

УДК 57.063.8:591.465.12

<https://doi.org/10.20538/1682-0363-2026-1-86-95>

Risk Assessment of Germline Transition of Adeno-associated Virus Vector of Chimeric Serotype PHP.eB into Mouse Oocytes

Malikova A.D.¹, Zhanataev A.K.¹, Esmagambetov I.B.², Anisina E.A.¹, Pligina K.L.¹, Chaika Z.V.¹, Ryabova E.I.², Dovgiy M.A.², Hossain R.M.², Durnev A.D.¹, Dorofeev V.L.¹

¹ Federal State Budgetary Research Institution Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies

8 Baltiyskaya St., 125315 Moscow, Russian Federation

² Federal State Budgetary Institution Gamaleya National Center for Epidemiology and Microbiology

18 Gamaleya St., 123098 Moscow, Russian Federation

ABSTRACT

Aim. To assess the risk of germline transmission of an adeno-associated virus vector of the chimeric serotype PHP.eB into the germ cells of female mice based on the development of an original method for obtaining mouse oocytes devoid of somatic cells and free vector particles.

Materials and methods. The vector under study was administered intravenously to female outbred CD-1 mice at a dose of 5×10^{10} vector genomes/mouse using an original technique which included hormonal superovulation of exposed animals, oocyte isolation, their purification from contaminating somatic cells, and direct quantitative PCR on lysed oocytes. The vector DNA content in the brain tissue, ovaries, and oocytes was assessed on days 1, 3, 7, 14, 30, and 90 after vector administration.

Results. Using the developed technique, we examined the ability of the adeno-associated virus vector of the chimeric serotype PHP.eB to undergo germline transmission into mouse oocytes. It was established that, despite persistence in brain and ovarian tissue for up to 3 months, vector DNA was not detected in oocytes at any time after vector administration.

Conclusion. The obtained data demonstrate the absence of germline transmission of the studied genetic construct into mouse oocytes. The detection of the adeno-associated vector in the ovaries, despite its absence in oocytes, confirms the effectiveness of the developed technique for obtaining mouse oocytes devoid of somatic cells.

Keywords: adeno-associated vector, chimeric serotype PHP.eB, germline transmission, oocytes, mice, direct PCR

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

Source of financing. This study was supported by the Ministry of Science and Higher Education of the Russian Federation as part of the research project "Health Preservation Technologies Based on the Methodology of Preclinical Studies of Drug Safety" (No. FGFG 2025-0003).

For citation: Malikova A.D., Zhanataev A.K., Esmagambetov I.B., Anisina E.A., Pligina K.L., Chaika Z.V., Ryabova E.I., Dovgiy M.A., Hossain R.M., Durnev A.D., Dorofeev V.L. Risk Assessment of Germline Transition of Adeno-associated Virus Vector of Chimeric Serotype PHP.eB into Mouse Oocytes. *Bulletin of Siberian Medicine*. 2026;26(1):86–95. <https://doi.org/10.20538/1682-0363-2026-1-86-95>.

Оценка риска вертикального переноса аденоассоциированного вектора химерного серотипа РНР.еВ в ооциты мышей

Маликова А.Д.¹, Жанатаев А.К.¹, Есмагамбетов И.Б.², Анисина Е.А.¹, Плигина К.Л.¹, Чайка З.В.¹, Рябова Е.И.², Довгий М.А.², Хоссаин Р.М.², Дурнев А.Д.¹, Дорофеев В.Л.¹

¹ Федеральный исследовательский центр (ФИЦ) оригинальных и перспективных биомедицинских и фармацевтических технологий
Россия, 125315, г. Москва, ул. Балтийская, 8

² Национальный исследовательский центр эпидемиологии и микробиологии (НИЦЭМ) им. почетного академика Н.Ф. Гамалеи
Россия, 123098, г. Москва, ул. Гамалеи, 18

РЕЗЮМЕ

Цель. Оценить риск вертикального переноса аденоассоциированного вирусного вектора химерного серотипа РНР.еВ в половые клетки самок мышей на основе разработки оригинального способа получения ооцитов мышей, неконтаминированных соматическими клетками и свободными векторными частицами.

Материалы и методы. Исследуемый вектор вводили внутривенно самкам аутбредных мышей CD-1 в дозе 5×10^{10} векторных геномов на мыш. Использовали оригинальную методологию, включающую гормональную суперовуляцию экспонированных животных, выделение ооцитов, их очистку от контаминирующих соматических клеток и прямую количественную полимеразную цепную реакцию (ПЦР) на лизированных ооцитах. Содержание векторной ДНК в ткани головного мозга, яичников и в ооцитах оценивали на 1, 3, 7, 14, 30 и 90-е сут после введения вектора.

Результаты. С использованием разработанного подхода исследована способность к вертикальному переносу в ооциты мышей аденоассоциированного вирусного вектора химерного серотипа РНР.еВ. Установлено, что несмотря на персистенцию в ткани головного мозга и яичников до 3 мес, векторная ДНК не выявляется в ооцитах ни на одном из сроков после введения вектора.

Заключение. Полученные данные демонстрируют отсутствие вертикального переноса в ооциты мышей исследуемой генной конструкции. Выявление аденоассоциированного вектора в яичниках при его обнаружении в ооцитах подтверждает эффективность разработанной методики получения ооцитов мышей, неконтаминированных соматическими клетками.

Ключевые слова: аденоассоциированный вектор, химерный серотип РНР.еВ, вертикальный перенос, ооциты, мыши, прямая ПЦР

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Исследование выполнено при поддержке Министерства науки и высшего образования в рамках финансирования по теме «Технологии сбережения здоровья на основе методологии доклинических исследований безопасности лекарственных средств» (№ FGFG2025-0003).

Соответствие принципам этики. Исследование одобрено ФИЦ оригинальных и перспективных биомедицинских и фармацевтических технологий (протокол № 3 от 21.02.2024).

