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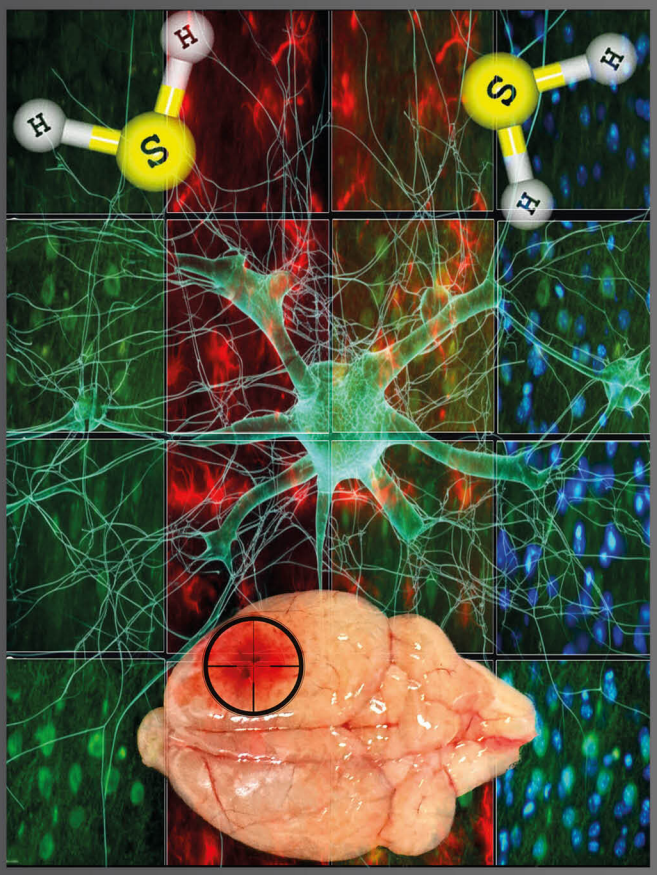

Hydrogen Sulfide as a Key Neuroprotective Gasotransmitter in Traumatic Brain Injury
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PARASITOLOGY
ПАРАЗИТОЛОГИЯ

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Original Empirical Research

Strategies of Equine Helminth Control in the Far East Region of Russia: A Pilot Study

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Abstract

Introduction. Equine helminthiasis, especially in the context of parasite resistance growth, remains an acute issue in the veterinary practices. In Russia, despite the availability of international guidelines, there are still no enough data on the parasite control measures really taken by the veterinary specialists and animal owners. The present pilot study aims at evaluating the level of awareness of horse owners and veterinary specialists about the advanced methods of combating helminth infections, as well as at analysing approaches to deworming and determining the possibility of using targeted treatment strategies in the Far East Region. The main objective is to identify the contradictions between the existing practices and scientific recommendations on prevention of anthelmintic resistance.

Materials and Methods. The study was conducted in September–October 2024 and was based on a questionnaire survey held among horse owners and veterinary specialists in the Far East Region of Russia. Several owners from other regions were interviewed to compare deworming practices. In total, 148 people participated in the survey. Online questionnaires and individual interviews were used to collect data. Questionnaires included the questions about frequency and methods of deworming, the use of diagnostic tests, knowledge about resistance and sources of getting information. To identify statistical relationships, the data were processed using the methods of descriptive statistics, chi-square tests and logistic regression.

Results. In the frame of the study, it was found that in the Far East the methods of interval deworming of horses were the most popular among the horse owners and veterinarians. Respondents were highly aware of the problem of anthelmintic resistance (73.13% among the owners and 78.57% among the veterinarians), but only 17.56% of the owners used the Faecal Egg Count (FEC) prior to deworming. Frequent consultations provided by the veterinarians did not much help to persuade the owners to implement the diagnostic tests or adhere to the quarantine measures. 72.39% of owners and 75% of veterinarians preferred using the macrocyclic lactones, which could exacerbate the risk of anthelmintic resistance development. However, the use of the anthelmintics rotation practices by the majority of owners (82.14%) and veterinarians (71.43%) makes it possible to feel optimistic. Logistic regression has revealed that knowledge on parasitic diseases has a positive effect on the use of FECs.

Discussion and Conclusion. The pilot study revealed the gaps between the knowledge on helminth control methods and their practical implementation. The majority of owners and veterinarians are aware of the problem of anthelmintic resistance, however diagnostic testing remains insufficient. To efficiently combat parasites, it is necessary to increase the quality of consultation services and implement the targeted deworming practices based on diagnostic tests.

Keywords: pilot study, equine helminthiasis, anthelmintic resistance, strategies of control, questionnaire survey, diagnostics, macrocyclic lactones, Far East

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Оригинальное эмпирическое исследование

Стратегии борьбы с гельминтами лошадей на Дальнем Востоке России: пилотное исследование

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Аннотация

Введение. Гельминтозы лошадей, особенно в условиях нарастающей резистентности паразитов, остаются актуальной проблемой ветеринарной практики. Несмотря на наличие международных рекомендаций, в России сохраняется дефицит данных о реально проводимых противопаразитарных мероприятиях ветеринарными специалистами и владельцами животных. Целью настоящего пилотного исследования было оценить уровень осведомленности владельцев лошадей и ветеринарных специалистов о современных методах борьбы с гельминтами, проанализировать подходы к дегельминтизации и определить возможность использования целенаправленных стратегий лечения на Дальнем Востоке. Основной задачей было выявить несоответствия между существующей практикой и научно обоснованными рекомендациями по предупреждению развития резистентности гельминтов.

