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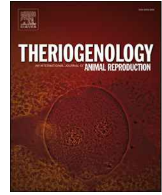

High efficient and non-invasive collection of ejaculates from rats using penile vibratory stimulation

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A B S T R A C T

Background: The rat is one of the most important experimental animals, which plays an indispensable role in biomedical research, particularly in reproduction. However, according to our best knowledge, there is no easy and efficient method available for semen collection from rats.
Results: In this study, we successfully collected semen through penile vibratory stimulation ejaculation (PVSE) from laboratory rats. This is an easier and more efficient method compared with rectal probe electro-ejaculation (RPE). We found that the ejaculation rate, volume, concentration and motility of semen collected with PVSE were substantially better than those of RPE. Although PVSE was time-consuming compared to RPE, the quality of semen was better; additionally, sperm concentration and motility of semen were significantly higher with a two-day interval between collections compared to a five-day interval. Moreover, we found that electrical stimulation, use of anesthesia and increased age of rats have a negative effect on sperm quality. In the last experiment, four fertile female rats were artificially inseminated with PVSE-collected semen, and healthy offspring were born.
Conclusion: Here, for the first time, we established the repeated collection of semen using the PVSE method in rats.

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1. Introduction

Rats have many valuable features that highlight their potential as experimental animals in biomedical research, including their genetic, anatomical, and biomechanical similarities with humans [1]. Historically, the first attempt to investigate the reproductive biology of rats was described by Hewer in 1914 [2]. Recently, sperm collection, *in vitro* fertilization, embryo transplantation and gene knock-out in rats have been successfully reported [3–5]. The demand for a genetically modified rat model for biomedical and genetic research has been increasing exponentially [6,7]; however, the purchasing and transportation of genetically modified rats are very expensive.

It is well established that much time and energy has been spent on exploring female reproductive issues whereas male issues have not received much attention [8].Sperm plays an irreplaceable role in assisted reproductive techniques [9].Sperm collection via an artificial vagina, squeezing or slicing of the epididymis, and electro-ejaculation are the most commonly used methods in small animals [10,11].However, the application of these methods in rats is limited. The vaginal washing method is time consuming and tedious [12]. The epididymal sperm collection method is costly, and the same animal cannot be used for the next sperm collection [13]. Rectal probe electro-ejaculation (RPE) is a potential sperm collection method, but the quality of sperm significantly decreases due to use of electro-stimulation and anesthesia, which limits its application [14].Therefore, an efficient and repeatable sperm collection method from rats is still needed.

Recently, pharmacologically induced ejaculation has been successfully reported in different species, such as stallions [15]. However, this technique is not successful in rats yet. Therefore, it is very

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important to introduce some new valuable and possible sperm collection methods for rats. Currently, penile vibratory stimulation ejaculation (PVSE) method has been reported as a repeatable noninvasive sperm collection method in marmoset monkeys [16]. It has been reported that the PVSE method is superior compared to other methods because it produced significantly higher semen samples with high sperm motility [17]. Additionally, successful application of PVSE has been reported in patients with spinal cord injuries [18]. However, the application of PVSE in rats has not yet been reported. Therefore, the current study was designed to explore the application of the PVSE method in rats and its effect on sperm quantity, quality and motility.

2. Materials and methods

2.1. Animals

A total of 50 fertile adult male rats (age 18 or 60 weeks) and four adult females (*Rattus norvegicus*) were obtained from a breeding colony at Southern Medical University Center. All animals were kept in individual cages (320 mm × 180 mm × 160 mm) with controlled temperature (20–25 °C) and humidity (40–60%), a cycle of 12 h light and 12 h darkness, and free access to water and food. Before the start of the experiment, sperm quality of all animals was measured, and then the animals were randomly divided into different experiment groups. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of South China Agricultural University. The protocol was approved by the Committee of the University on the Ethics of Animal Use in Experiments (protocol approved number: 2016019).

Protocol: 1. Sodium pentobarbital was injected intramuscularly only for the RPE method. 2. Animals were placed in a homemade holder. 3. The prepuce area was cleaned. 4. To arouse erection, external genitals were mildly rubbed for 1 min with wetted cotton ball. 5. Semen was collected with the VSE and RPE methods. 6. Semen was collected into 1.5 mL tubes and evaluated.