Для цитирования: Маликова А.Д., Жанатаев А.К., Есмагамбетов И.Б., Анисина Е.А., Плигина К.Л., Чайка З.В., Рябова Е.И., Довгий М.А., Хоссаин Р.М., Дурнев А.Д., Дорофеев В.Л. Оценка риска вертикального переноса аденоассоциированного вектора химерного серотипа рНР.еВ в ооциты мышей. *Бюллетень сибирской медицины*. 2026;26(1):86–95. <https://doi.org/10.20538/1682-0363-2026-1-86-95>.

INTRODUCTION

Gene therapy is a cutting-edge, innovative method for treating hereditary, oncological, cardiovascular, hematological, and other diseases by means of restoring the functions of defective genes or expressing

new genes in cells using genetically engineered constructs based on recombinant nucleic acids [1, 2]. Like any drug therapy, gene therapy is associated with certain risks that are specific to the nature of the active agent. For DNA-based drugs designed for *in vivo* gene therapy, one potential and significant safety risk

is the germline (unintentional) transmission of vector DNA sequences into germ cells [3–5]. The discovery of the potential for germline transmission limits the applicability of gene therapy drugs for patients with reproductive potential [6, 7].

According to regulatory guidelines, if vector DNA is detected in the gonads at three consecutive time points (i.e., it persists) during a biodistribution study, additional studies are required to determine its transduction directly into germ cells [7–9]. The irreversibility of transduction and, consequently, a high risk of germline transmission are evidenced by the persistence of vector DNA in spermatozoa for more than 3 cycles of spermatogenesis [8, 9]. The identification of vector DNA in oocytes at any time point is considered as a high risk of germline transmission, since the entire non-renewable ovarian reserve is affected [8, 9].

Despite strict regulatory restrictions on conducting gene therapy when the risk of germline transmission is identified, there is currently no scientifically based, standardized methodology for its assessment. This problem is particularly relevant for female germ cells, since, unlike male germ cells, risk assessment for them can be based only on data of preclinical studies [9]. The EMA guidelines specifically note that the development and validation of animal models for these purposes are a pressing issue [9].

To identify target DNA sequences in germ cells, nucleic acid amplification methods (polymerase chain reaction (PCR) or more sensitive quantitative PCR) are recommended [9]. Alternatively, *in situ* hybridization or immunohistochemistry may be used, provided their sensitivity and reliability are properly validated [9]. However, these methods are costly, labor-intensive, and difficult to standardize. The main disadvantage of PCR-based methods is the high risk of false-positive results due to contamination of germ cells with free vector particles and/or somatic cells (spermatozoa by epididymal or vas deferens cells and oocytes by cumulus cells of the oocyte-cumulus complex) when standard isolation methods are used. In the case of oocytes, the analysis is further complicated by the extremely small amount of cellular material available for testing.

The aim of this study was to assess the risk of germline transmission of an adeno-associated viral vector of the chimeric serotype PHP.eB into the germ cells of female mice based on the development of an original method for obtaining mouse oocytes uncontaminated with somatic cells and free vector particles.

MATERIALS AND METHODS

Production of PHP.eB

In this study, we used the recombinant adeno-associated virus rAAV9-PHP.eB-CASI-Luc, which is an AAV of the chimeric PHP.eB serotype, constructed in the laboratory of Dr. Viviana Gradinaru [10] and carrying the firefly luciferase gene under the control of a modified chicken β -actin (CASI) promoter.

The rAAV9PHP.eB-CASI-Luc virus was constructed using the pAAV-CASI-Luc, pAAV2/9-PHP.eB, and pHelper Vector plasmids (Cell Biolabs, USA). The pAAV-CASI-Luc was constructed using the CASI promoter sequence together with the cloning sites and the WPRE sequence and the polyadenylation signal that were synthesized at Evrogen (Russia) with subsequent cloning of the synthesized sequence into the pAAV-CMV-Vector plasmid (Cell Biolabs, USA) between the left and right ITRs, replacing the existing expression cassette. Next, the codon-optimized sequence of the firefly luciferase gene used in our previous study [11] was cloned between the CASI promoter sequence and the WPRE element sequence. The pAAV2/9-PHP.eB plasmid was derived from the pREPCAP2/9 plasmid using insertional mutagenesis by PCR with specific primers in order to introduce the appropriate amino acid substitutions into the capsid protein sequence of the serotype 9 AAV [10].

To produce the PHP.eB vector, the HEK293 cell line (from the cell culture collection of Gamaleya National Research Center for Epidemiology and Microbiology) was used. Cultivation and transfection were carried out under adherent conditions at 37°C and 5% CO₂, as described previously [12, 13]. The PHP.eB Viral preparations were purified using affinity chromatography (AC) on the POROSTM CaptureSelectTM AAVX Affinity Resin (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Additional purification and buffer exchange were performed using size-exclusion chromatography (SEC) on an XK 26/100 column packed with Superdex 200 sorbent (Cytiva, USA). Final vector formulation was performed on Amicon Ultra-15 100 kDa centrifugal concentrators (Merck, USA). The content of viral genomes in the preparation was assessed as described previously [11–13].

Work with Laboratory Animals

The study was conducted on mature female outbred CD-1 mice weighing 20–22 g and aged 8–9 weeks, obtained from Andreevka animal breeding

facility (Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency of Russia). The animals were kept in the vivarium of the Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies with a 12-hour light cycle, free access to water, and a standard complete diet. The conditions for animal housing and working with the animals complied with the requirements of Directive 2010/63/EU of the European Parliament and the EU Council on the protection of animals used for scientific purposes, as well as with Recommendation No. 33 of the Eurasian Economic Commission (November 14, 2023) "Guidelines for Working with Laboratory (Experimental) Animals in Preclinical (Non-Clinical) Studies".

Administration of Vector to Mice

A suspension of PHP.eB vector particles at a dose of 5×10^{10} vector genomes/mouse in a volume of 50 μ l was injected into mice via the lateral tail vein at a flow rate of 70 μ l/min using an MD-1001 BASi Bee Baby syringe drive (BASi Corporate Headquarters, USA).