Материалы и методы. Исследование, проведенное в сентябре–октябре 2024 г., основывалось на анкетировании владельцев лошадей и ветеринарных специалистов в Дальневосточном регионе России. Для сравнения практик дегельминтизации опрошено несколько владельцев из других регионов. Всего в исследовании приняли участие 148 человек. Для сбора данных использовались онлайн-опросы и индивидуальные интервью. Анкеты включали вопросы о частоте и методах дегельминтизации, использовании диагностических тестов, знании о резистентности и источниках получения информации. Данные были обработаны с использованием описательной статистики, критерия хи-квадрат и логистической регрессии для выявления статистических взаимосвязей.

Результаты исследования. В ходе исследования было установлено, что среди владельцев лошадей и ветеринарных врачей на Дальнем Востоке распространены интервальные методы обработки лошадей против гельминтов. Осведомленность у респондентов о проблеме резистентности была высокой (73,13 % у владельцев и 78,57 % у ветеринаров), но только 17,56 % владельцев использовали тест FEC перед дегельминтизацией. Частое проведение консультаций ветеринарами не оказывает существенного влияния на применение диагностических исследований или соблюдение карантина владельцами. Макроциклические лактоны предпочитают использовать 72,39 % владельцев и 75 % ветеринаров, что может усугубить риск развития резистентности. Однако обнадеживает использование ротации антигельминтиков большинством владельцев (82,14 %) и ветеринаров (71,43 %). Логистическая регрессия показала, что знания о паразитарных болезнях положительно влияют на использование тестов FEC.

Обсуждение и заключение. Проведенное пилотное исследование выявило пробелы между знанием о методах борьбы с гельминтами и их практическим применением. Большинство владельцев и ветеринаров осведомлены о проблеме резистентности, однако проведение диагностических тестов остаётся недостаточным. Для эффективной борьбы с паразитами необходимо повышать уровень консультативного взаимодействия и внедрять целевые методы дегельминтизации на основе диагностических исследований.

Ключевые слова: пилотное исследование, гельминтозы лошадей, антигельминтная резистентность, стратегии борьбы, анкетирование, диагностика, макроциклические лактоны, Дальний Восток

Для цитирования. Дёмкина О.В. Стратегии борьбы с гельминтами лошадей на Дальнем Востоке России: пилотное исследование. *Ветеринарная патология.* 2025;24(2):7–18. https://doi.org/10.23947/2949-4826-2025-24-2-7-18

Introduction. The problem of bacterial and parasite resistance to various chemotherapeutic agents is becoming increasingly challenging in recent years. Anthelmintic resistance is not as widely known as antibiotic resistance, but it is a global problem that requires international cooperation and data exchange to develop efficient control strategies [1]. After the information about registering the signs of anthelmintic resistance in equine nematodes was disseminated across the scientific community worldwide, the issues of finding the causes and determining follow-up measures for this phenomenon became urgent [2]. Traditional methods, which include intensive use of anthelmintics and interval treatment without taking into account the diagnostic tests, are no longer efficient for preventing resistance [3]. Currently, equine cyathostomins and Parascaris have been proved to be resistant to benzimidazoles and pyrantel on all six continents. Cases of Parascaris resistance to macrocyclic lactones are also described. Since no new class of anthelmintics has been introduced during the last 40 years, it is important to know the current risk factors of using the antiparasitic agents to maintain their efficacy [4].

The advanced treatment strategies focus on diagnostics and targeted use of medicines. Selective therapy implies treatment of only those horses, which were diagnosed with infestation of high intensity [5]. Key techniques for assessing parasite load and anthelmintic resistance status are the Faecal Egg Count (FEC) and the Faecal Egg Count Reduction Test (FECRT) [6]. These techniques are the gold standard for determining the resistance status in practice, they enable selection of the optimal medicines and reduce the frequency of their use [7].

Surveys aimed at investigating the current helminth control strategies were conducted among the horse owners and veterinarians in a number of countries. The studies revealed that many owners were not fully aware about the new horse treatment methods [8]. The owners in the European part of the world were more keen in following the best practices of anthelmintic resistance control than on the American continent and in island countries (Australia, New Zealand, England, Ireland and Scotland). In Lithuania, at most of the stud farms, pasture rotation and scheduled deworming were the usual practice [9]. On stud farms in Germany, nematode infestation in adult horses decreased as a result of decreased frequency of treatments [10]. In Denmark, 97% of owners, who participated in the survey, used the FEC for diagnosis and monitoring, and 67% regularly changed medicines to prevent resistance [11]. In the United States, only 22% of respondents used Faecal Egg Counts, and less than 10% applied this test regularly [12]. In Australia, only 2.6% of horse owners carried out preliminary diagnostics, and the majority of respondents adhered to the traditional interval treatment method [13]. The surveys conducted in New Zealand revealed that racehorse trainers applied a six-times-a-year treatment schedule, whereas only 20.1% of respondents used the FEC [14]. In Brazil, 95.03% of owners regularly dewormed their animals, however only 54.30% of survey participants sought veterinary advice on the selection of the appropriate anthelmintic [15]. In Ireland, at 81.4% of horse farms, horses were treated 4–5 times a year. In Germany, 74.2% of veterinarians calculated the dosage of a medicine only by visual assessment of horse weights [16]. At horse yards in Scotland, macrocyclic lactones were the most often prescribed medicines; ivermectins and moxidectins were chosen for treatment in 90% of cases [17]. A study in the UK revealed that horse owners' intention to use the FEC for targeted treatment was influenced by social and veterinary service pressure, rather than by potential risk of animals being infected or resistance development [18].

