

DEVELOPMENT OF A NON-TERMINAL TECHNIQUE FOR SPERM COLLECTION IN WISTAR RATS TO STUDY THE INFLUENCE OF ENVIRONMENTAL FACTORS ON REPRODUCTION

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ABSTRACT

Background. In more than 30 % of cases, infertility is attributed to male factors. The diagnosis of male infertility is based on semen analysis. The assessed semen parameters include sperm concentration, motility, and morphology. Laboratory animal models enable the study of various biological processes, including the effects of external and internal factors on reproduction processes. However, to date, there is no simple, safe, non-terminal method for sperm collection in animals.

Aim. To develop a non-terminal sperm collection technique using optimal electrical stimulation parameters in Wistar rats to study the influence of environmental factors on the reproductive function.

Materials and methods. The study was performed on 30 male Wistar rats weighing 280–300 g. For sperm collection, a rectal electrical stimulator was used to obtain ejaculate. A hemocytometer and two types of microscopes were used for sperm analysis and counting: a Micromed P-1 LED (Russia) and a Levenhuk D90L LCD digital microscope (China).

Results. All animals were divided into 6 groups of five individuals each, according to the electrical stimulation protocol. All stimulation protocols resulted in erection in all groups of animals. However, the safest and most cell-saving method was found to be rectal electrical stimulation of the seminal colliculus of rats at settings of 60 Hz and 4 V.

Conclusion. A method for sperm collection in Wistar rats with an optimized electrical stimulation protocol has been developed, enabling the study of various factors affecting male fertility.

Keywords: non-terminal ejaculate collection, rectal electrostimulator, optimal electrical stimulation parameters, sperm count and morphology, environmental factors, reproduction

Received: 30.01.2026

Accepted: 13.07.2026

Published: 23.07.2026

For citation: Khasanshina Z.R., Bogacheva N.V. Development of a non-terminal technique for sperm collection in Wistar rats to study the influence of environmental factors on reproduction. *Acta biomedica scientifica*. 2026; 11(3): 182-191. doi: 10.29413/ABS.2026-11.3.22

РАЗРАБОТКА МЕТОДИКИ ПРИЖИЗНЕННОГО ПОЛУЧЕНИЯ СПЕРМАТОЗОИДОВ У КРЫС WISTAR ДЛЯ ИЗУЧЕНИЯ ВЛИЯНИЯ ФАКТОРОВ ВНЕШНЕЙ СРЕДЫ НА РЕПРОДУКЦИЮ

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РЕЗЮМЕ

Более чем в 30 % случаев проблема фертильности обусловлена мужскими факторами бесплодия. Диагностика мужского бесплодия основана на анализе эякулята (спермы). К исследуемым параметрам спермы относятся: концентрация и подвижность сперматозоидов, а также их морфология. Модели лабораторных животных позволяют изучать различные биологические процессы, включая воздействие внешних и внутренних факторов на процессы репродукции. Однако до настоящего времени отсутствует простой, безопасный клеточно-сберегающий способ получения сперматозоидов у животных.

Цель. Разработать методику прижизненного получения сперматозоидов с использованием оптимальных параметров электростимуляции у крыс Wistar для изучения влияния факторов внешней среды на репродуктивную способность животных.

Материалы и методы. Исследование выполнено на 30 крысах-самцах Wistar массой 280–300 г.

Для получения сперматозоидов использовали ректальный электростимулятор для получения эякулята у животных. Для анализа и подсчета сперматозоидов использовали камеру Горяева и микроскопы 2 видов: «Микромед Р-1 LED», Россия; цифровой микроскоп «Levenhuk D90L LCD», Китай.

Результаты. Все животные были поделены на 6 групп по пять особей в каждой в зависимости от режима электростимуляции. Все используемые режимы электростимуляции позволяли добиться эрекции во всех группах животных. Однако было установлено, что наиболее безопасным и клеточно-сберегающим способом получения сперматозоидов является ректальная электростимуляция семенных бугорков крыс при показателях электрического тока 60 Гц и 4 В.

Вывод. Разработан метод получения сперматозоидов у крыс Wistar с обоснованным режимом электростимуляции, позволяющим изучать влияние различных факторов на мужской фактор фертильности.