Mice were divided into 7 groups of 5–6 animals each: I (control), II (1 day after injection), III (3 days after injection), IV (7 days after injection), V (14 days after injection), VI (1 month after injection), and VII (3 months after injection). Forty-eight hours prior to vector injection, mice of group II were intraperitoneally administered equine chorionic gonadotropin (eCG, Folligon, MSD Animal Health, Netherlands) at a dose of 5 IU/mouse. Seven hours after vector injection, mice were intraperitoneally administered human chorionic gonadotropin (hCG, PG600, MSD Animal Health, Netherlands) at a dose of 5 IU/mouse and euthanized 17 hours later. Mice of group III were administered eCG together with vector injection. After 55 hours, the mice were administered hCG and euthanized 17 hours later. Mice of groups I, IV, V, VI, and VII were administered PGFA and hCG 65 and 17 hours prior to euthanasia, respectively.

Isolation and Purification of Oocytes

The animals were euthanized by cervical dislocation. Ovaries with oviducts were transferred to drops of M2 medium (Sigma-Aldrich, USA) on a Petri dish, and the oviduct ampullae were dissected using a dissecting needle under a stereomicroscope (Stemi DV4, Carl Zeiss, Germany). For denudation, oocytes released in the oocyte-cumulus complex were transferred using an EZ-Grip microcapillary pipette (RI, UK) into a 100 μ l drop of medium containing

150 IU/ml hyaluronidase Type II (Sigma, USA) and incubated for 20 min at room temperature. Oocytes were washed from the enzyme and cumulus cells by sequentially transferring them through 6–8 drops of fresh M2 medium. To remove the zona pellucida, oocytes were incubated in 0.025% collagenase Type I solution (Sigma-Aldrich, USA) for 20 min, followed by sequential washes in 6–8 drops of M2 medium to remove the enzyme.

For final purification from cumulus cells, the oocytes were transferred to the lower part of a drop of Percoll (Sigma-Aldrich, USA) at varying concentrations (10–70%) in a Petri dish and gently mixed with a circular motion of the microcapillary tip (Fig. 1). Within approximately 10 min, oocytes floated to the surface of the drop, after which they were collected and transferred to a fresh drop of Percoll, and the procedure was repeated. The oocytes were then washed in a drop of phosphate-buffered saline (pH 7.4), transferred to microtubes in a minimal volume of the buffer, frozen and stored at -20°C until real-time PCR. To assess contamination with somatic cells, a part of the oocytes was fixed, stained, and examined under a microscope according to a previously described protocol [14].

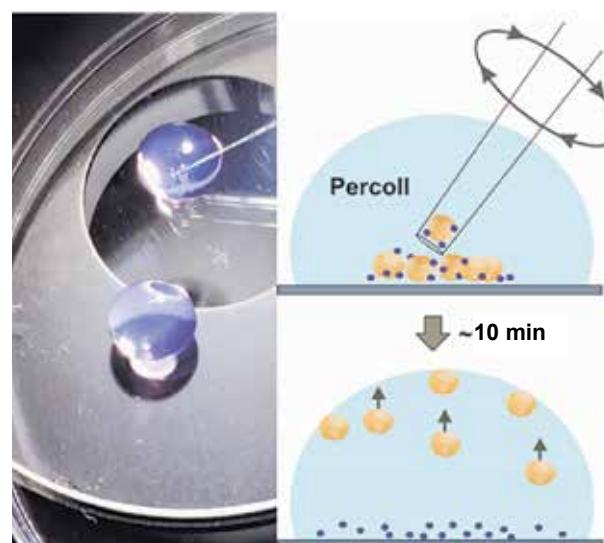


Fig. 1. Separation of oocytes and cumulus cells in a Percoll drop. A 100 μ l drop of Percoll solution is placed on a polystyrene Petri dish, and the oocytes are transferred to the lower part of the drop. Using the tip of a microcapillary positioned at an angle of approximately 60° , the oocytes are gently distributed throughout the lower part of the drop by circular motions. After approximately 10 min, the oocytes that have floated to the surface of the drop are collected.

After the release of oocyte-cumulus complexes, total DNA was extracted from ovaries with oviducts and brain tissue using the D-Tissues kit (Biolabmix,

Russia). The concentration of isolated DNA in the samples was measured using the dsDNA BR kit on a Qubit 4 mini fluorometer (Thermo Fisher Scientific, USA). DNA was frozen and stored at -20°C until real-time PCR was performed.

Real-time PCR Procedure

To detect DNA sequences, direct PCR on lysed oocytes was performed [15]. Microtubes containing the oocyte suspension were thawed, an equal volume of lysis solution (500 $\mu\text{g}/\text{ml}$ polyadenylic acid, 20 mM EDTA- Na_2 , 500 mM dithiothreitol, and 1% N-lauryl sarcosine) was added, and the mixture was incubated at 37°C for 30 min. The lysate was then

thoroughly resuspended and used for PCR. To assess the efficiency of direct PCR on lysed oocytes, PCR was performed for the sequences of both nuclear DNA gene (*Ptger2*) and mitochondrial DNA gene (*Cox-1*) of the mouse. The primer sequences are listed in Table 1.

Real-time PCR was performed on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA). The reaction mixture included 1X qPCRmix-HS LowROX mixture (Eurogen, Russia), 2 μM SYBR Green I, 500 nM each of forward and reverse primers (DNA Synthesis, Russia), 5 μl of lysed oocyte suspension or 4 ng of DNA isolated from ovaries (PCR control).

Table 1

Primers Used in the Study for Real-time PCR			
Gene Name	Primer Sequence		Amplicon size (bp)
Ptger2	Forward	5'-CCTGCTGCTTATCGTGGCTG-3'	186
	Reverse	5'-GCCAGGAGAATGAGGTGGTC-3'	
Cox-1	Forward	5'-ATTACAGCCGTACTGCTCCTAT-3'	150
	Reverse	5'-CCCAAAGAATCAGAACAGATGC-3'	
Luc	Forward	5'-GAGGCGAACTGTGTGTGAGA-3'	149
	Reverse	5'-GTGTTCTGCTTCGTCCAGT-3'	

Thermal cycling conditions were as follows: 98°C for 1 min, then 35 cycles of 98°C for 10 s, 61°C for 30 s, and 72°C for 15 s. Melting curve was generated by increasing the temperature from 65°C to 95°C within 1 min (HRM mode: 20 data points per 1°C). PCR efficiency (E , %) was calculated using the formula:

$$E = (10^{(-1/a)} - 1) \times 100$$

where a is the slope of the regression line describing the dependence of the threshold cycle C_q on the logarithm of the number of oocytes in the reaction (5, 10, 20, 40, 80, and 160 oocytes).