At present, the issues of implementing the diagnostic tests, the anthelmintics used, their rotation, pasture rotation and other routine measures of preventing and combating equine helminthiasis are relevant for other countries and conditions too. With regard to the Russian Federation, such survey-based studies have never been conducted, although the problem of anthelmintic resistance in horses in Russia has already been recognised [19].

By conducting the survey among the horse owners and veterinary specialists in the Far East, the pilot study is targeted to achieve the following objectives: to assess the level of awareness about the anthelmintic resistance phenomenon; to obtain data on the use of the FEC and its influence on the choice of treatment strategy; to identify contradictions between the current practices of horse deworming and science-based recommendations; to evaluate the communication of the veterinary specialists and horse owners when choosing medicines and treatment strategies; to identify regional differences in preventing and combating helminth infections.

Materials and Methods. The questionnaire survey, conducted in September–October 2024, involved 148 respondents: private horse owners, owners of equestrian clubs, trainers and veterinary specialists living in the Far East Region, and in some other regions (to compare deworming practices). The main method of data collection was an online survey platform. Links to the platform were disseminated via the social networks and equestrian forums. Additional method used was conducting the individual interviews with respondents by completing a paper form.

The horse owner questionnaire included 19 questions, which were divided into thematic sections: general information (number of horses owned, geographic location, use of horses), deworming methods (frequency of using anthelminthics, types of medicines used, pasture management methods, methods of obtaining information on anthelmintics, dosages and timing of deworming, quarantine measures), diagnostic measures (use of the Faecal Egg Count (FEC) prior to deworming, carrying out the diagnostic procedures, influence of results obtained on treatment decisions and awareness (sources of getting information and advice on deworming, knowledge about the problem of anthelmintic resistance, concerns about horse health).

The questionnaire for veterinary specialists included 13 questions divided into sections: general information (geographic location, frequency of work with horses), awareness (knowledge about the problem of anthelmintic resistance), recommended and used methods of helminth control in horses (medicines used, schedules and timing of deworming, use of the FEC before deciding on treatment), communication with horse owners (consulting on equine helminthiasis and on choice of an anthelmintic). Deworming practices were assessed according to the answers received in the questionnaire regarding the frequency of using the anthelmintics, types of medicines used and rotation of anthelmintics. The respondents were asked to indicate the number of times a year they treat their horses against parasites, what medicines they used and whether they rotated them to prevent resistance. In the frame of the survey the questions about the sources of information on the

antiparasitic agents, dosages, timing of deworming and quarantine measures were asked. These data were used to assess compliance with the recommended deworming schedules and potential influence on helminth control efficiency. Most questions were closed-ended with multiple choice, in some cases there was an opportunity to express personal opinion in an extra response box. All participants were informed about the aim of the study, voluntary participation and anonymity.

The obtained data were processed in the *AtteStat* software using the descriptive statistics techniques (mean and median values for numerical variables), comparative analysis (chi-square tests to study the correlation of the factors within and across the groups), logistic regression (creating a model of using the FEC if observing the recommendations). The significance of discrepancies was accepted at a p-value of < 0.05.

Research Results

Descriptive analysis

Usually, deworming is referred to the preventive measures, and its frequency is determined by the animals' condition, the epizootic situation and the Regulations of the local veterinary services. There are currently no clear instructions in the acting Orders, Rules and Regulations of the Russian Federation stipulating a specific schedule of deworming in horses. The text of the "Veterinary Rules for Keeping Horses for the Purposes of Reproduction, Rearing, Sale, and Use" refers to the veterinary rules, list of anti-epizootic measures and decisions on regionalization[1] [20]. The "Veterinary Regulation on the Transportation of Sport Horses on the Territory of the Russian Federation" stipulates the procedure for conducting diagnostic tests and deworming only during transportation of horses[2]. Thus, the need to evaluate the current helminth control strategies focusing on the extent of their compliance with the science-based recommendations is quite evident.

The total number of respondents, who participated in the survey, was 134 — among the horse owners, and 14 — among veterinary specialists. We planned to conduct the survey in the Far East Region (Amur Region, Khabarovsk and Primorsky Territories, Yakutia, Jewish Autonomous Region), however online links to the survey got disseminated in the other regions and even countries — one horse owner from Belarus and one from Europe took part in the survey (unfortunately, the owner did not specify the country he was from). The questionnaires of owners from other regions were included for comparison of deworming practices. In the "owners" category, 23 questionnaires were incomplete: the number of missing answers varied from one to four, which averaged 11%. Such questionnaires were not excluded from the analysis. Thus, the number of questionnaires suitable for processing equalled to 89%. In the "veterinarians" category, one questionnaire was incomplete with one answer missing, however it was not excluded from the analysis either.