Experimental studies were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Anesthesia was used only for experiments 1 and 2.

2.2. Media preparation

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise indicated. The sperm diluent extender referred to as HTF contained NaCl 101.6 mM, KCl 4.70 mM, $CaCl_2$ 5.14 mM, KH_2PO_4 0.40 mM, $MgSO_4$ 0.20 mM, $NaHCO_3$ 25.0 mM, Sodium lactate 18.36 mM, Sodium pyruvate 0.34 mM, Glucose 2.78 mM, BSA (Fraction V) 4.0 mg/mL, Streptomycin sulfate 50 μg/mL, and Potassium penicillin 75 μg/mL, according to the method described by Aoto et al. [15]. All the extenders were kept in a 37 °C water bath before use.

2.3. Electrode used for Experiment 2

Homemade electrodes consisted of two 1.5 v batteries and a battery case lined with two sheets of aluminum.

2.4. Rectal probe electro-ejaculation

Rectal probe electro-ejaculation was performed via intramuscular injection of sodium pentobarbital anesthesia at a dose of 30–50 mg per kg of body weight. The electro-ejaculator (model AC-1, Beltron Instruments, Longmont, CO) with a custom probe with a diameter of 7.5 mm was used. The probe was lubricated with water-based non-spermicidal lubricant and inserted into the rectum at the level of the prepuce with the electrodes facing ventrally. Proper placement was verified by palpating the inguinal area. After the probe was inserted into its hub, the current was increased incrementally in a pulsatile fashion until an ejaculate was obtained or a maximum parameter was attained [19].

2.5. Penile vibratory stimulation ejaculation

Penile vibratory stimulation ejaculation was performed using a battery-operated FertiCareR personal vibrator (Multicept, Rungsted, Denmark). The basic modifications were similar to those described by Kuederling et al. [16]. Additionally, the plastic collecting 1.5 mL-EP-tube attached to the silicon rubber holder was 20 mm long with 5 mm internal diameter. This provided a better contact with the target and was more effective in achieving the required level of stimulation. Animals were placed in a homemade holder, as shown in Fig. 1a and b. Before we carried out sperm collection, the penis was gently washed with warmed saline by using sterile cotton. To arouse erection, external genitals were mildly rubbed for 1 min with wetted cotton ball. Then, the vibrating tube was placed on the genitals. Vibration parameters were set as follows: from an initial stimulation at 80 Hz and two mm amplitude, intensity was increased in 1 min. This stimulation protocol was repeated once (following a 2–3 min rest period) until ejaculation was completed. As an indicator of adequate stimulation, a rhythmic contraction and expansion of the penis occurred prior to ejaculation. The ejaculate could be seen as a clear droplet on the urogenital orifice, as show in Fig. 1c and d.

2.6. Semen evaluation assays

Semen samples collected with the RPE and PSE methodswere kept at 37 °C for 30 min to allow for liquefication. A 10-*μL*sperm sample was taken to examine sperm motility and acrosomal integrity as a non-frozen control. The motility of each sperm sample was evaluated by counting with an erythro-cytometer. A 5 μL sample of 100xdiluted semen with HTF was placed on the erythrocytometer and sperm cells were counted under light microscopy (Motic BA2000, Shanghai, China). Motility was assessed by two qualified technicians individually. Thawed sperm motility was evaluated immediately after thawing and washing. To minimize evaluator bias, the evaluator was blinded as to the identity of the samples.

2.7. Experiment 1: Comparison of ejaculation characteristics between the PVSE and RPE methods in mature rats

In this study, we sought to compare whether the PVSE method is better than the RPE method. Ten sexually mature male rats were randomly divided into two groups for PVSE and RPE. Successful ejaculation and the time taken for each rat were recorded. Semen samples were collected in 2-mL-EP tubes with 495 μLHTF. Thus, semen was incubated in a 37 °C in water-bath for 10 min before evaluating semen volume, concentration, motility and malformation.