To detect vector DNA in the ovaries and oocytes, primers specific to the sequence of the luciferase gene cloned into the vector were used (Table 1). The reaction mixture included 1X BioMaster UDG HS-qPCR Lo-ROX SYBR (2X) mixture (Biolabmix, Russia), 600 nM each of forward and reverse primers (DNA Synthesis, Russia), 4 ng of DNA isolated from the ovaries or 5 μl of lysed oocyte suspension. Thermocycling conditions were as follows: 50°C for 2 min (anti-contamination treatment), 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 62°C for 15 s, and 72°C for 15 s. Melting curve was generated by increasing the temperature from 65°C to 95°C within 1 min (HRM mode: 20 data points per 1°C). The number of vector genome copies per microgram of total DNA (ovaries) or per haploid genome

(oocytes) was determined based on a calibration curve constructed using control solutions with known vector concentrations. PCR efficiency was calculated using the formula provided above. The matrix effect and recovery were determined in accordance with the recommendations [16]. Amplification of the target DNA sequence was confirmed by the melting curve of the product.

RESULTS

In laboratory mice, the number of oocytes ovulating during one estrous cycle ranges from 8 to 12, depending on the strain and age [17]. To obtain a larger number of oocytes, hormonal superovulation is induced by sequential administration of eCG which stimulates follicle growth and hCG which resumes meiotic divisions of oocytes and causes ovulation [15]. In female CD-1 mice, the number of ovulated oocytes under conditions of superovulation was 20.1 ± 6.4 per mouse.

If the standard isolation procedure is used, after denudation the oocyte suspensions with an intact zona pellucida contain a large number of cumulus cells (Fig. 2, a). Removal of the zona pellucida followed by repeated washing significantly reduces contamination, but it does not allow to completely remove cumulus cells from the suspension (Fig. 2, b), since oocyte selection and transfer with

a microcapillary are performed in a small amount of medium/buffer. In a study by H. Okada et al. [18], a method of centrifugation in 22.5% Percoll was proposed for the purification of *in vitro* fertilized oocytes and two-cell mouse embryos from cumulus cells and spermatozoa. However, this approach was found to be unsuitable for unfertilized oocytes with removed zona pellucida. Centrifugation in Percoll resulted in the loss of over 90% of oocytes due to their mechanical destruction (own data).

In this study, we tested an approach based on the separation of oocytes and cumulus cells using differences in buoyancy in a Percoll drop (Fig. 1). In 10, 20, 30, and 40% Percoll, oocytes remained

in the lower part of the drop, whereas in 50, 60, and 70% Percoll, they floated to the surface within approximately 10 min and remained on the surface of the drop. Cumulus cells remained in the lower part of the drop in all Percoll solutions. Collecting oocytes from the surface of the drop using a microcapillary proved to be most convenient in 70% Percoll, due to the decrease in surface tension of the Percoll solution with increasing density. To assess contamination, we conducted a series of four experiments with oocyte purification in 70% Percoll (a total of 119 oocytes). In none of the cases were cumulus cells detected on oocyte micropreparations after their purification (Fig. 2, b).

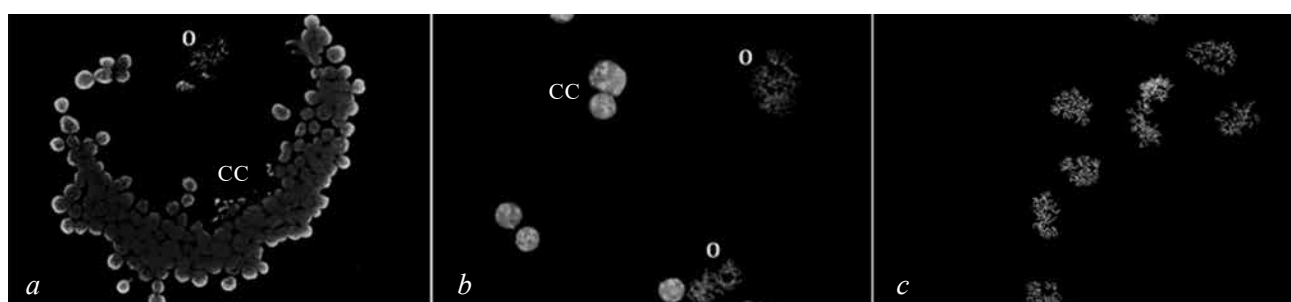


Fig. 2. Cytogetic preparations of mouse oocytes. *a* – with intact zona pellucida, *b* – after removing the zona pellucida, *c* – after washing in 70% Percoll. O – oocyte (metaphase MII), CC – cumulus cells. Stained with Hoechst 33258; magnification $\times 400$

Traditional DNA extraction methods (for PCR analysis) are not applicable to oocytes due to the limited availability of the material. To detect DNA (or RNA) sequences in oocytes, it is advisable to perform PCR directly on lysed cells. This method makes it possible to analyze the entire pool of isolated cells without loss, increasing the accuracy and sensitivity of detection. We used the “spanning” protocol developed by S.Tsuchita et al. [15], which involves cell lysis and DNA deproteinization using the detergent N-lauryl sarcosine at a concentration that does not inhibit PCR. The authors demonstrated high efficiency and a low

false-positive rate when using this protocol to amplify DNA regions in single blastomeres and lymphocytes.