Demographic data

When processing the demographic part of the questionnaires in the "owners" group, the geographic distribution of respondents revealed their concentration in the Far East (63.8%), followed by the Central Russia (30.7%), then the South of Russia, Moscow Region, Bashkortostan, Yakutia, Belarus and a European country (5.5%) (Fig. 1).

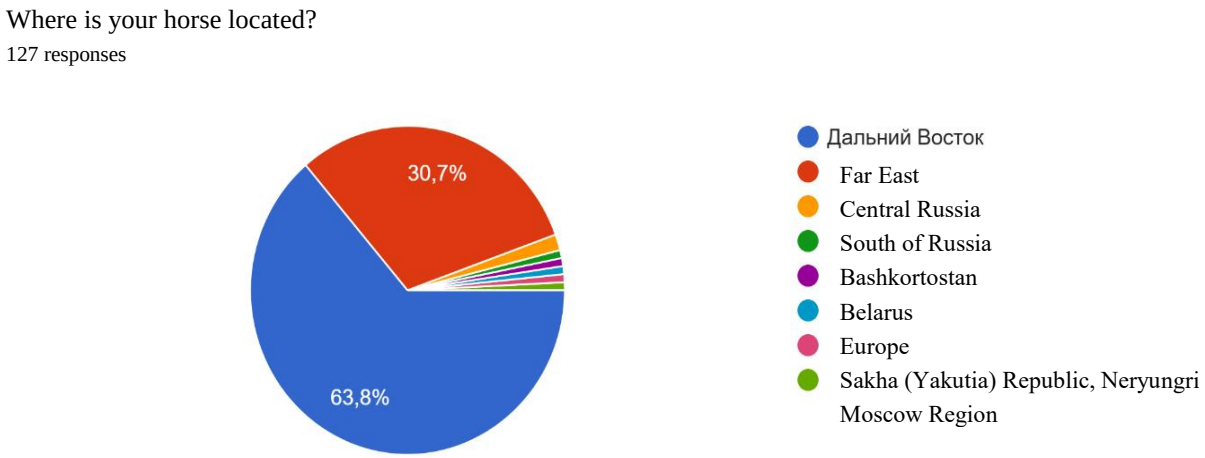

Fig. 1. Geographic distribution of horse owners

[1] On the Approval of the Veterinary Rules for Keeping Horses for the Purposes of Reproduction, Rearing, Sale, and Use. Order No. 939 of December 26, 2023. (In Russ.) URL: http://publication.pravo.gov.ru/document/0001202402070015 (accessed: 07.06.2025).

[2] Veterinary Regulation on the transportation of sport horses on the territory of the Russian Federation. (Adopted by the Ministry of Agriculture of Russia on September 28, 2005). (In Russ.) URL: https://www.consultant.ru/law/podborki/veterinarnye_pravila_peremescheniya_sportivnyh_loshadej_po_territorii_rossijskoj_federacii/(accessed: 07.06.2025).

In the “veterinarians” group, 35.74% of doctors were geographically located in the Far East, the rest were distributed among other regions (Moscow Region, Moscow, Irkutsk, Khanty-Mansi Autonomous Area, Nizhny Novgorod, Orenburg, Kurgan Region, Tambov Region, Vladimir Region). All veterinarians, who participated in the survey, were constantly (50%) or periodically (50%) working with horses.

Most respondents in the “owners” group identified themselves as private horse owners (87.2%), a smaller part were club managers (7.2%) and trainers (5.6%) (Fig. 2).

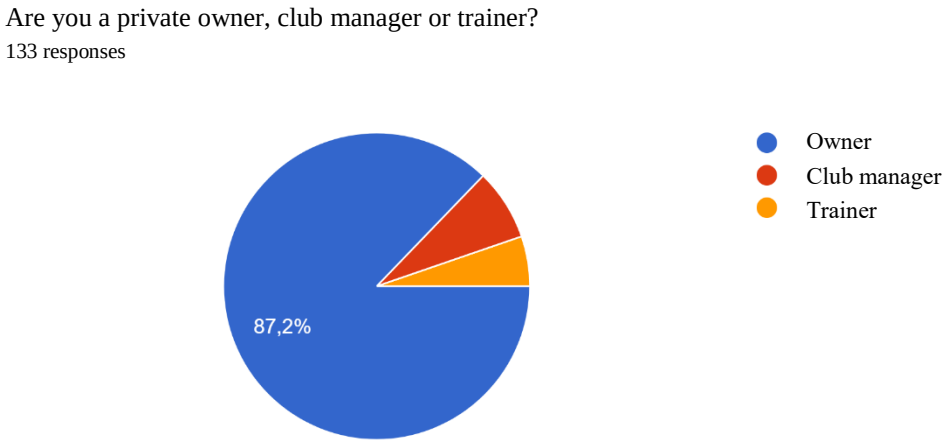

Fig. 2. Identification of respondents

44.44% of owners owned one horse, and 27.41% owned two horses. 28.15% of respondents owned more than three horses, of which 8 people owned 10–25 horses, and one had a herd of 100 horses. Five trainers and one club manager answered that they did not own horses.