Ключевые слова: прижизненное получение эякулята, ректальный электростимулятор, оптимальные параметры электростимуляции животных, количество и морфология сперматозоидов, факторы внешней среды, репродукция

Статья поступила: 30.01.2026
Статья принята: 13.07.2026
Статья опубликована: 23.07.2026

Для цитирования: Хасаншина З.Р., Богачева Н.В. Разработка методики прижизненного получения сперматозоидов у крыс Wistar для изучения влияния факторов внешней среды на репродукцию. *Acta biomedica scientifica*. 2026; 11(3): 182–191. doi: 10.29413/ABS.2026-11.3.22

INTRODUCTION

Currently, the problem of fertility impairment is a major medical and social issue that is prevalent in society. According to the latest statistics from the World Health Organization, 50 to 80 million people worldwide suffer from infertility [1, 2].

Infertility is defined as a reproductive system disorder characterized by the inability to achieve clinical pregnancy after 12 or more months of regular unprotected sexual intercourse [3]. It has been established that one in six individuals worldwide is affected by infertility [4]. Fertility problems are attributed to female factors in 50 % of cases, to male factors in more than 30 %, and to combined disorders in both partners in approximately 20 % of cases [5, 6].

Among the causes of male infertility, the most important include urogenital and endocrine disorders, immunological and genetic defects, lifestyle factors (smoking, alcohol and drug use), as well as adverse environmental factors [7–10].

The diagnosis of male infertility is based on ejaculate (semen) analysis. The assessed semen parameters include sperm concentration, motility, and morphology [11, 12]. Laboratory animal models enable the study of various biological processes, the reproduction of specific pathological conditions, the assessment of the safety of pharmaceutical agents on quantitative and qualitative sperm parameters, as well as the efficacy of novel drugs developed to enhance reproductive function [13].

Among the existing methods for obtaining ejaculate in animals, the following are used: testicular tissue dissection [14], sperm retrieval from the cauda epididymidis [15], and sperm extraction from testicular tissue [16]. The aforementioned techniques are applicable only to deceased animals or following euthanasia. These methods are not suitable for the non-terminal collection of germ cells and cannot be used to evaluate ejaculate parameters dynamically in response to environmental factors.

Among the promising methods for experimental animal studies when natural semen collection is not feasible, electroejaculation should be considered [17]. The advantages of electroejaculation include the collection of maximum ejaculate volume with minimal cell damage. Furthermore, this technique allows the study of the effects of various agents on the male reproductive system in rats dynamically, without the need for euthanasia. Despite the existence of numerous modifications of the seminal tubercle electrostimulation method [18, 19], to date, no standardized stimulation protocol (frequency, voltage, current intensity, and duration of exposure) has been developed, which justifies the relevance of developing a step-by-step method for sperm collection in male rats.

AIM

To develop a non-terminal sperm collection technique using optimal electrical stimulation parameters

in Wistar rats to study the influence of environmental factors on the reproductive function.

MATERIALS AND METHODS

The study was performed on 30 male Wistar rats weighing 280–300 g. All experiments were conducted in accordance with the Interstate Standard GOST 33215–2014 “Guidelines for the Care and Maintenance of Laboratory Animals. Rules for Equipping Premises and Organizing Procedures” and GOST 33216–2014 “Guidelines for the Care and Maintenance of Laboratory Animals. Rules for Conducting Procedures Using Animals”. All animals were housed at the Medical and Biological Center for Biomodeling of the Kirov State Medical University under identical standard conditions, in cages measuring 25 × 19 × 40 cm, one animal per cage, with free access to food and water, on a 12-hour light cycle (light on from 8:00 AM to 8:00 PM, and off from 8:00 PM to 8:00 AM). All rats underwent a two-week adaptation period prior to the experiment. Experimental manipulations were performed at the same time each day, at 9:00 AM. The experiment was approved by the local ethics committee of the Kirov State Medical University (Protocol No. 16/2022 dated October 24, 2022).

For sperm collection, a rectal electrical stimulator for animal ejaculate collection was used [20]. Male rats were divided into six groups of five animals each. Group allocation was based on the electrical stimulation parameters (frequency, current intensity, and voltage).