The amplification efficiency using oocyte lysate as a template for the mitochondrial DNA gene (*Cox-I*) and for the nuclear DNA gene (*Ptger2*) comprised 98.2% and 90.3%, respectively. The higher efficiency and earlier threshold cycles of *Cox-I* amplification are due to the large number of mitochondrial DNA copies (200,000–400,000) in mature oocytes [19]. PCR for the *Ptger2* gene showed linearity ($R^2 = 0.975$) with the number of oocytes in the reaction ranging from 5 to 160 (Fig. 3). With the

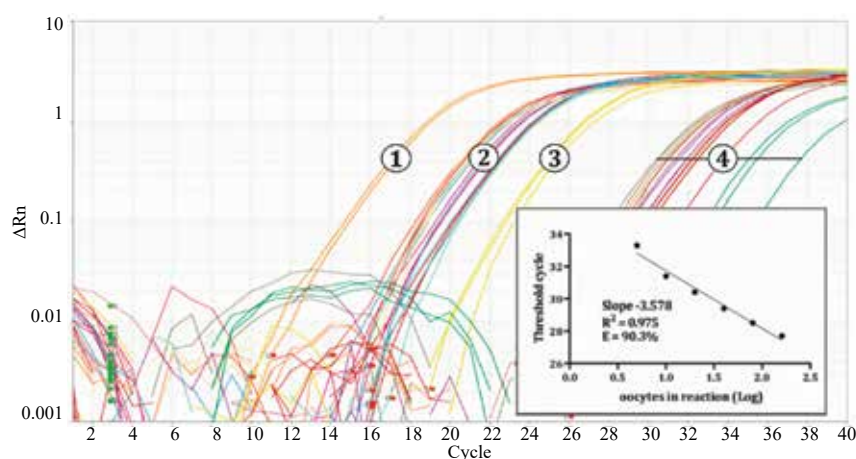


Fig. 3. Amplification curves for the *Ptger2* gene with ovarian DNA as a template (1), for the *Cox-I* gene with oocyte lysate (2), for the *Cox-I* gene with ovarian DNA (3), and for the *Ptger2* gene with oocyte lysate (4). The X-axis shows the amplification cycle, and the Y-axis shows the normalized change in the fluorescent signal at each amplification cycle (ΔR_n). The inset shows the curve of dependence of the threshold cycle on the logarithm of the number of oocytes in the reaction for the *Ptger2* gene (slope of the regression line (slope), correlation coefficient (R^2), and reaction efficiency (E)).

number of oocytes less than 5, the linearity of the reaction significantly decreased, although the signal identified by the melting curve of the product was still detectable even with 3 oocytes in the reaction, which corresponds to the theoretically achievable sensitivity of the quantitative PCR method [16, 20].

The amount of vector DNA in mouse brain and

ovarian tissues was similar at various time points after vector administration (Fig. 4). On days 1 and 3, vector genome copies were detected in all exposed animals, while on day 7 – in 5 out of 6 animals. Vector persistence was observed in both tissues for up to 3 months after administration. Vector DNA was not detected in the oocytes of any of the animals.

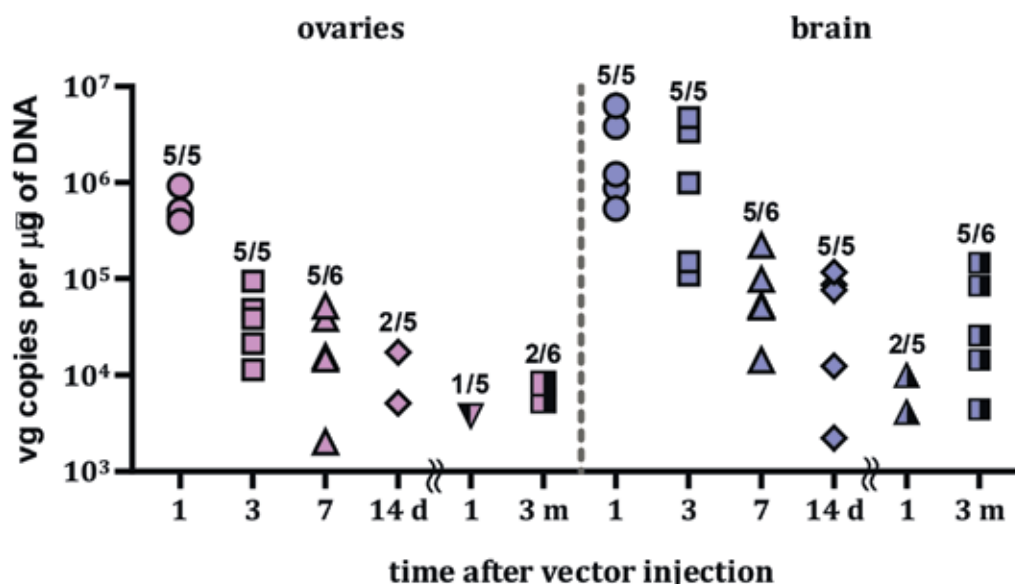


Fig. 4. The content of vector genomes (vg) in the ovaries and brain of mice. The numbers indicate the number of mice in which vector DNA was detected, relative to the total number of animals in the group

DISCUSSION

Immature oocytes in primordial ovarian follicles are protected from potentially dangerous agents by regulated transport through the basal membrane and the layer of granulosa cells, as well as by the absence of direct vascularization [21]. As oocytes mature, which is accompanied by structural and transport changes in the follicles, the blood–follicle barrier is progressively formed. The barrier includes the zona pellucida and cumulus cells surrounding the oocyte, as well as granulosa and theca cells of the antrum [21]. Theoretically, the “molecular sieve” of the blood–follicle barrier is impermeable to viral vectors due to their large size (total virion mass exceeds 5 MDa) [22]. However, AAVs have been shown to be capable of passing through the zona pellucida and transducing pre-implantation embryos [23, 24]. When injected into the ovarian stroma, AAVs are capable of penetrating the blood–follicle barrier via transcytosis and transducing the cumulus cells surrounding the oocyte [25]. In antral follicles, intercellular exchange between oocytes and somatic cells, in addition to transzonal projections,

is carried out by circulating in the follicular fluid extracellular vesicles (EVs) that can transfer large molecules and viruses into the oocyte [21, 26]. For instance, the capacity for germline transmission into human oocytes and embryos has been demonstrated for the hepatitis B virus [27]. Taken together, this indicates a potential risk of germline transmission of viral and non-viral vectors into female germ cells and emphasizes the need to develop methodological approaches to risk assessment. For this purpose, we developed and validated an original methodology that includes hormonal superovulation of vector-exposed animals, oocyte isolation, their purification in a Percoll drop, and direct quantitative PCR on lysed oocytes.