By the conditions of keeping horses, the answers had distributed as follows: 59.7% of horses were kept in equestrian clubs; 32.09% — on the private farms and farmsteads; 8.21% — in herds.

Awareness of respondents

When answering the questions about the level of knowledge on equine parasitic diseases, about harm they can have to the health of animals and the problem of anthelmintic resistance in horse breeding, the majority of owners (98%) said that they understood the harm of helminthic diseases to the health of horses. Awareness of the anthelmintic resistance was high in both groups of respondents: 78.57% of veterinarians were aware of the problem, whereas some contradiction was found in the owners group: 73.13% responded that they were familiar with anthelmintic resistance as a highly specific problem for horse breeding, but when it came to answering a control question about parasitic diseases, 66.4% of owners responded that their knowledge in this area was limited, 5.2% knew nothing, and only 28.4% were well informed (Fig. 3).

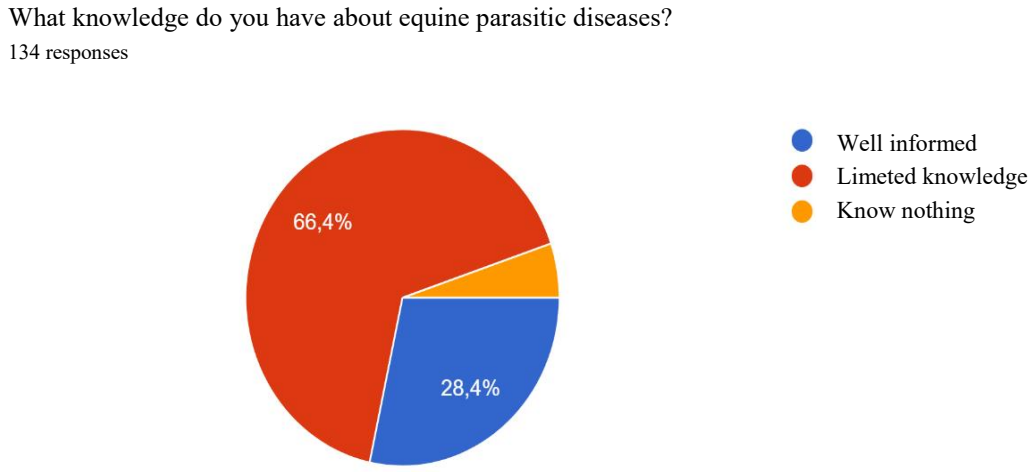

Fig. 3. Owners’ awareness of equine parasitic diseases

Deworming Practices

The questions of this section were aimed at identifying the schedule of deworming, the use of drug rotation, and the types of anthelmintics. In the "owners" group, 49.2% of respondents treated their horses twice a year, in spring and autumn; 42.4% — four times a year; 6.6% — once a year; and 1.8% — did not do this at all (Fig. 4).

Among veterinarians, the approach to treatment of horses suffering from helminthic diseases was rather different: 50% performed and recommended deworming four times a year; 35.7% – twice a year; 7.1% — three times a year; 7.2% — 3–4 times a year (Fig. 5).