Prior to the procedure, animals were anesthetized by inhalation anesthesia using medical ether (Kuzbas-sorgkhim LLC, Russia). The animal was placed under a 3-L glass jar containing cotton wool soaked in ether and kept there until immobilization was achieved. The onset of anesthesia was determined by the loss of motor activity and decreased muscle tone. To confirm the absence of pain sensitivity and distress during electrical stimulation, light pressure was applied to the paws and skin: muscle relaxation, absence of protective reflexes, and lack of resistance indicated the animal was ready for the procedure. Following anesthesia, the animal was placed in the supine position and secured in a restrainer for safe manipulation. Fixation ensured a stable body position, prevented injury, and allowed accurate insertion of the rectal electrical stimulator. Next, the rectum was emptied by palpation. The anal orifice and the rectal probe were lubricated with vaseline oil and inserted into the rectum to a depth of 2.5–3 cm (Fig. 1).

After confirming that the probe was fully inserted into the animal's anal orifice, the electrical stimulator, pre-set to the required parameters, was switched on. Erection served as evidence of stimulation of the seminal colliculus. In its absence, the rectal probe was moved within the rectum, pressing against its anterior wall until erection was achieved. Electrical stimulation of male rats was continued until erection and ejaculate were obtained. The following parameters were recorded:

duration of electrical stimulation required to obtain ejaculate, severity of erection and muscle contraction, and the presence or absence of ejaculation.

The following visual criteria were used to assess erection quality: "+" – weak erection: partial filling of the corpora cavernosa, an increase in penile volume without pronounced rigidity or extrusion from the prepuce. "++" – moderate erection: characterized by full penile rigidity and extrusion from the preputial sac; however, the elevation angle relative to the animal's body axis is less than 90°. "+++" – pronounced erection: maximum rigidity, complete penile extrusion accompanied by characteristic color changes and a specific position facilitating ejaculate collection.

The degree of muscle response to electrical stimulation was assessed by the involvement of muscle groups: "+" – moderate contraction: localized muscle response limited to the pelvic girdle and hind limbs. "++" – pronounced contraction: generalized muscle response involving the back and forelimb muscles, with pronounced trunk arching requiring additional animal fixation to maintain the rectal probe in the correct position.

The obtained ejaculate was collected using a single-channel variable-volume pipette with 200 µL plastic tips (Thermo Scientific, USA) and mixed with Sil-Select Plus Sperm Washing medium (FertiPro, Belgium) in Eppendorf tubes. Microscopy of the obtained samples was then performed to assess sperm count and morphology.



FIG. 1.
Animal preparation for transrectal electrical stimulation

Sperm concentration was counted using a Goryaev counting chamber. A 10-fold diluted ejaculate sample (10 µL) was introduced with a pipette into the chamber at the edge of the coverslip so that the ejaculate filled the entire chamber. Subsequently, at ×200 magnification (Micromed P-1 LED, Russia), cells were counted in 50 large squares of the chamber, while noting the presence or absence of sperm motility.

The following formula was used to calculate sperm concentration [21]:

$$C = \frac{a \times 250 \times 10}{50 \times 1000} \text{ million/mL,}$$

where

a – total number of cells counted in 50 large squares of the Goryaev chamber;

250 – volume of one large square of the Goryaev chamber;

10 – dilution factor of the original ejaculate;

1000 – conversion factor from 1 µL to 1 mL;

50 – number of large squares in which sperm were counted.

For sperm morphology assessment, microscopy was performed using a digital microscope with a ×40 objective (Levenhuk D90L LCD, China).

Sperm motility was evaluated in accordance with WHO guidelines, with three categories distinguished: progressively motile (PR) – actively moving linearly or in large circles; non-progressively motile (NP) – movement in place; and immotile (IM) – complete absence of movement.

Statistical analysis was performed using non-parametric methods. Quantitative data (sperm concentration) are presented as median and interquartile range – Me (25%–75%). Motility data are presented as mean ± standard error ($M \pm m$). The Kruskal – Wallis test was used to detect differences between groups. Differences were considered statistically significant at $p < 0.05$.

RESULTS

The current parameters used to obtain ejaculate samples from rats by transrectal electrical stimulation of the seminal tubercles are presented in Table 1.