Hormonal superovulation is a crucial step in the study. The estrous cycle in mice lasts for 4–5 days and culminates in the ovulation of 8–12 oocytes [15]. Hormonal stimulation synchronizes ovulation, allowing to collect an increased number of oocytes in a narrow time frame. For maximum effectiveness, it is recommended to design the experiment so that at the time of superovulation induction the animals are in the

diestrus phase, when the ovaries are most responsive to hormonal stimulation [28]. In addition to the increased number of oocytes, superovulation also results in more than a twofold increase in the size of the ovaries and oviducts, significantly simplifying their visual identification and retrieval during necropsy.

Mature oocytes contain a significant amount of lipids in the form of triglycerides in lipid droplets and membrane phospholipids which together constitute 10 to 30% of the oocyte's dry weight [29]. This property, along with the low cytoplasmic density and large size of oocytes, ensures their buoyancy in high-concentration Percoll solutions, in which somatic cells and free vector particles remain immobile. Purification in a Percoll drop completely removes cumulus cells that contaminate oocytes. As mentioned above, vectors are capable of transducing cumulus cells, which may lead to false-positive results. Performing direct quantitative PCR without a DNA extraction step allows to avoid material loss, which increases the accuracy and sensitivity of the analysis and reduces the likelihood of false-negative results [15, 16].

Using the developed methodology, we investigated an AAV of the chimeric serotype PHP.eB capable of effectively crossing the blood – brain barrier and transducing brain and spinal cord cells in mice. The choice of a synthetic AAV capsid was based on the fact that AAV engineering is currently actively developing and new variants of artificially created chimeric AAV serotypes with cell-type- and tissue-specific tropism appear every year [30]. In 2024, the drug BEQVEZ™ (fidanacogene elaparovvec-dzkt) based on the chimeric serotype rh74var was approved for the treatment of hemophilia B.

Thus, the study of the tropism and biodistribution of chimeric AAV serotypes is of particular scientific interest. Considerable data have accumulated on the efficacy of chimeric capsids of the PHP family in penetrating the blood – brain barrier in various animal species and even mouse strains [31]. However, there are no data on the ability of such chimeric AAVs to transduce oocytes or perform germline transmission. In the present study, the vector under investigation persisted for up to 3 months both in the targeted brain tissue and in the ovaries; however, vector DNA was not detected in oocytes at any time point after the exposure. These findings indicate a low risk of germline transmission of AAVs of the chimeric serotype PHP.eB into female germ cells.

AAVs have high tropism for ovarian tissue [32]. J. Zhao et al. studied the biodistribution of AAV

of serotype 9 in mice using the high-precision RNAscope® *in situ* hybridization method [33]. Following intravenous administration, vector DNA was detected in the medulla and corpus luteum of the ovaries, as well as in the theca cells of the follicles, but not in oocytes. AAV of serotype 2 administered intravenously or into the spleen was detected in the ovaries and testes of mice but was not transmitted to their offspring [34]. AAV of serotype 8 persisted in the ovaries of mice for 150 days after intravenous administration [35]. Using *in situ* hybridization, vector DNA was identified in oocytes. However, a study of the offspring of vector-exposed females did not confirm the risk of germline transmission, raising questions about the accuracy and reliability of *in situ* hybridization for its assessment.

To date, there are only a few studies on the risk assessment of germline transmission of viral and non-viral vectors into female germ cells, which is obviously due to the methodological challenges in conducting such studies. For these purposes, the methodology we developed and described in this article can be used.

CONCLUSION

An original methodology for preclinical assessment of the risk of germline transmission of genetic constructs into the germ cells of female mice was developed and validated using an adeno-associated viral (AAV) vector of the chimeric serotype PHP.eB. The methodology is based on direct quantitative PCR; it is characterized by high sensitivity, accuracy, and relatively low labor intensity. It does not require specialized equipment or materials and can be integrated into biodistribution studies. With appropriate adaptation, it can be used in experiments on other mammalian species.

REFERENCES

1. Arabi F., Mansouri V., Ahmadbeigi N. Gene therapy clinical trials, where do we go? An overview. *Biomedicine & Pharmacotherapy*. 2022;153:113324. DOI: 10.1016/j.biopha.2022.113324.
2. Makarevich P.I. Three Decades of Gene Therapy Development: Milestones and Prospects. *Tissue and Organ Regeneration*. 2023;1(1):16–24. (In Russ.). DOI: 10.60043/2949-5938-2023-1-16-24.
3. Bittlinger M., Hoffmann D., Sierawska A.K., Mertz M., Schambach A., Strech D. Risk assessment in gene therapy and somatic genome-editing: An expert interview study. *Gene and Genome Editing*. 2022;3–4:100011. DOI: 10.1016/j.ggedit.2022.100011.
4. Astapova O.V., Berchatova A.A. Gene Therapy Medicinal Products: Non-clinical Safety Studies. *Safety and Risk*

