphology was supported by a clear statistical difference between groups. The positive control group (K2) showed a significant reduction in normal sperm morphology compared to the negative control (K1) ($p < 0.05$), indicating the detrimental effect of cigarette smoke. In contrast, all treatment groups receiving Qustul Hindi extract (K3, K4, K5) demonstrated significantly higher percentages of normal morphology compared to K2 ($p < 0.05$), with the highest improvement observed in the 200 mg/kgBW group (K5). This indicates a dose-dependent protective effect of the extract.

Normal and abnormal morphology were evaluated according to the WHO Laboratory Manual for the Examination and Processing of Human Semen, 2010, in which abnormal forms include defects of the head (e.g., amorphous, tapered, double head), midpiece (bent, irregular, cytoplasmic droplet), and tail (short, coiled, double tail) (WHO, 2010). Although human criteria were used as reference standards, these parameters are commonly adapted for rodent morphology assessment. According to WHO standards, a normal morphology of ≥4 % (strict criteria) is required for humans to be considered within normal fertile range. Earlier WHO criteria (pre-2010) recommended ≥30–50% normal forms; however, current laboratory practice follows the 5th edition threshold. A supporting reference is recommended to maintain scientific accuracy. Abnormal morphology was most prevalent in Group K3 (22.00 ± 0.11 %) and least in the negative control (K1) group (3.00 ± 0.05

%). Since the data were not normally distributed. a Kruskal-Wallis test was conducted. revealing a significant difference in spermatozoa morphology among the groups (p = 0.013).

Spermatozoa Count

The sperm count was evaluated using a hemocytometer (Improved Neubauer chamber), which is the standard tool for manual sperm concentration assessment. According to the World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Semen, a sperm concentration greater than 15 million/mL is considered within the normal reference range, whereas values below 15 million/mL are categorized as oligozoospermia and reflect impaired sperm quality. Although these reference values are established for human semen, they are widely adapted in rodent studies to indicate relative changes in sperm concentration following toxicological or therapeutic interventions.

The mean spermatozoa count was highest in the treatment group receiving Saussurea lappa extract at 200 mg/kg BW (Group K4). with a value of 32.69 ± 6.43 $\times 10^6$/mL. The lowest count was found in Group K5 (extract 300 mg/kg BW) with 11.87 ± 6.76 $\times 10^6$/mL.

One-way ANOVA showed a statistically significant difference in spermatozoa count across the groups (p = 0.000). Post-hoc LSD analysis revealed significant differences between Group K1 and K4 (p = 0.007). K1 and K5 (p = 0.003). K2 and K4 (p = 0.000). K3 and K4 (p = 0.014). K3 and K5 (p = 0.001). and K4 and K5 (p = 0.000).

Spermatozoa Viability

Spermatozoa viability refers to the proportion of live sperm cells that remain intact after leaving the male reproductive tract. Viability is commonly assessed using vital stains such as eosin–nigrosin, where live sperm remain unstained and dead sperm take up the eosin dye. According to the World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Semen, 5th Edition, a normal viability value is defined as greater than 58% live spermatozoa. Although this threshold is established for human semen, it is frequently adapted in rodent reproductive studies as an indicator of sperm cell membrane integrity and overall quality. Group K4 also showed the highest mean viability (69.00 ± 0.07 %). while Group K5 had the lowest (42.00 ± 0.14 %). One-way ANOVA indicated a significant difference in viability among the groups (p = 0.000). Post-hoc LSD test showed significant differences between K1 and K5 (p = 0.000). K2 and K5 (p = 0.000). K3 and K5 (p = 0.000). and K4 and K5 (p = 0.000).

Discussions

Cigarette smoke exposure has long been known to cause disturbances in sperm quality in men. with significant decreases in parameters such as sperm count. morphology. and viability. This is due to increased oxidative stress that occurs due to exposure to harmful compounds in cigarette smoke. such as nicotine. tar. and other reactive oxygen compounds. This study assessed the effect of administering qustul hindi root extract on sperm quality in male Wistar rats exposed to cigarette smoke. focusing on three main variables. namely sperm morphology. count. and viability.