During the study, erection was observed in all six experimental animal groups upon electrical stimulation, manifesting as an increase in penile volume and, in some cases, cyanosis due to blood engorgement (Fig. 2).

As a result of transrectal electrical stimulation of the seminal colliculus, ejaculate was obtained from animals in experimental groups No. 2, 3, 4, 5, and 6. In group No. 1, ejaculation did not occur even after 3 minutes of electrical stimulation (Table 2).

The data presented in Table 2 indicate that all stimulation protocols resulted in erection in all animal groups. The minimum stimulation time required to obtain ejaculate was observed in groups No. 3–6: up to 1 minute. In these same groups (No. 3–6), pronounced erection was observed. However, in groups No. 5 and 6, a more active response in the form of generalized muscle contractions of the entire body was observed at a frequency of 70 Hz, which was considered a negative factor. Despite the rigidity of the rectal probe (a metal probe was used for stimulation), intense muscle contractions caused physical displacement of the probe relative to the projection of the rectal nerve plexuses, making it difficult to maintain stable stimulation even with constant manual control of its position. At a frequency of 60 Hz, no excessive motor activity or generalized muscle contractions were observed, indicating adequate anesthetic depth and absence of pain sensitivity. This protocol ensured a stable neuromuscular status in the animals during the experiment, providing a physiological background for transrectal stimulation under anesthesia.

Ejaculate samples from animal groups No. 3–6 were collected for analysis. The results of sperm concentration and motility assessment are presented in Table 3.

Based on the obtained data, the highest mean sperm concentration was observed in experimental groups No. 3 and 4 – 8.70 (8.10; 9.40) million cells/mL and 7.45 (6.90; 8.15) million cells/mL, respectively. Motility analysis revealed that in group No. 3, progressively motile sperm predominated ($PR = 62.4 \pm 5.1\%$). In group No. 4, a decrease in the proportion of progressively motile sperm to $34.8 \pm 4.4\%$ was observed, accompanied by an increase in the number of non-progressively motile sperm. In groups No. 5 and 6, no motile cells were detected. To exclude the influence of external factors (temperature shock, waiting time), sample collection was performed according to a uniform protocol: analysis was performed no later than 5 minutes after ejaculate collection, and tubes were kept in a thermostat at 37°C. Given the presence of motile sperm in groups No. 3 and 4, the absence of activity in groups No. 5 and 6 is directly related to excessive high-frequency current

exposure (70 Hz) to the rat seminal colliculus tissue at the time of ejaculation.

In groups No. 5 and 6, the sperm counts were lower than in groups No. 3 and 4 – 6.05 (5.50; 6.70) million cells/mL and 4.90 (4.45; 5.40) million cells/mL, respectively. Morphological assessment of cells in these animal groups revealed morphological abnormalities, manifested as detachment of the sperm head from the tail (Fig. 3).



FIG. 2.
Erection induced in a male rat

TABLE 1

ELECTRICAL STIMULATION PARAMETERS FOR TRANSRECTAL EJACULATE COLLECTION FROM THE SEMINAL COLLICULUS IN RATS

Characteristics of electroejaculation parameters	Animal group					
	1 (n=5)	2 (n=5)	3 (n=5)	4 (n=5)	5 (n=5)	6 (n=5)
Frequency, Hz	50		60		70	
Voltage, V	4	9	4	9	4	9
Current intensity, A	≤8					

TABLE 2

OUTCOMES OF TRANSRECTAL ELECTRICAL STIMULATION OF THE SEMINAL COLLICULUS IN MALE RATS

Parameters	Characteristics of electroejaculation parameters					
Animal group	1 (n=5)	2 (n=5)	3 (n=5)	4 (n=5)	5 (n=5)	6 (n=5)
Frequency, Hz	50		60		70	
Voltage, V	4	9	4	9	4	9
Current intensity, A	≤8					
Duration of electrical stimulation to obtain ejaculate	Up to 3 min	Up to 2 min	Up to 1 min	Up to 1 min	Up to 1 min	Up to 1 min
Median time to ejaculation (Me (25%–75%), min)	No ejaculate obtained	1.7 (1.5; 2.1)	0.5 (0.4; 0.6)	0.6 (0.4; 0.7)	0.7 (0.5; 0.8)	0.6 (0.4; 0.7)
Muscle response severity	+	+	+	+	++	++
Erection quality	+	++	+++	+++	+++	+++
Ejaculation achieved	-	+	+	+	+	+
Sperm present	-	+	+	+	+	+

Notes.

