Clin. Lab. 2021;67:XXX-XXX
©Copyright

ORIGINAL ARTICLE

Molecular Mechanism and Anti-Fertility Effect of Plant Complex Sterility Agent on Targeted Gene DPY19L2 in Rats

Chun Shi and Chenxi Yang

College of Biological Science and Engineering, North Minzu University, Yinchuan, China

SUMMARY

Background: **Damage caused by rats is a serious hazard to the environment and crop irrigation. It not only results in great damage to the ecological environment, but also seriously affects the growth and yield of crops and forests. Controlling damage caused by rats is the primary task for improving the environment and maintaining ecological balance.**
Methods: **Basing on the related gene DPY19L2 in the maturation and development of seminal vesicles in rats, our study explored the effect of non-toxic and pollution-free plant complex sterility agent on rat fertility.**
Results: **The results showed that the plant complex sterility agent could effectively reduce the genital organ index of male rats, change the sperm morphology, reduce the testosterone content and sperm motility, inhibit the expression of spermatogenesis genes DPY19L2, SPATA16, SUN5, and ZPBP, and achieve the effective sterility control of rat pests.**
Conclusions: **Plant complex sterility agent with high concentration can effectively target rat sperm gene DPY19L2 and realize effective sterility control of rat pests.**
(Clin. Lab. 2021;67:xx-xx. DOI: 10.7754/Clin.Lab.2020.200322)

Correspondence:
Chun Shi
College of Biological Science and Engineering
North Minzu University
Yinchuan, 750021, Ningxia
China
Phone: +86 9512066807
Email: sc191206@yeah.net

Manuscript accepted May 13, 2020

KEY WORDS

damage caused by rats, plant complex sterility agent, DPY19L2, fertility, mechanism

INTRODUCTION

Infertility control is one of the new techniques to control the number of harmful rats in the world [1,2]. In recent years, some relevant research and applications have been conducted in Australia, the United States, Canada, India, and other countries [3-5]. According to the ecological model study, it was found that in the population of 10,000 mice, if 70% of the individuals were killed for three generations, the population could still recover to the original level after 26 generations. However, if 70% of individuals were infertile for 3 consecutive generations, the population would be extinct after 19 generations [6]. Therefore, the use of sterility control is more likely to achieve sustainable rodent control than traditional chemical control [7]. Infertility control is the use of sterility drugs administered by oral bait. Rodent feed-

ing causes the decrease of fertility or sterility of female or male individuals, ultimately leading to the decline of the population number of rodents, to achieve the purpose of effective rodent control. Moreover, sterility drugs especially plant complex sterility agent do not pollute the environment and have no effect on non-targeted animals [8]. Our study explored the anti-fertility effect and molecular mechanism of plant complex sterility agent containing refined gossypium acetate, crude trichosanthin, zedoary rhizome powder, bait on targeted gene DPY19L2 in rats.

MATERIALS AND METHODS

Materials

Male SD rats were purchased from Changchun Yisi Experimental Animal Technology Company. The main ingredients of the compound sterility agent are refined gossypium acetate, crude trichosanthin, zedoary rhizome powder, and bait (Nanjing Shenglide Biotechnology Company). Materials and reagents used include 10% SDS (Solabao Biotechnology Company); rabbit anti-rat primary antibody (Tianjin Sanjian Biotechnology Co., LTD., China); Beyotime Biotechnology, ELISA; PBS (Invitrogen); cell culture dish: 6/12/48 plate (Thermo fisher); sterile pipette: 5 mL/10 mL (Costar, USA); liquid transfer gun: 10 μL/20 μL/200 μL/1,000 μL (Eppendorf); centrifuge tube: 10 mL/50 mL (Thermo fisher); western blotting instruments (Eppendorf); confocal microscope (Olympus, Germany); transmission electron microscope (Jem-2000ex; JEOL Co., Japan).

Methods

Grouping and administration

Thirty male SD rats (6 - 7 weeks old), weighing 33.28 ± 2.76 g, were purchased and bred at room temperature in a light environment for 24 hours. After one week of adaptive feeding, the rats were randomly divided into three groups. One group was set as control group without treatment. One group was set as the low dose group by intragastric administration (low dose of plant complex sterility agent 10 mg/kg), and the other group was set as the high dose group by intragastric administration (high dose of plant complex sterility agent 20 mg/kg) for 10 days. The activity and mental status of rats were recorded every day.