Sperm Morphology

Abnormal sperm morphology can indicate disturbances in the sperm maturation process. which in turn can reduce fertilization ability. In this study. the group of rats exposed to cigarette smoke showed an increase in the number of spermatozoa with abnormal morphology. such as deformed sperm heads and imperfect flagella. This is in line with the findings by Omolaoye et al. (2020). which states that exposure to cigarette smoke increases the number of spermatozoa with abnormal morphology. due to damage to the structure of sperm cells and DNA that occurs due to oxidative stress (Omolaoye et al., 2020). In addition, the nicotine in cigarette smoke causes damage to the spermatozoa membrane and increases the number of abnormal sperm (Khan et al. 2019).

However, the group of mice treated with Qustul Hindi extract showed a significant decrease in the number of abnormal spermatozoa. This indicates that qustul hindi extract can protect sperm morphology from damage caused by exposure to cigarette smoke. This decrease in the number of abnormal spermatozoa may be related to the ability of qustul hindi to reduce lipid peroxidation. protect sperm cell membranes. and improve DNA repair mechanisms in spermatozoa (Hidayati et al., 2021). Previous studies have also shown that administering natural antioxidants can improve spermatozoa morphology in animal models exposed to toxic compounds (Agarwal et al., 2014).

Sperm Count

A low spermatozoa count can be an indication of a disturbance in the spermatogenesis process. The decrease in the number of spermatozoa in the group of mice exposed only to cigarette smoke is in line with research conducted by Alahmar (2019) which showed that exposure to cigarette smoke disrupts spermatogenesis through increased oxidative stress that damages DNA and testicular cells (Alahmar, 2019). This study also showed that administration of qustul hindi extract significantly increased the number of spermatozoa in the group of mice exposed to cigarette smoke. This can be explained by the presence

of active compounds in qustul hindi which have high antioxidant activity. such as flavonoids and alkaloids. which function to reduce oxidative stress (El-Demerdash et al., 2009; Subash-Babu et al. 2018). Several previous studies also support these results. where administration of herbal plants with antioxidant properties such as Saussurea lappa can increase the number of spermatozoa in mice exposed to environmental toxins or toxins (Goyal et al., 2016; Ahmad et al., 2020). The content of antioxidant compounds in qustul hindi helps neutralize free radicals produced by cigarette smoke. as well as prevent damage to testicular tissue and seminiferous tubules. Therefore. it can be concluded that qustul hindi extract has the potential to protect sperm quality through antioxidant mechanisms.

Sperm Viability

Sperm viability. which measures the percentage of live sperm in a sample. is an important indicator in determining the success of fertilization. In this study. the group of mice exposed to cigarette smoke showed a significant decrease in spermatozoa viability. This can be explained by increased oxidative stress that causes damage to the cell membrane and DNA of spermatozoa. which ultimately leads to sperm cell death. A study by Agarwal et al. (2014) showed that smoking can reduce spermatozoa viability through the mechanism of increased lipid peroxidation and sperm DNA damage (Agarwal et al., 2014). In addition. a study by Sharma et al. (2019) revealed that exposure to cigarette smoke decreases spermatozoa viability through increased apoptosis in sperm cells. (Sharma et al., 2019; Youssef et al., 2022).

However, administration of qustul hindi root extract to a group of mice exposed to cigarette smoke was able to significantly increase spermatozoa viability. This increase in viability may be related to the ability of qustul hindi extract to reduce lipid peroxidation and protect sperm cell membranes from damage. Flavonoid and phenolic compounds found in qustul hindi are known to have high antioxidant activity. which functions to neutralize free radicals and reduce damage to sperm (Liu et al., 2017). This is in line with research by Youssef et al. (2022). which shows that administration of herbal plant extracts can increase spermatozoa viability in animals exposed to oxidative stress.