1. In group No. 1, no ejaculate was obtained within 10 minutes of stimulation.

2. Muscle contraction: “+” – moderate (local contractions of the pelvic girdle); “++” – pronounced (generalized contractions of the trunk musculature).

3. Erection: “+” – weak (partial filling of the corpora cavernosa); “++” – moderate (complete rigidity and extrusion from the prepuce); “+++” – pronounced (maximum rigidity with hyperemia).

TABLE 3

ASSESSMENT OF SPERM CONCENTRATION, MOTILITY, AND MORPHOLOGY IN EJACULATE SAMPLES

Parameters		Characteristics of electroejaculation parameters			
Animal group		3 (n=5)	4 (n=5)	5 (n=5)	6 (n=5)
Frequency, Hz		60		70	
Voltage, V		4	9	4	9
Current intensity, A		≤8			
Median sperm count (Me (25%-75%), million/mL)		8.70 (8.10; 9.40)	7.45 (6.90; 8.15)	6.05 (5.50; 6.70)	4.90 (4.45; 5.40)
Sperm motility, %	progressively motile (PR)	62.4±5.1	34.8±4.4	0	0
	non-progressively motile (NP)	15.2±2.4	28.6±3.2	0	0
	immotile (IM)	22.4±4.8	36.6±5.4	100	100

The analysis revealed statistically significant differences between the study groups in both sperm concentration ($p < 0.05$) and motility parameters ($p < 0.01$). The mean sperm concentration in group No. 3 (8.70 million cells/mL) was significantly higher than in groups No. 4, 5, and 6. Analysis of cell motility in these groups also revealed significant differences. Thus, in group No. 3, progressively motile sperm significantly predominated ($PR = 62.4 \pm 5.1 \%$), which was significantly higher than in group No. 4 ($PR = 34.8 \pm 4.4 \%$) ($p < 0.05$) and groups No. 5 and 6, where motile cells were completely absent ($p < 0.01$). In group No. 4, a statistically significant increase in the proportion of non-progressively motile sperm was observed compared to group No. 3: $28.6 \pm 3.2 \%$ vs. $15.2 \pm 2.4 \%$.

The low sperm concentration in groups No. 5 and 6 is explained by the fact that only intact cells are counted. Sperm destruction at 70 Hz results in a significant portion of the biological material being converted into cellular debris, which cannot be quantified. The presence of morphological abnormalities in cells indicates the traumatic effect of the 70 Hz stimulation protocol at both voltage settings. Given this fact, these electrical stimulation parameters should not be used for sperm collection to avoid compromising sperm morphology.

DISCUSSION

The electrostimulation method is the most promising for obtaining sperm from male animals when studying their fertility level and analyzing the role of the male factor in reproduction. Its key advantage lies in the ability to obtain native ejaculate rather than epididymal suspension. Unlike work with suspension, ejaculate collection allows assessment of the functional status of cells in the presence of accessory sex gland secretions, ensuring maximum accuracy of semen parameters close to physiological norms. Furthermore, the use of this method complies with international bioethical principles. Non-terminal sample collection eliminates the need for animal euthanasia, enabling studies on the same individuals and increasing the human nature of the experiment. The ability to select male animals with equal sperm cell potential for experimental work allows the exclusion of the male infertility factor and assessment of the role of the female organism in reproduction under the influence of various external and internal factors.

Literature analysis revealed that various stimulation protocols have been used in experimental studies on transrectal electrical stimulation of the seminal colliculus (Table 4).