Effects of plant complex sterility agent on reproductive organs

Rats were anesthetized with intraperitoneal injection of 1% pentobarbital sodium (50 mg/kg) and killed after 10 days administration. The testis and seminal vesicle tissues of rats were collected immediately, and the testis and seminal vesicle tissues in each group were weighed to reflect the viscera index of genitals.

HE staining analysis of testicular tissue morphology of rats

The experimental procedure is as follows [9,10]: testicular tissue of adult male rats was completely removed and weighed after the rats were sacrificed. The testicle was washed with 0.9% sodium chloride to remove blood and other debris. The testicle was fixed with formalin fixation solution (10 mL formaldehyde solution + 90 mL distilled water). Fixation method: soak and fix for 3 ~ 5 days, and cut the testicles on the second day of soaking, and put back the fixation solution to continue soaking. Pathological sections: routine dehydration was done step-wise using 70%, 80%, 90%, and anhydrous ethanol, made transparent with xylene, immersed in wax and embedded in paraffin, and 4 mm thick sections were prepared for HE staining (hematoxylin-eosin staining). Morphological changes of testicular mesenchymal cells, spermatogenic tubules, spermatogenic cells at all levels, and supporting cells were observed under an optical microscope.

The content of testosterone in the blood were analyzed by ELISA

The content of testosterone in the blood of the rats in each group was detected by using an ELISA kit as follows [11]: The reagent used was kept at room temperature (18 ~ 25°C) for 0.5 hour. An appropriate amount of concentrated washing liquid was taken and diluted with distilled water at the ratio of 1:20. To each well, 50 μL liquid was added, and no liquid was added to the blank well. To all wells, 50 μL enzyme labeled antigen and 50 μL antibody were added, except the blank one. The mixture was incubated at 37°C for 1 hour after shaking and mixing. The liquid in the wells was vertically shaken off, and 330 μL washing liquid was added to each well, which was allowed to stand for 10 second and then dried. It was repeated three times and patted dry on filter paper. After washing, 50 μL chromogenic agent A and 50 μL chromogenic agent B were added to each well. After shaking and mixing, the solution was placed at 37°C in the dark for 15 minutes for color development. OD values of each well were read by the filter of the microplate analyzer after the wavelength was adjusted to 450 nm.

Sperm count and sperm motility were observed and analyzed

Sperm count [12]

The two sides of testicles were removed and put into 1 mL normal saline, then cut into pieces, and made into sperm suspension. The sperm were killed and mixed after putting into a water bath at 60°C then filled into a CBC board. The number of sperm in the 5 middle squares were counted according to the erythrocyte count method, marked as R. The formula: R x 5 = sperm count (x 10^5 /mL).

Table 1. Semen quality parameters according to different experimental groups.

	I	II	III	IV	Sperm motility (%)
Control group	**109.0 ± 3.6**	**31.3 ± 2.1**	**8.0 ± 1.0**	**22.7 ± 2.6**	**86.5**
Low dose group	**83.0 ± 7.9**	**23.7 ± 3.2**	**8.7 ± 1.5**	**40.3 ± 2.1**	**74.4**
High dose group	**61.0 ± 8.5**	**25.3 ± 4.5**	**7.0 ± 1.0**	**52.3 ± 3.5**	**64.1**

Sperm motility [13]
The two sides of testicles were removed and put into 1 mL normal saline, then cut into pieces, and made into sperm suspension. The mixture was incubated at 37°C for 20 minutes, mixed well, and then filled into a CBC board. The number of motile sperm in 200 spermatozoa was counted at high magnification. Sperm motility is classified according to the method recommended by the WHO. Sperm motility is divided into 4 levels according to the swimming state of sperm. Level I: sperm movement is active and linear; Level II the movement is more lively but the direction is uncertain, not linear movement; Level III sperm motility is slow, turning or swimming in place; Level IV no movement.
Sperm motility = (I + II + III/(I + II + III + IV) x 100%.

Sperm morphology [14]
The liquid semen was centrifuged (1,000 r/min) for 10 minutes, and the supernatant was discarded. The precipitate was retained and then 2 ~ 3 mL normal saline was added. The mixture was mixed and dyed for 10 minutes with Wright-Giemsa Stain, washed with PBS, dried naturally, sealed with optical resin, and observed by confocal microscope.