2.8. Experiment 2: Effect of electrical stimulation and anesthesia on sperm motility

To determine the effect of electrical stimulation and anesthesia on sperm motility and to avoid semen contamination with urine during the process of ejaculation, 10 epididymal sperm samples were collected using a flushing method and incubated at 37 °C for 30 min, after which,5 mL semen samples were transferred into

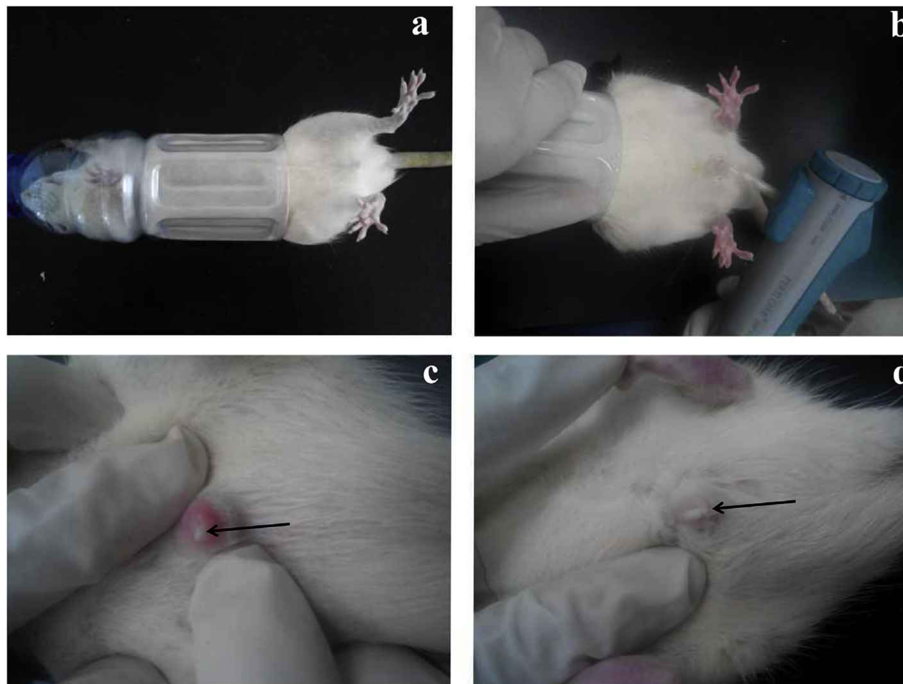

Fig. 1. The photographs showing the processing of penile vibratory stimulation method.
a. Rat was held in a self-made bottle.
b. Rat was stimulated by PVSE.
c and d. Ejaculation occurred after the PVSE protocol. Arrows showed the semen.

9-cm-diameter glass petri dishes and placed at 37 °C. Positive and negative poles were inserted under the surface of the semen samples1 cm from the edge of petri dish on a straight line which crossed the center of the petri dish. Five micro liter semen samples were taken from the positive and negative poles and the center of the petri dish, at 1 min, 3 min, and 5 min after electrical stimulation, respectively. Sperm motility and malformation were measured according to the methods mentioned above.

We added 5 μL and 10 μL pentobarbital sodium (Sigma Co, St. Louis, USA) solution (1%) which was diluted ten times into two 2-mL-EP tubescontaining1 mL semen samples. After incubation at 37 °C for 5 min, sperm motility and malformation were measured according to the method mentioned above.

2.9. Experiment 3: Comparison of ejaculation characteristics between young and old male rats using PVSE

In this study, we employed ten young (18 weeks) and ten old (60 weeks) male rats to collect semen with the PVSE method. The details about total time, ejaculation, volume, concentration, motility and malformation were measured as mentioned above.

2.10. Experiment 4: Comparison of ejaculation characteristics between two-day and five-day intervals using PVSE

In this study, we employed 20 mature male rats for PVSE, repeatedly collecting semen at two-day intervals and another ten rats at five-day intervals. On the better interval, we selected 3 elite rats with the PVSE method for repetitively collecting semen for 3 months. The details about total time, ejaculation, volume, concentration, motility and malformation were measured as mentioned above.