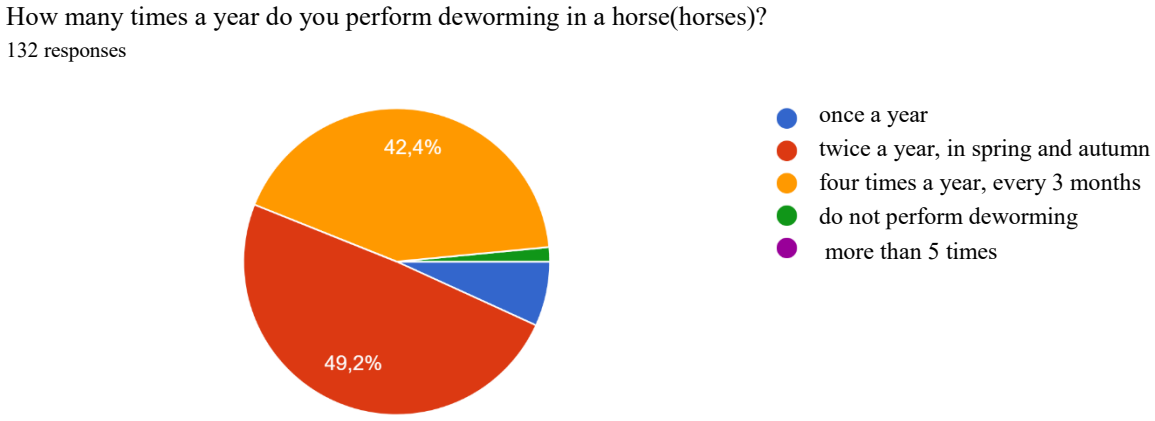

Fig. 4. Horse deworming schedules used by the owners

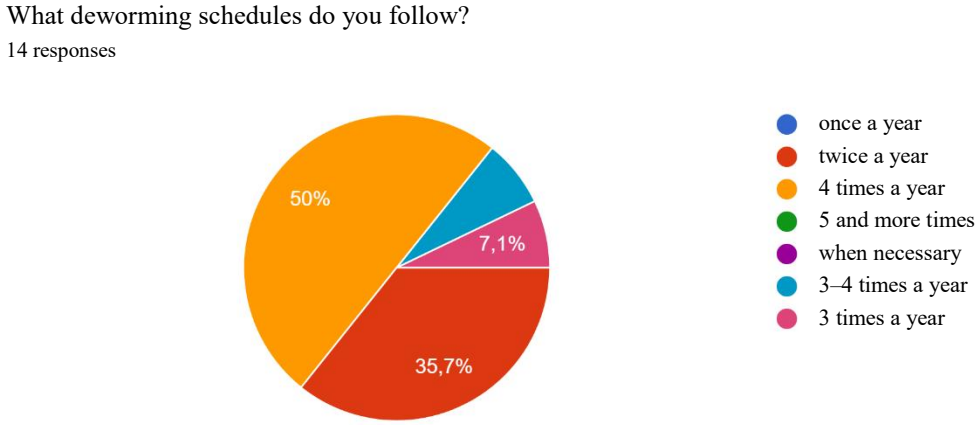

Fig. 5. Horse deworming schedules used by the veterinarians

Of the therapeutic agents, 75% of veterinarians preferred using macrocyclic lactones. However, in an open-ended question, most doctors indicated that in addition to lactones, they also used anthelmintics of other groups (tetrapyrimidines, benzimidazoles, pyrazinoisoquinolines). Two doctors used only lactones, two — only tetrapyrimidines. Such active use of lactones by the veterinarians goes in compliance with the global trends, and, at the same time, raises concerns about the possibility of resistance development due to frequent use [21]. Among horse owners, ivermectin-containing drugs proved to be also popular, with 72.39% using them on a regular basis. Anthelmintics of other groups were preferred by 12% of horse owners, who participated in the survey. 15.67% of respondents did not know, what medicine was used to treat their horses. Bearing in mind that the main part of the study sample was represented by the livestock in private ownership, in the future, constant monitoring of the therapeutic efficacy of macrocyclic lactones and possible use of alternative anthelmintics, where necessary, is desirable.

To calculate the dosage of anthelmintics, the owners commonly determined the horse's weight by visual inspection, by eye. This method was used by 59.5% of respondents. This popular method of calculating the dosage can lead to overestimation or underestimation of the required amount of the medicine for treatment [22]. Only 13.7% of owners used scales to determine the exact weight of horses, and 26.7% used a measuring tape for calculations.

Communication of veterinarians and owners

The questionnaires included questions on consultating, seeking consultation, and impact of consultations on the diagnostic procedures, choice of an anthelmintic, and quarantine decisions. The majority of owners (80%) said they consulted with veterinarians. Veterinarians also reported about providing regular consultations to the owners on prevention of equine helminthiasis (92.86%).

The majority of veterinarians reported to be aware of the anthelmintic resistance problem, but only half of those who participated in the survey performed the FEC prior to deworming (52.86%) and recommended this to the owners (50%). Although, the majority of the owners claimed being aware of the problem of anthelmintic resistance, only 17.56% of respondents noted performing the FEC prior to deworming; 6.14% did not know the reason for diagnostic testing of a horse; 76.3% did not perform the diagnostic tests because no one offered them. Only 14.5% of the owners received recommendations from the veterinarians to perform the necessary diagnostics to enable optimal selection of an anthelmintic. However, veterinarians noted that in 85.71% of cases, the owners sought their advice on purchasing a medicine without preliminary diagnostics.

Only 32.3% of the owners buy medicines for treating horses based on the advice of the veterinarians, the remaining 67.7% use information from the Internet, advice of other horse owners, trainers, or did not participate in choosing an anthelmintic because the club purchased the medicine for all horses at once (Fig. 6).

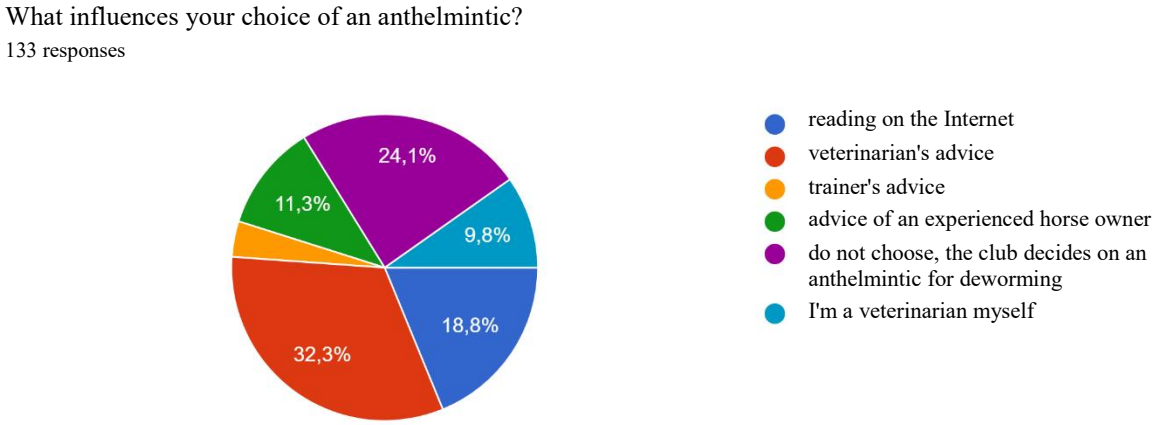

Fig. 6. Sources of information used by the owners for choosing an anthelmintic

Measures for the prevention and control of helminthiasis

Despite the consultative communication, such an important measure as quarantine of newly arrived animals was often neglected — 85.71% of veterinarians answered negatively to the question about the owners adhering to quarantine. However, horse owners took care of daily cleaning of horse loose boxes (95%) and rotation of pastures (65%) for reducing the parasite load. Despite the preference for lactones, a high percentage of horse owners (82.14%) and veterinarians (71.43%) reported about rotating the anthelmintics. This method is recommended for reducing the risk of resistance development, although its efficacy depends on correct application and compliance with recommendations.