Western blot analysis of DPY19L2, SPATA16, SUN5, ZPBP expression
Total proteins of testicular tissue in rats were extracted from tissue and 20 μg proteins were sampled. A 5% concentrated gel and 12% isolated gel were prepared to isolate proteins by SDS-PAGE. DPY19L2, SPATA16, SUN5, ZPBP proteins were transferred to NC membrane, then incubated with 5% skimmed milk powder sealing fluid for 2 hours at room temperature. Rabbit-anti-rat primary antibody DPY19L2, SPATA16, SUN5, ZPBP, (1:500), rabbit-anti-rat primary antibody β-actin (1:1,000) were added and incubated at 4°C overnight. Then the gels were washed 4 times with TBST, then HRP labeled rabbit-anti-rat secondary antibody (1: 5,000) was added and incubated at 37°C for 1 hour and then washed again 4 times with TBST. Color was developed with ECL luminescent solution, protein bands were exposed by gel image analysis system, and images were photographed and quantitatively analyzed. The experiment was repeated three times.

Statistical analysis
All experiments were repeated independently at least three times. One-way analysis of variance (ANOVA) was used to analyze the data, which was expressed as the mean ± standard deviation (S.D.). Statistical significance was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Condition of rats
The rats in the control group had normal diet, normal activity, good spirit, and no obvious abnormality. There were no obvious abnormal changes in the first 3 days of low-dose administration. The rats began to eat less and exercise less on the fourth day. The rats in the high-dose group began to eat less and exercise less on the third day after administration, and showed significant anorexia, sleepiness, and fatigue on the fifth day.

Effects of plant complex sterility agent on reproductive organs
The viscera index of male testis and seminal vesicles in different experimental groups were shown in Figure 1. As shown in Figure 1, the viscera index of male testis and seminal vesicles of male rats in the experimental group with a concentration of 20 mg/kg were significantly lower than those in the control group ($F = 6.49$, $p < 0.01$; $F = 4.81$, $p < 0.01$). The results of the viscera index of testis and seminal vesicle showed that the plant complex sterility agent had an anti-fertility effect, and the effect was positively correlated with the concentration.

HE staining analysis of testicular tissue morphology of rats
The mesenchymal cells in the testicular tissues of the control group showed normal morphology and regular arrangement and were clearly visible. Spermatogenic basilar membrane was continuous and complete (Figure 2A). Mesenchymal cells in the low dose group were still visible, and the basal membrane of spermatogenic tubules began to break, with sparse structure, thinner layers, wider tubule gap, and decreased mesenchymal cells, as shown in Figure 2B. In the high dose group, some of the mesenchymal cells disappeared, most of the basal membrane of spermatogenic tubules broke off, the lumen of spermatogenic tubules was irregular, sper-

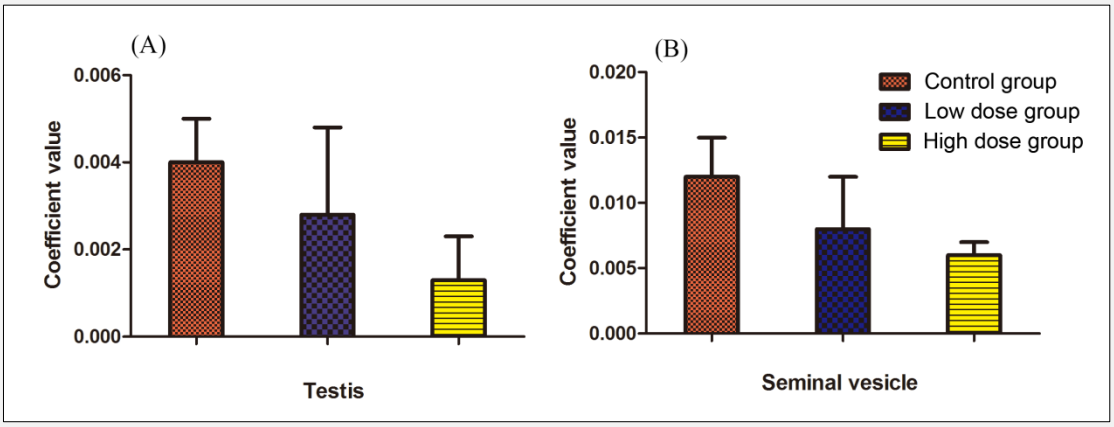

Figure 1. Coefficient values of testis and seminal vesicle in SD male rats of different experimental groups at different concentrations.

(A): Testis; (B): Seminal vesicle.