2.11. Experiment 5: Artificial insemination

The basic protocol was similar to that described by Nakata et al. [20]. Fertile female rats were selected as recipients and mated with vasectomized male rats to induce pseudo-pregnancy. After inducing pseudo-pregnancy, insemination was done within 1 h by using nonsurgical artificial insemination method. An otologic speculum (Welch Allyn, New York, USA) illuminated with a lamp was used to visualize the exterior of the cervix. A square-tipped, blunted, 22-gauge needle (length 8 cm) with a 1-mL-plastic syringe (Pingan, Hunan, China) was used to inseminate. The insertion of the needle was less than 2 cm beyond the vaginal end of the speculum, and semen from PVSE was pushed into cervix.

2.12. Statistical analysis

Data are presented as the means ± sem. Statistical differences in time to ejaculation, volume, sperm density, sperm motility and malformation rate between the two groups were determined by the independent simple *t*-test. A *P*-value <0.05 was regarded as statistically significant. The statistical analysis was performed using SPSS (ver. 13.0 for Windows; SPSS, Chicago, IL).

3. Results

3.1. Experiment 1: Comparison of ejaculation characteristics between the PVSE and RPE methods in mature rats

The ejaculation rate, time taken, seminal volume, concentration, motility and malformation of sperm from PVSE and RPE are shown in Table 1. All the animals underwent penile vibratory stimulation ejaculation or rectal probe electro-ejaculation once. The successful ejaculation rate was slightly high with PVSE compared with that of the RPE method, although the difference was not significant

Table 1
Comparison of ejaculating characters between PVSE and RPE method in mature rats (n = 10).

Method	First ejaculation rate*	Motility (%)	Malformation (%)	Time-consuming(s)	Volume (mg)	Concentration (10^6/ml)
RPE	40% (2/5)	40.9 ± 4.3^a	21.0 ± 1.3^a	71.0 ± 12.0^a	8.0 ± 1.1^a	87.3 ± 8.8^a
PVSE	60% (3/5)	60.6 ± 3.1^b	16.6 ± 1.4^b	236.2 ± 25.2^b	13.6 ± 1.9^b	106.8 ± 18.2^a

*successful ejaculation/used animals.
Values are means ± SEM.
Values within a column with different superscripts are significantly different (P < 0.0.5).

(P > 0.05). Ejaculation time with PVSE was significantly higher (P < 0.01) compared with RPE. The total semen volume and sperm concentration with PVSE was significantly (P < 0.05) higher than that of RPE. Sperm concentration was higher with PVSE compared with RPE, although the difference was not significant (P > 0.05). Furthermore, we found that PVSE resulted in an increase in total sperm motility and malformation compared with the RPE method (P > 0.05) (Table 1).

3.2. Experiment 2: Effects of electrical stimulation and anesthetic treatments on sperm quality

The effects of electrical stimulation on sperm motility are shown in Fig. 2a. The sperm motility of the control group did not change at different times (P > 0.05). However, the sperm motility was significantly decreased with 1 min electrical stimulation, and it further decreased with increased time (P < 0.01). Moreover, the sperm motility was similar at the center of the petri dish and the negative pole side, while it was substantially decreased at the positive pole.

To explore the effect of anesthesia on sperm motility, we added pentobarbital sodium at different doses to semen. The motility of sperm significantly (P < 0.01) decreased with the addition of anesthesia compared with the control group. Similarly, sperm motility decreased with increased pentobarbital sodium concentration, but the difference was not significant (P > 0.05) (Fig. 2b).

3.3. Experiment 3: Comparison of ejaculation characteristics between young and old male rats using PVSE

As show in Table 2, 7/10 young males rats (aged 18 weeks) were ejaculated with PVSE; the ejaculation time, seminal volume and sperm concentration were 152.0 ± 24.3 s, 12.0 ± 1.9 mg and 103.8 ± 19.4106/mL, respectively. All ten old male rats (aged 60 weeks) failed to ejaculate with the PVSE method.

3.4. Experiment 4: Comparison on different intervals of PVSE

In this study, we hypothesized that sperm quality, quantity and concentration may be influenced by repetitive ejaculation frequency. Surprisingly, we found that volume of semen was high at five-day ejaculation intervals, but the concentration of semen decreased (Table 2). Similarly, there was higher sperm motility at two-day intervals as compared with five-day intervals (Table 2). Interestingly, the ejaculation rate and sperm motility among semen collected in August were lower than those collected in September and October, but the concentration of semen collected with PVSE were stable among the three months (Table 3).