When asked a question: "Is it necessary to replace the traditional horse deworming schedule (2 or 4 times a year) by an individual one (depending on the parasite load)?", 37.5% of the veterinarians answered positively, and 35.7% answered that the change of the approach would be impossible due to additional costs for the owners and a time delay. It has to admitted, for horse owners, changing traditional treatment and prevention schedules can result in additional costs on diagnostics. This is one of the reasons why only 42% of the owners, who participated in the survey, considered implementation of new deworming schedules to be expedient, while 58% were quite satisfied with the current situation.

Statistical processing of the obtained data

The questions and answers about the number of horses owned were processed using descriptive statistics. The mean value, confidence intervals and median value were calculated (Table 1).

Table 1

Descriptive statistics data on the number of horses owned by the owners

Sample size	Mean	Lower Confidence Bound, 95%	Upper Confidence Bound, 95%	Median
134	3,4	1,8	5,0	1

The obtained median indicates that half of the owners have one or less horses, which reflects the central tendency for the sample. The obtained data are distributed asymmetrically, the mean is significantly higher than the median. This confirms the presence of several owners with a large number of horses in the sample. Confidence intervals show that with a 95% probability, the true mean number of horses for each owner in the general totality is between 1.8 and 5.0.

To compare and ascertain the statistical significance of parameters within the groups, the data were processed using the chi-square test. A threshold for statistical significance was set at a 0.05 p-value. For the "owners" group, it was found, how the factor of awareness of parasitic diseases was related to the other factors that were important for the development of resistance (Table 2).

Table 2

Relationship between the awareness of equine parasitic diseases and various factors: results of the analysis obtained among the owners using the chi-square test

Factor	chi-square	p-value	Statistical significance
Performing the FEC prior to deworming	5,32	0,021	yes
Seeking veterinarian's advice	3,14	0,077	no
Rotation of anthelmintics	2,47	0,116	no
Regular rotation of pastures	4,68	0,030	yes
Regular cleaning of horse loose boxes	1,92	0,165	no
Understanding the need of changing a deworming schedule	6,09	0,014	yes
Use of ivermectins	24,95	< 0,001	yes

Significant results ($p < 0.05$) were obtained for the following factors: performing the FEC prior to deworming, regular pasture rotation, need to change the deworming schedule and use of Ivermectin by the owners. The owners who have knowledge on equine parasitic diseases understand the importance of diagnostic tests and pasture rotation for reducing helminth egg contamination. However, despite their knowledge, such owners use Ivermectin more intensively to deworm horses and do not see the need to rotate the medicines. Regular cleaning of loose boxes did not prove its relation to the knowledge on parasitic diseases. The routine nature of this procedure, must have prevented the owners from relating it to solving the problem of animal infestation. The owners did not receive their knowledge from veterinarians — no statistical relationship between these two factors was found. This opens up prospects for improving consultative communication.

To identify statistically significant differences in horse deworming practices used by the owners from different regions, Analysis of Variance (ANOVA) was used to determine whether the approach to deworming differed across the regions. For this purpose, the answers to the questions "How many times a year do you perform deworming?" and "Where is your horse kept?" were used. The null hypothesis was the statement that the mean values of deworming frequency are the same in all regions. After processing the data, we obtained the following results: F-statistics: 1.19 and p-value: 0.073. Since the p-value is greater than the significance level (0.05), we cannot reject the null hypothesis. This means that although there may be some small differences in the frequency of procedures across the regions, they are not big enough to be considered statistically significant. Owners in all the regions have similar deworming practices.

To establish a statistical relationship of the factors between the "owners" and "veterinarians" groups, the answers to the questions were analysed using the chi-square test. Due to the small sample in the "veterinarians" group, it would be more appropriate to use the Fisher's criterion for statistical processing, however not all the answers in both groups were binary, which does not meet the requirements for using this criterion (Table 3).

Table 3

Relationships of the factors for conducting the diagnostics and treatment between the "veterinarians" and "owners" groups

Factors		chi-square test	p-value
veterinarians (n=14)	owners (n=134)		
Performing FEC			
Do you perform FEC prior to deworming?	Have you ever performed FEC prior to deworming?	3,827	0,0504
Recommendation and use of FEC			
Do you suggest the owners to perform FEC prior to deworming?	Have you ever performed FEC prior to deworming?	0,0	1,0
Frequency of deworming			
How many times a year do you perform deworming?	How many times a year do you perform deworming?	10,73	0,0133

The veterinarians and the owners have different attitudes towards performing the FEC prior to deworming, even though the difference reached the threshold of statistical significance. No statistically significant relationship was found between the recommendation of the veterinarians to perform the FEC and its actual performing by the owners. Possibly, that was because the owners did not always follow the recommendations, or the recommendations did not affect significantly their behaviour. Statistically significant differences were found between the veterinarians and the owners in the frequency of deworming in the schedules used: the value of 10.73 means that the veterinarians and the owners have different approaches to this issue.