- of *Pharmacotherapy*. 2023;11(1):73–96. (In Russ.). DOI: 10.30895/2312-7821-2023-11-1-329.
5. Moffit J.S., Blanset D.L., Lynch J.L., MacLachlan T.K., Meyer K.E., Whiteley L.O. et al. Regulatory consideration for the nonclinical safety assessment of gene therapies. *Hum. Gene Ther.* 2022;33(21–22):1126–1141. DOI: 10.1089/hum.2022.090.
 6. ICH guideline S12 on nonclinical biodistribution considerations for gene therapy products (EMA/CHMP/ICH/318372/2021).
 7. Quality, preclinical and clinical aspects of gene therapy medicinal products – Scientific guideline. (EMA/CAT/80183/2014. EMA, 2018).
 8. ICH Considerations. General principles to address the risk of inadvertent germline integration of gene therapy vectors (CHMP/ICH/469991/2006).
 9. Non-clinical testing for inadvertent germline transmission of gene transfer vectors – Scientific guideline. (EMA/273974/2005. EMA, 2006).
 10. Chan K.Y., Jang M.J., Yoo B.B., Ravi N., Wu W.L., Sanchez-Guardado L. et al. Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems. *Nat. Neurosci.* 2017;20(8):1172–1179. DOI: 10.1038/nn.4593.
 11. Esmagambetov I.B., Ryabova E.I., Derkaev A.A., Shcheblyakov D.V., Dolzhikova I.V., Favorskaya I.A. et al. rAAV expressing recombinant antibody for emergency prevention and long-term prophylaxis of COVID-19. *Front. Immunol.* 2023;14. DOI: 10.3389/fimmu.2023.1129245.
 12. Derkaev A.A., Ryabova E.I., Esmagambetov I.B., Shcheblyakov D.V., Godakova S.A., Vinogradova I.D. et al. rAAV expressing recombinant neutralizing antibody for the botulinum neurotoxin type A prophylaxis. *Front. Microbiol.* 2022;13:960937. DOI: 10.3389/fmicb.2022.960937
 13. Ryabova E.I., Derkaev A.A., Esmagambetov I.B., Shcheblyakov D.V., Dovgii M.A., Byrikhina D.V., Prokofiev V.V., Chemodanova I.P. Comparison of Different Technologies for Producing Recombinant Adeno-associated Virus on a Laboratory Scale. *BIOpreparations. Prevention, Diagnosis, Treatment.* 2021;21(4):266–278. (In Russ.). DOI: 10.30895/2221-996X-2021-21-4-266-278.
 14. Pligina K.L., Zhanataev A.K., Chaika Z.V., Durnev A.D. Method of Cytogenetic Analysis of Mouse Oocytes. *Bulletin of Experimental Biology and Medicine.* 2013;156(7):128–132. (In Russ.). DOI: 10.1007/s10517-013-2292-0.
 15. Tsuchiya S., Sueoka K., Matsuda N., Tanigaki R., Asada H., Hashiba T. et al. The “spanning protocol”: a new DNA extraction method for efficient single-cell genetic diagnosis. *J. Assist. Reprod. Genet.* 2005;22(11–12):407–414. DOI: 10.1007/s10815-005-7482-x.
 16. Ma H., Bell K.N., Loker R.N. qPCR and qRT-PCR analysis: Regulatory points to consider when conducting biodistribution and vector shedding studies. *Mol. Ther. Methods Clin. Dev.* 2021;20:152–168. DOI: 10.1016/j.omtm.2020.11.007.
 17. Martín-Coello J., Gonzalez R., Crespo C., Gomendio M., Roldan E.R.S. Superovulation and in vitro oocyte maturation in three species of mice (*Mus musculus*, *Mus spretus* and *Mus spicilegus*). *Theriogenology.* 2008;70(6):1004–1013. DOI: 10.1016/j.theriogenology.2008.06.002.
 18. Okada H., Hirose Y., Manonmani P., Ito M., Sankai T. Zona-float method for separating mouse eggs from other cells. *Exp Anim.* 2004;53(4):355–359. DOI: 10.1538/expanim.53.355.
 19. Mahrous E., Yang Q., Clarke H.J. Regulation of mitochondrial DNA accumulation during oocyte growth and meiotic maturation in the mouse. *Reproduction.* 2012;144(2):177–185. DOI: 10.1530/REP-12-0113.
 20. Forootan A., Sjöback R., Björkman J., Sjögreen B., Linz L., Kubista M. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomol. Detect. Quantif.* 2017;12:1–6. DOI: 10.1016/j.bdq.2017.04.001.
 21. Andrade G.M., del Collado M., Meirelles F.V., da Silveira J.C., Perecin F. Intrafollicular barriers and cellular interactions during ovarian follicle development. *Anim. Reprod.* 2016;16(3):485–496. DOI: 10.21451/1984-3143-AR2019-0051.
 22. Zhou H., Ohno N., Terada N., Saitoh S., Fujii Y., Ohno S. Involvement of follicular basement membrane and vascular endothelium in blood follicle barrier formation of mice revealed by “in vivo cryotechnique.” *Reproduction.* 2007;134(2):307–317. DOI: 10.1530/REP-07-0062.
 23. Krivonogova A.S., Bruter A.V., Makutina V.A., Okulova Y.D., Ilchuk L.A., Kubekina M.V. et al. AAV infection of bovine embryos: Novel, simple and effective tool for genome editing. *Theriogenology.* 2022;193:77–86. DOI: 10.1016/j.theriogenology.2022.09.007.
 24. Sato M., Sato-Yamamoto N., Wakita A., Haraguchi M., Shimonishi M., Okuno H. Direct Injection of recombinant AAV-containing solution into the oviductal lumen of pregnant mice caused *in situ* infection of both preimplantation embryos and oviductal epithelium. *Int. J. Mol. Sci.* 2022;23(9):4897. DOI: 10.3390/ijms23094897.
 25. Kanatsu-Shinohara M., Lee J., Miyazaki T., Morimoto H., Shinohara T. Adeno-associated-virus-mediated gene delivery to ovaries restores fertility in congenital infertile mice. *Cell Rep. Med.* 2022;3(5):100606. DOI: 10.1016/j.xcrm.2022.100606.
 26. Urbanelli L., Buratta S., Tancini B., Sagini K., Delo F., Porcellati S. et al. The Role of Extracellular Vesicles in Viral Infection and Transmission. *Vaccines (Basel).* 2019;7(3):102. DOI: 10.3390/vaccines7030102.
 27. Hu X.L., Zhou X.P., Qian Y.L., Wu G.Y., Ye Y.H., Zhu Y.M. The presence and expression of the hepatitis B virus in human oocytes and embryos. *Hum. Reprod.* 2011;26(7):1860–1867. DOI: 10.1093/humrep/der103.
 28. Ajayi A.F., Akhigbe R.E. Staging of the estrous cycle and induction of estrus in experimental rodents: an update. *Fertil. Res. Pract.* 2020;6:5. DOI: 10.1186/s40738-020-00074-3.
 29. Aizawa R., Ibayashi M., Mitsui J., Tsukamoto S. Lipid droplet formation is spatiotemporally regulated in oocytes during follicular development in mice. *J. Reprod. Dev.* 2024;70(1):18–24. DOI: 10.1262/jrd.2023-055.
 30. Pupo A., Fernández A., Low S.H., François A., Suarez-Amaran L., Samulski R. J. AAV vectors: The Rubik’s cube of human gene therapy. *Mol. Ther.* 2022;30(12):3515–3541. DOI: 10.1016/j.ymthe.2022.09.015.
 31. Batista A.R., King O.D., Reardon C.P., Davis C., Shankara-