3.5. Experiment 5: Artificial insemination

In this experiment, we determined the use of semen collected with PVSE in artificial insemination. Four fertile female rats were inseminated with semen from PVSE using a nonsurgical artificial insemination method. After 22 days, 2 recipient female rats successfully gave birth to 15 offspring.

4. Discussion

Siosteen was the first person to report penile vibratory stimulation ejaculation method to collect semen from spinal-cord injury patients [21].PVSE has also been successfully reported in common marmosets [16]. However, this technique had not yet been reported in rats. Here, for the first time, we report PVSE in rats as an alternative to RPE. Our results show that PVSE is a highly efficient, noninvasive and simple method for collecting semen in rats, as shown in the recorded video. Moreover, it provides substantially higher quality motile sperm compared with those collected with the RPE method. Furthermore, our findings indicate that the sperm collected with the PVSE method was safe from the adverse effects of electrical stimulation and anesthetic agents. In this experiment,

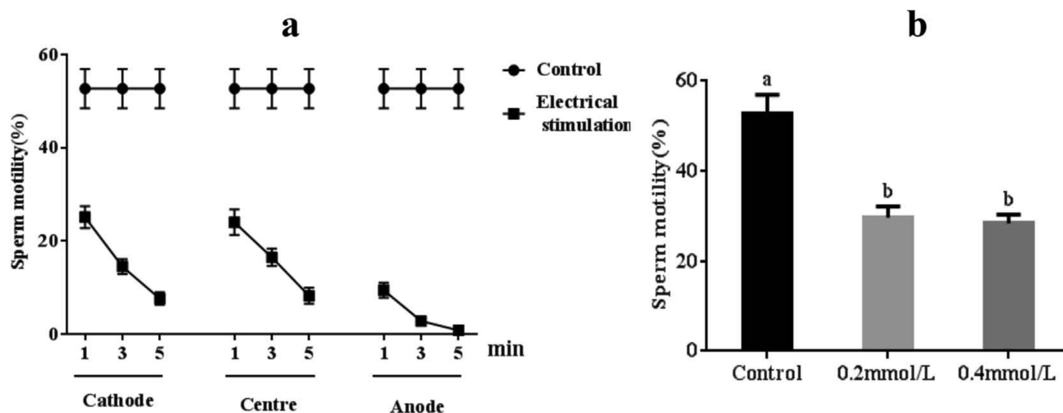

Fig. 2. Effect of electrical stimulation and anesthesia on sperm motility.
a. The effect of different electrode and stimulation time on the motility of rat sperm. Values with different letters are significantly different (P < 0.05).
b. The effect of different molar concentration of pentobarbital sodium on the motility of rat sperm culturing for 3 min in vitro.

Table 2
Comparison of ejaculating characters between two-day and five-day intervals using PVSE in young male rats (n = 20).

Interval	Ejaculation rate*	Motility (%)	Malformation (%)	Time-consuming (s)	Volume (mg)	Concentration (10^6/mL)
Two days	70 (7/10)	60.5 ± 3.7^a	19.3 ± 2.2^a	169.1 ± 13.0^a	8.74 ± 1.2^a	103.8 ± 19.4^a
Five days	50 (5/10)	43.4 ± 5.6^b	30.4 ± 3.7^b	255.5 ± 32.8^b	16.09 ± 4.0^b	37.2 ± 8.4^b

*Successful ejaculation/attempt.
Values are means ± SEM.
Values within a column with different superscripts are significantly different (P < 0.0.5).

Table 3
Characteristics of PVSE ejaculating with two-days interval for continuously three months in mature rats (n = 3).

Month	Ejaculation rate*	Motility (%)	Time-consuming (s)	Concentration (10^6/mL)
August	46.7% (14/30)	51.2 ± 9.1^a	262.1 ± 30.7^a	105.6 ± 7.9^a
September	63.3% (19/30)	53.5 ± 15.2^a	154.6 ± 19.5^b	98.4 ± 12.2^a
October	60.0% (18/30)	56.3 ± 12.6^a	161.3 ± 14.2^b	103.3 ± 17.5^a

*Successful ejaculation/attempt.
Values are means ± SEM.
Values within a column with different superscripts are significantly different (P < 0.0.5).