Results of this study confirm that the 100 mg/kg BW dose demonstrated the most optimal improvement in viability because it provides sufficient antioxidant capacity to counteract cigarette-smoke–induced oxidative damage without exceeding the threshold at which plant extracts may lose efficiency or exhibit diminishing returns. At this moderate dose, the concentration of active flavonoid and phenolic compounds appears to be ideal for stabilizing sperm membranes, maintaining mitochondrial function, and preventing apoptosis, resulting in the highest proportion of viable spermatozoa compared to lower or higher doses.

Protection Mechanism by Qustul Hindi

Qustul hindi root extract is known to contain active compounds such as flavonoids. terpenoids. and alkaloids. which have strong antioxidant properties (Zhang, 2021). These compounds can neutralize free radicals produced by exposure to cigarette smoke. thereby reducing oxidative stress that damages spermatozoa quality. Some possible protective mechanisms include increased activity of endogenous antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx). as well as inhibition of lipid peroxidation that can damage sperm membranes (Choudhury et al., 2020; Adedara et al., 2018)

Research conducted by Subash-Babu et al. (2018) showed that flavonoids from herbal plants can inhibit lipid peroxidation and protect sperm cells from damage caused by free radicals (Subash-Babu P et al., 2018. Abdel-Rahman HG et al., 2019). In addition. saponins contained in qustul hindi are also known to increase blood circulation to the testes. which has the potential to increase nutrition and oxygenation in testicular tissue exposed to toxins (Raji et al., 2021).

Comparison with Previous Research

Several previous studies have shown that qustul hindi (Saussurea lappa) has the potential to improve sperm quality. For example. research by Khan et al. (2020) showed that administering Saussurea lappa extract for 28 days increased the number and motility of sperm in rats exposed to bisphenol A (Khan et al., 2020). In addition. a study by Alotaibi et al. (2019) proved that giving herbal plants containing flavonoids can increase reproductive hormone activity and reduce oxidative stress that damages spermatogenesis (Alotaibi et al., 2019).

An experimental study by Yusran et al. (2021) also showed the hepatoprotective and antioxidant effects of qustul hindi on reproductive organs. including the testes. which are damaged by exposure to toxic substances such as cigarette smoke and pesticides (Yusran et al., 2021). Another study by Hassan et al. (2023) also supports these results by showing that the active compounds of *Saussurea lappa* can improve fertility parameters in animal models experiencing oxidative stress (Hassan et al., 2023)

Research by Shaikh et al. (2022) even states that qustul hindi roots have the ability to increase the expression of anti-apoptotic genes and reduce oxidative stress biomarkers in the testes of mice

(Shaikh et al., 2022). This indicates that the mechanism of action of qustul hindi is not only limited to scavenging free radicals but also supports the endogenous cellular defense system.

Clinical Implications

This study shows that administration of qustul hindi root extract can protect spermatozoa quality from damage caused by cigarette smoke exposure. This protective effect is reflected in the improvement of morphology, quantity, and viability of spermatozoa in rats exposed to cigarette smoke. These results support the potential use of qustul hindi as a therapeutic agent in overcoming fertility disorders caused by oxidative stress due to cigarette smoke exposure. Although this study was conducted in an animal model the results provide an important foundation for potential clinical exploration. Given the increasing prevalence of male infertility due to lifestyle and exposure to environmental pollutants such as cigarette smoke, herbal-based interventions such as qustul hindi can be a safe and economical therapeutic alternative. However, further research is needed including long-term toxicity tests, molecular mechanisms involved, and clinical trials in humans.

CONCLUSION

This study shows that Qustul Hindi root extract can affect the morphology. count and viability of spermatozoa in male Wistar rats exposed to cigarette smoke at a dose of 100 mg/kg BW is the most effective in improving the normal morphology. count and viability of rat spermatozoa. These findings indicate that Qustul Hindi extract has potential as a protective agent against spermatozoa quality affected by oxidative stress.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest in this work.

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