The identification of numerous modifications of the seminal colliculus electrostimulation method and the absence of a standardized stimulation protocol (frequency, voltage, current intensity, duration) justified the need to develop a step-by-step method for sperm

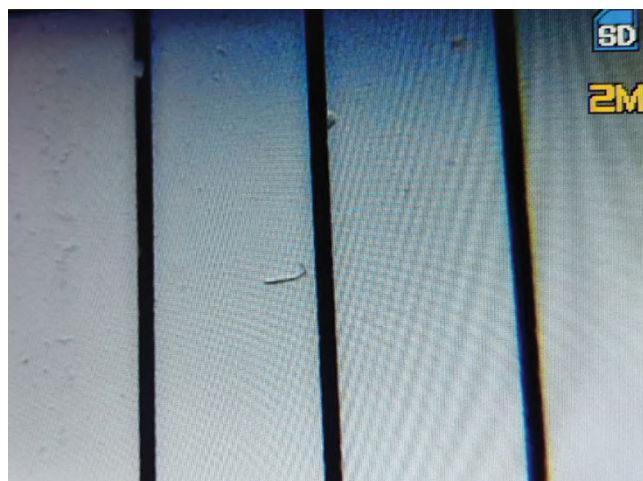


FIG. 3.
Sperm head in the Goryaev chamber, at $\times 400$ magnification

collection in male rats. To determine the most favorable stimulation protocol, based on the results of previous studies, two frequency settings (60 Hz and 70 Hz) and two voltage settings (4 V and 9 V) were applied at one current intensity level. The criteria for selecting the optimal stimulation protocol were minimal time required to obtain ejaculate. Compared to previously published methods, the proposed protocol significantly reduces the stimulation time required to induce erection and successfully collect material to 1 minute. This not only increases experimental productivity but also minimizes stress exposure to the animal, prevents muscle contractions, and preserves sperm morphological integrity.

The study established that the safest and most cell-sparing method for obtaining active, morphologically intact sperm is transrectal electrical stimulation of the rat seminal colliculus at 60 Hz and 4 V. Conversely, exceeding the frequency threshold above 60 Hz triggers a cascade of irreversible destructive processes in the cell structure. It is known that at 70 Hz, high-frequency microvibrations resonate with elements of the sperm cytoskeleton. Due to the inertial mass difference between the head and tail, excessive mechanical stress is generated in the vulnerable neck region, leading to massive decapitation of cells [22]. In addition to mechanical rupture, the intense electric field induces membrane electroporation and oxidative stress, disrupting ionic homeostasis and leading to rapid loss of motility [23]. An additional negative factor is local tissue heating, causing denaturation of axonemal proteins and the tail motor apparatus [24].

The use of optimal parameters of 60 Hz and 4 V allows the collection of a greater number of cells (8.7 (8.10; 9.40) million cells/mL) within 1 minute, not only compared to other stimulation protocols within this experimental study but also compared to previously published studies using various current parameters.

TABLE 4

COMPARATIVE EVALUATION OF TRANSRECTAL ELECTRICAL STIMULATION METHODS FOR THE RAT SEMINAL COLLICULUS [17, 18, 19]

Electrical stimulation protocol	Frequency, Hz	Voltage, V	Current intensity, A	Stimulation duration, min
1	60	8.1	No information available	10.0
2	60	5.8	No information available	5.5
3	60	7.4	No information available	5.0
4	60	3	0.5	until ejaculate obtained
5	No information available	2–10	No information available	until ejaculate obtained

CONCLUSIONS

A non-terminal sperm collection technique has been developed for Wistar rats, with the key advantage being the optimization of electrical parameters – 60 Hz and 4 V. The selection of these parameters allowed a significant reduction in the duration of the procedure, which favorably distinguishes the proposed method from existing analogues and minimizes stress exposure to laboratory animals.

The scientifically validated stimulation protocol ensures the collection of morphologically intact and functionally active sperm, opening broad opportunities for the application of the obtained results: in studying the etiology and pathogenesis of various forms of male infertility; for analyzing the efficacy and safety of novel immunobiological agents aimed at improving male fertility; for developing and refining *in vitro* fertilization protocols in laboratory animals; and for investigating the effects of complex environmental and internal factors on reproductive potential.

The developed technique represents a reliable tool for standardizing andrological research, ensuring high reproducibility of results while strictly adhering to bioethical principles.

Funding

This work was supported by a grant from the UMN program grant from the Foundation for Assistance to Small Innovative Enterprises (FASIE) (agreement No. 18682GU/2023) and a grant from the Kirov State Medical University of the Ministry of Health of the Russian Federation No. 7-2026–GRANT.

Conflicts of interest

The authors declare no conflicts of interest.

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