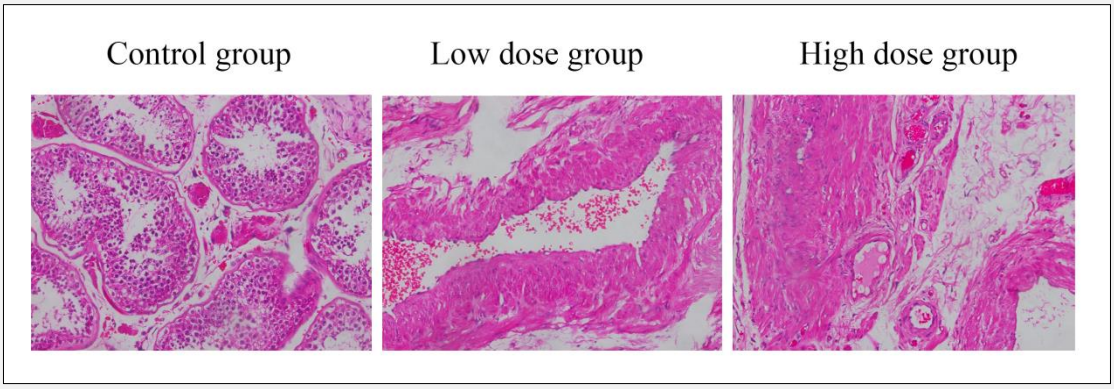

Figure 2. HE staining analysis of testicular tissue morphology of rats x 40.

matogenic cells were arranged in disorder, the layer of spermatogenic epithelial cells decreased and became thinner, and the cell structure was unclear, as shown in Figure 2C.

The content of testosterone in the blood was analyzed by ELISA

A testosterone ELISA kit was used to detect the concentration of testosterone in the serum of rats in each group, and the detection results were shown in Figure 3. Compared with the control group, plant complex sterility agent had an obvious effect on serum testosterone concentration. There were significant statistical differences between the experimental group and the control group, $p < 0.05$.

Sperm count and sperm motility were observed and analyzed

Sperm count

The sperm count in control group was 20.3 x 10^5, the sperm count in low dose plant complex sterility agent was 10.9 x 10^5, and the sperm count in high dose plant

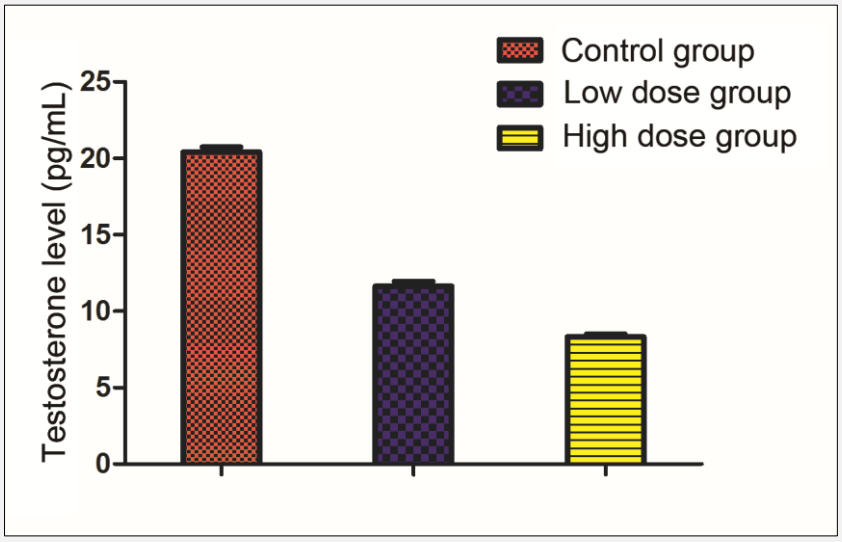

Figure 3. ELISA results of testosterone level in different experimental groups.