- charya, Philip V. et al. Ly6a Differential Expression in Blood-Brain Barrier Is Responsible for Strain Specific Central Nervous System Transduction Profile of AAV-PHP.B. *Hum. Gene Ther.* 2020;31(1–2):90–102. DOI: 10.1089/hum.2019.186.
32. Walkey C.J., Snow K.J., Bulcha J., Cox A.R., Martinez A.E., Ljungberg M.C. et al. A comprehensive atlas of AAV tropism in the mouse. *Mol. Ther.* 2025;33(3):1282–1299. DOI: 10.1016/j.ymthe.2025.01.041.
33. Zhao J., Yue Y., Patel A., Wasala L., Karp J.F., Zhang K. et al. High-resolution histological landscape of AAV DNA distribution in cellular compartments and tissues following local and systemic injection. *Mol. Ther. Methods Clin. Dev.* 2020;18:856–868. DOI: 10.1016/j.omtm.2020.08.006.
34. Sarkar R., Xiao W., Kazazian H.H. A single adeno-associated virus (AAV)-murine factor VIII vector partially corrects the hemophilia A phenotype. *J. Thromb. Haemost.* 2003;1(2):220–226. DOI: 10.1046/j.1538-7836.2003.00096.x.
35. Ferla R., Alliegro M., Marteau J.B., Dell'Anno M., Nusco E., Pouillot S. et al. Non-clinical safety and efficacy of an AAV2/8 vector administered intravenously for treatment of mucopolysaccharidosis type VI. *Mol. Ther. Methods Clin. Dev.* 2017;6:143–158. DOI: 10.1016/j.omtm.2017.07.004.

Author Contribution

Malikova A.D. – conception and design, administration of the vector into the animals, selection of experimental conditions, data analysis and interpretation, and drafting of the initial manuscript. Zhanataev A.K. and Esmagambetov I.B. – conception, analysis of results and literature sources, drafting of the initial manuscript, and scientific editing. Anisina E.A. – work with laboratory animals, conducting PCR assays, processing of the obtained data, and revision of the manuscript. Pligina K.L. – work with laboratory animals, oocyte isolation, processing of the obtained data, and revision of the manuscript. Chaika Z.V. – DNA isolation, processing of the obtained data, and revision of the manuscript. Ryabova E.I., Dovgiy M.A., and Hossain R.M. – production and purification of vector particles and revision of the manuscript. Durnev A.D. – conception, analysis of results, revision of the manuscript, critical revision for important intellectual content. Dorofeev V.L. – critical revision for important intellectual content and final approval of the manuscript for publication.

Author Information

Malikova Aleksandra D. – Junior Researcher, Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies, Moscow, malikova_ad@academpharm.ru, <https://orcid.org/0009-0001-2417-6620>

Zhanataev Aliy K. – Cand. Sci. (Biol.), Leading Researcher, Head of the Laboratory of Genetic and Reproductive Toxicology, Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies, Moscow, zhanataev_ak@academpharm.ru, <https://orcid.org/0000-0002-7673-8672>

Esmagambetov Ilias B. – Cand. Sci. (Biol.), Leading Researcher, Head of the Laboratory of Stromal Cell Immune Regulation, Gamaleya National Research Center for Epidemiology and Microbiology, Moscow, iesmagambetovib@yandex.ru, <https://orcid.org/0000-0002-2063-2449>

Anisina Elena A. – Senior Researcher, Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies, Moscow, anisina_ea@academpharm.ru, <https://orcid.org/0000-0002-7542-5658>

Pligina Kira L. – Cand. Sci. (Biol.), Senior Researcher, Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies, Moscow, pligina_kl@academpharm.ru, <https://orcid.org/0000-0003-2026-5260>

Chaika Zlata V. – Researcher, Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies, Moscow, chajka_zv@academpharm.ru, <https://orcid.org/0000-0001-5366-4917>

Ryabova Ekaterina I. – Junior Researcher, Gamaleya National Research Center for Epidemiology and Microbiology, Moscow, ryabovaei96@gmail.com, <https://orcid.org/0000-0002-2687-5185>

Dovgiy Mikhail A. – Junior Researcher, Gamaleya National Research Center for Epidemiology and Microbiology, Moscow, imhail@yandex.ru, <https://orcid.org/0000-0002-0017-7784>

Hossain Rosa M. – Junior Researcher, Gamaleya National Research Center for Epidemiology and Microbiology, Moscow, xossain2013@gmail.com, <https://orcid.org/0009-0009-4483-2697>

Durnev Andrey D. – Dr. Sci. (Med.), Professor, Full Member of the Russian Academy of Sciences, Head of the Department of Toxicology, Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies, Moscow, durnev_ad@academpharm.ru, <https://orcid.org/0000-0003-0912-7684>

Dorofeev Vladimir L. – Dr. Sci. (Pharm.), Professor, Acting Director General, Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies, Moscow, dorofeev_vl@academpharm.ru, <https://orcid.org/0009-0004-3584-3742>

(✉) **Malikova Aleksandra D.**, malikova_ad@academpharm.ru

Received on October 16, 2025;
approved after peer review on October 27, 2025;
accepted on October 30, 2025