sperm concentration decreased with an increased interval between ejaculations, contrary to what we expected. A potential reason sperm concentration decreased with an increased interval between ejaculations might be that rats are more adapted to short intervals and can therefore produce a higher concentration of semen. Another hypothesis is that the experiments were not repeated enough. Young male rats can have semen repeatedly collected at less than three months of age without pain and with high sperm quality. Finally, we show that sperm collected with the PVSE method can be used for artificial insemination. The substantially high quality semen achieved with PVSE may relate to a more physiologic stimulation involving the natural reflexive sequence of emission and ejaculation. In this experiment, we took all precautionary measures to minimize the stress on the experimental animal, for example, we used a special plastic bottle to restrain the rat. The design of the restraint bottle fulfilled the drill holes habit of rats, and the bottle was opened from the front to ensure proper breathing during the sperm collection process.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.theriogenology.2017.10.024.

One of the important advantages of PVSE is the potential for regular semen collection. Currently, most researcherspreferto collect and use epididymal sperm of rats, but the drawback is that one cannot use the same animal the next time because it must be killed for epididymal sperm collection [22]. Recently, frozen-thawing of rat sperm has been successfully reported, but sperm quality significantly decreases after recovery, which limits its uses [23]. Therefore, the present study explores the potential of PVSE for regular semen collection with a convenient protocol requiring no anesthesia or recovery time [12].

In this study, we recruited sexually active male rats for semen collection in a crossover paradigm, and our results showed that quality, density and motility of sperm collected with PVSE were significantly better than those of RPE. This high quality of semen from PVSE was due to higher concentration of spermatozoa, larger volume, higher density, higher motility grade and lower level of sperm malformation than those of sperm collected with the RPE method. Another most important advantage is that PVSE is done without electrical stimulation and anesthesia. Therefore, no pain is caused to the animal. In contrast, RPE is done via the rectum with electric stimulation under anesthesia, which causes stress to the animal. We found that quality of sperm decreased with the use of anesthesia and electrical stimulation because anesthesia can depress afferent neural responsiveness, which is important for the ejaculatory reflex. Therefore, using electric stimulation and anesthesia may affect the quality of sperm during artificial collection processing.

After successful high quality semen collection, we designed another experiment to determine whether this semen can lead to conception. We recruited four fertile female rats and inseminated them using nonsurgical artificial insemination. Two female rats were successfully impregnated and gave birth to fifteen offspring. In this study, the pregnancy rate was 50%, which is lower than previously reported [20]. However, it is similar to natural mating results. Therefore, it is likely that the sperm collected by PVSE have normal fertility. There have been few reports of artificial insemination in rats, maybe due to the lack of studies reporting repeated semen collection. Artificial insemination technique was first reported in mice by several researchers in the 1960s [24]. Another route of insemination in mice has been successfully reported in which semen is dropped into a space near the infundibulum between the ovary and ovarian bursa, and fertilization occurred at the ampulla [25]. Transgenic and mutant mice, which are normally very difficult to breed [26], have been successfully produced with this technique. Nonsurgical artificial insemination has been established in large domestic animals. It is a desirable technology for livestock, as it is cheap and less risky for the recipients [27]. However, the application of both nonsurgical artificial insemination and traditional artificial insemination is still not clear in rats. Moreover, the cryopreservation of rat semen is still at the experimental stage, and cryopreservation has not yet been used for resource conservation. Detailed experimental research is needed to improve and complete the cryopreservation of rat semen.

In conclusion, the PVSE method in rats produces high quality motile sperm and can potentially facilitate studies involving artificial insemination, *in vitro* insemination, and spermatozoa cryopreservation in this species. In this study, we showed the application of PVSE for regular semen collection with high speed. We also demonstrated that live young can be produced from rat sperm obtained by PVSE with traditional nonsurgical insemination.

Authors' contributions

Xiaoxing Liu, Zulqarain baloch, Senren Xue, Ge Wang, and Sien Qiu had done the experiments. Zulqurain Baloch, Xiaoxing Liu, took part in the data collection and analyzed the data and wrote the paper. Prof. Shihua Yang and Qunshan Huang designed and supervised the study. All of the authors read and approved the final

manuscript.

Competing interests

All authors declare that they have no actual or potential competing interests.

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