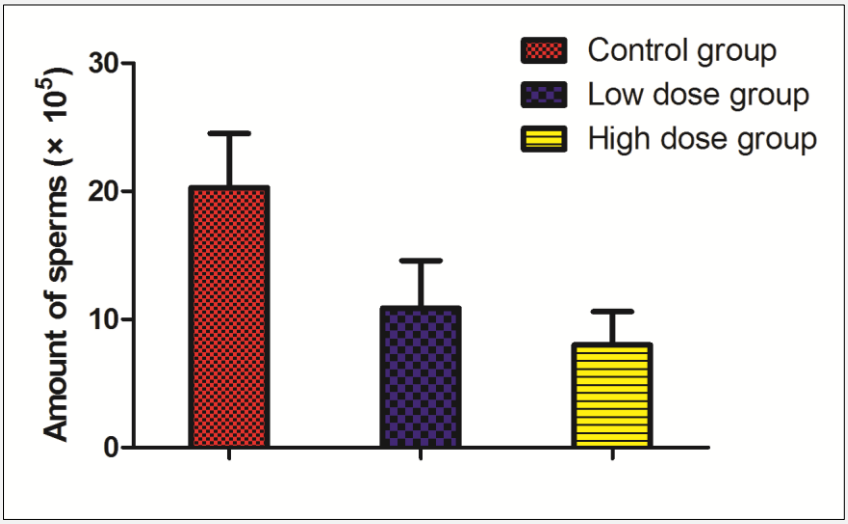

Figure 4. Amount of sperm in different experimental groups.

complex sterility agent was 8.0 x 10^5, which was statistically different from that of the control group ($p < 0.01$).

Sperm motility

As shown in Table 1, high and low dose of plant complex sterility agent can cause decreased sperm motility compared with the control group. Sperm motility of the control group was 86.5%. Sperm motility of the low

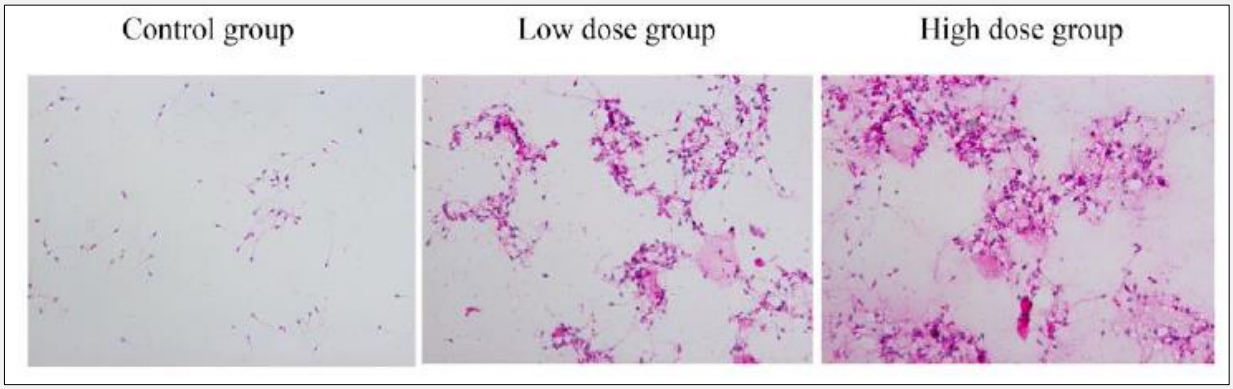

Figure 5. Sperm morphology was determined by confocal microscopy.

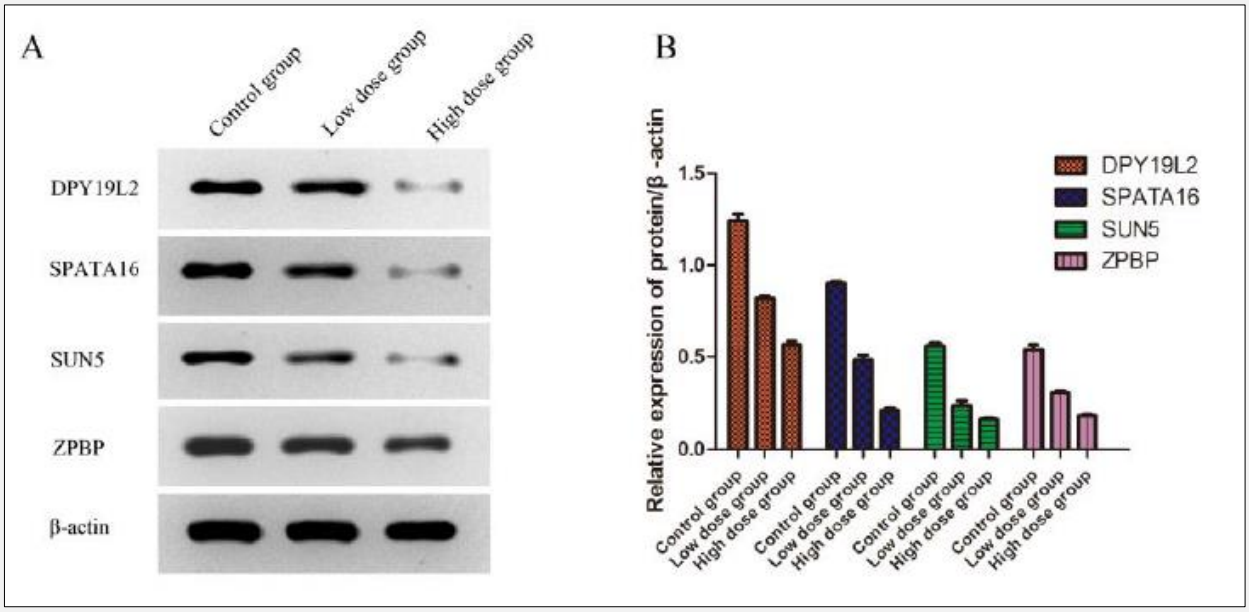

Figure 6. Protein level of DPY19L2, SPATA16, SUN5, ZPBP in different groups determined by Western blotting (mean ± SD, n = 3).

dose group decreased by 12.1% and that of the high dose group decreased by 22.4% compared with the control group.

Sperm morphology

Sperm head morphology and ultrastructure were not changed in the control group, which had good morphology and even distribution. There was no acrosome, and the head nucleus was dark and full. However, sperm in the low dose group were disordered and clustered, with

morphological changes, uneven distribution and deepened color. The most serious cases were severely disordered sperm aggregation in the high dose group, with morphological changes, uneven distribution, and deepened color. Therefore, it can be seen that high dose of plant complex sterility agent can effectively destroy and prevent sperm morphology and maturation.

Western blot analysis of DPY19L2, SPATA16, SUN5, ZPBP expression

The expression of DPY19L2, SPATA16, SUN5, ZPBP in testicular tissue of rat tissue was shown in Figure 6. The expression of protein in the control group was higher than that of low dose group ($p < 0.05$) and much higher than that of the high dose group ($p < 0.01$). The DPY19L2 (dpy-19-like 2) gene is involved in the physiological activities of spermatogenesis, development, and maturation. It is a gene encoding a protein of transmembrane domain, which is interregulated with protein genes SPATA16, SPACA1, SUN5, ZPBP, and GUK1, and co-participates in the process of spermatogenesis and maturation. Deletion of the DPY19L2 gene is the main cause of sterility in rats and is also the cause of clinical round head spermatozoosis. Round head spermatozoa is a condition in which sperm have a rounded head and cannot pass through the zona pellucida to fuse with oocyte due to the lack of acrosome, resulting in sterility [15]. DPY19L2 is a member of DPY19 gene family, consist of 22 exons that encode proteins with the 9 - 11 transmembrane domain [16]. DPY19L2 stabilized the connection between the acrosome anchoring disc and the outer membrane of the nucleus. It would facilitate the connection of the acrosome and sperm caudate to the nucleus, allowing the sperm to be a normal shape [15,17]. In addition to the DPY19L2 gene, there are also SPATA16, PICK1, GOPC, Hrb, and SMAP2 genes. Among them, SPATA16 gene [18] was located at 3q26. It contained 11 exons and encoded a highly conserved protein containing a TPR domain. The TPR domain could regulate protein interactions and assemble multi-protein complexes, so SPATA16 played a key role in the formation of acrosomes. Whether the other two mutations SUN5 and ZPBP are also involved in sperm activity and development needs further study.

CONCLUSION

Plant complex sterility agent with high concentration can effectively target the rat sperm gene DPY19L2 and realize effective sterility control of rat pests.

Funding:
Key Project of North Minzu University (No. 2019KJ 23).

Declaration of Interest:
All authors declared no conflict of interest related to the study.

References:

1. Zhang ZB. The ecological fundamentals of rodent control by contraception. Acta Theriologica Sinica 1995;15(3):229-34. http://en.cnki.com.cn/Article_en/CJFDTOTAL-SLXX503.010.htm

2. Bos PMJ, Gosens I, Geraets L, Delmaar C, Cassee FR. Pulmonary toxicity in rats following inhalation exposure to poorly soluble particles: The issue of impaired clearance and the relevance for human health hazard and risk assessment. Regul Toxicol Pharmacol 2019;109:104498 (PMID: 31604110).

3. Jacob J, Rahmini J, Sudarmaji J. The impact of imposed female sterility on field populations of rice-field rats (Rattus argentiventer). Agriculture Ecosystems & Environment. 2006;115(1): 281-4. https://doi.org/10.1016/j.agee.2006.01.001

4. Tran TT, Hinds LA. Fertility control of rodent pests: a review of the inhibitory effects of plant extracts on ovarian function. Pest Manag Sci 2013;69:342-54 (PMID: 22753347).

5. Mandal R, Dhaliwal PK. Antifertility effect of Melia azedarach Linn. (dharek) seed extract in female albino rats. Indian J Exp Biol 2007;45:853-60 (PMID: 17948733).

6. Knipling EF, McGuire JU. Potential role of sterilization for suppressing rat population: A theoretical appraisal. Technology Bull Agriculture Resources Service, US Department Agriculture 1972; 2(3):1455. http://ageconsearch.umn.edu/record/171904/files/tb1455.pdf

7. Buckland A, Nattrass N. Understanding Preferences for Humane and Cruel Treatment of Pest Rodents in Site C, Khayelitsha, South Africa. J Appl Anim Welf Sci 2019 (PMID: 31524532).

8. Kashkouli M, Fathipour Y, Mehrabadi M. Potential Management Tactics for Pistachio Stink Bugs, Brachynema germari, Acrosternum heegeri and Acrosternum arabicum (Hemiptera: Pentatomidae): High Temperature and Chemical Surface Sterilants Leading to Symbiont Suppression. J Econ Entomol 2019;112: 244-54 (PMID: 30329064).

9. Razavi S, Khadivi F, Hashemi F, Bakhtiari A. Effect of Zinc on Spermatogenesis and Sperm Chromatin Condensation in Bleomycin, Etoposide, Cisplatin Treated Rats. Cell J 2019;20:521-6 (PMID: 30123998).

10. Meng SL, Liu T, Song C. Construction of Suppression Subtractive Hybridization Library for Testis of Male Tilapia Under the Stress of Methomyl. Agricultural Biotechnology. 2019;8(3):73-7. http://www.cnki.com.cn/Article/CJFDTotal-AGBT201903019.htm

11. Kim N, Kim J, Yoo C, Lim K, Akimoto T, Park J. Effect of acute mid-intensity treadmill exercise on the androgen hormone level and uncoupling protein-1 expression in brown fat tissue of mouse. J Exerc Nutrition Biochem 2018;22:15-21 (PMID: 29673 241).

12. Cai XL, Guo H, Li F. The effect of Mangamese on morphology of sperm in rats and its significance. Acta Academiae Medicinae Zunyi. 2009;32(6):551-3. http://www.en.cnki.com.cn/Article_en/CJFDTOTAL-ZYYB200906006.htm

13. Shi WX, Ou CL, Lu FL, et al. Chronotoxicology of manganese on sperm quality and reproductive hormones in male rats. Modern Preventive Medicine. 2018;45(11):2039-46. http://en.cnki.com.cn/Article_en/CJFDTotal-XDYF201811028.htm

14. Santiago J, Silva JV, Alves MG, Oliveira PF, Fardilha M. Testicular Aging: An Overview of Ultrastructural, Cellular, and Molecular Alterations. J Gerontol A Biol Sci Med Sci 2019;74:860-71 (PMID: 29688289).

15. Harbuz R, Zouari R, Pierre V, et al. A recurrent deletion of DPY19L2 causes infertility in man by blocking sperm head elongation and acrosome formation. Am J Hum Genet 2011;88:351-61 (PMID: 21397064).

16. Alvarez Sedo C, Rawe VY, Chemes HE. Acrosomal biogenesis in human globozoospermia: immunocytochemical, ultrastructural and proteomic studies. Hum Reprod 2012;27:1912-21 (PMID: 22511613).

17. Pierre V, Martinez G, Coutton C, et al. Absence of Dpy19l2, a new inner nuclear membrane protein, causes globozoospermia in mice by preventing the anchoring of the acrosome to the nucleus. Development 2012;139:2955-65 (PMID: 22764053).

18. Dam AH, Koscinski I, Kremer JA, et al. Homozygous mutation in SPATA16 is associated with male infertility in human globozoospermia. Am J Hum Genet 2007;81:813-20 (PMID: 17847006).