To forecast FEC performing by the owners under the influence of various factors, a logistic regression model was built. The variable value in this model was the FEC, and the constant values were awareness of parasitic diseases and resistance, as well as seeking veterinarian's advice for choosing an anthelmintic (Table 4).

Table 4

Logistic model indicators of FEC performing by the owners under the influence of the factors

Factors	Coefficients	Influence	Model accuracy
Knowledge on equine parasitic diseases	0,334	positive	87 %
Awareness of the problem of anthelmintic resistance	0,104	weak positive	
Consultations with veterinarians	-0,036	weak negative	

The positive coefficient of knowledge on parasitic diseases means that the better the owner's knowledge, the higher is probability of performing FEC by him. The anthelmintic resistance awareness factor has a weak positive coefficient indicating its small effect on the probability of performing FEC. The owners were aware of resistance but might not understand the importance of regular testing. Consultation with veterinarians reduced the probability of performing FEC. This could be due to the fact that the owners relied on drug recommendations received and saw no need in diagnostic testing. This logistic regression model forecasts with 87% accuracy that as the effects of the factors increase, the owners will more often perform diagnostic tests in horses prior to deworming.

Discussion and Conclusion. This pilot study aimed to evaluate the existing helminth control practices used by the horse owners and veterinary specialists in the Russian Far East. The obtained results indicate predominance of the traditional deworming practices among the veterinarians and horse owners consisting of the routine interval treatments. Such kind of an interval schedule is considered a factor contributing to the development of parasite resistance to anthelmintics.

Despite the high level of awareness of anthelmintic resistance in both groups, there is a significant gap between the knowledge and implementation of the science-based strategies, such as the use of the Faecal Egg Count (FEC) prior to deworming. Only 17.56% of the owners performed the FEC prior to deworming, and just over half of veterinarians performed the FEC or recommended it to be performed prior to treatment. Consultations of the owners with veterinarians, do not significantly affect owners' adherence to diagnostic testing or quarantine. It makes feel optimistic that major part of the owners and veterinarians reported using drug rotation, however, in the absence of diagnostic tests, which should be a basis for making decisions on treatment and drug selection, mere rotation becomes insufficient for reducing the risk of resistance development.

The owners usually estimate the weight of their horses visually when calculating the medicines, which can lead to incorrect dosage and reduce the efficacy of therapy. Excessive use of macrocyclic lactones by the owners and veterinarians can accelerate the emergence of resistant parasite populations.

The statistical processing of data allows us to make several significant conclusions. Descriptive statistics showed that half of the private owners, who participated in the survey, owned one horse, and the other half owned more than three horses. The average number of horses in the general totality with a probability of 95% is within 1.8–5.0.

Analysis using the chi-square criterion revealed statistically significant relationships between performing FEC prior to deworming, regular pasture rotation, the need to change deworming schedules and the use of Ivermectin ($p < 0.05$) in the group of owners who have knowledge on parasitic diseases. Aware owners more often perform the diagnostic tests and understand the importance of pasture rotation for reducing the level of infestation. The widespread use of Ivermectin indicates the gaps in understanding importance of comprehensive approaches to helminthiasis prevention and control. The lack of correlation between veterinarians' consultations and the level of owners' knowledge highlights the importance of improving communication.

Analysis of Variance (ANOVA) ($F=1.19$, $p=0.073$) showed absence of any significant differences in the frequency of deworming accepted among the owners from different regions.

Comparison of the "owners" and "veterinarians" groups showed that differences in performing FEC are close to statistical significance. At the same time, a weak relationship was found between the recommendations of the veterinary specialists to perform FEC and actual performing the test by the owners. Between the groups, frequency of deworming differs more significantly.

Logistic regression showed that knowledge on parasitic diseases has a positive effect on the probability of performing FEC, whereas the awareness of resistance and consultations with veterinarians have a weak effect. The model with an accuracy of 87% allows us to forecast that the owners will more often conduct diagnostics prior to deworming in case the significance of these factors increases.

The following limitations of this study should be mentioned: relatively small number of respondents, which makes it difficult to apply the generalized results to a wider sample, and does not fully reflect the general totality. Using questionnaire data may also cause an inaccuracy, as participants may have misrepresented the real data perceiving them to be "incorrect" and indulge in wishful thinking. In addition, the data obtained reflect the status quo at the time of the study and do not reflect changes over time. Future survey studies should expand the scope of topics covered by the questions and statistical methods for data processing to gain a more detailed understanding of the situation.

The findings revealed the need to increase the level of knowledge among the owners, implement the targeted methods of deworming preceded by the diagnostic tests, calculate the drug dosage based on the accurate weight measurements, and adhere to quarantine measures. The veterinary specialists play a leading role in communication with the owners, therefore strengthening partnership is required for efficient combating the parasites. In conclusion, it should be noted that the present study was a pilot one and can be used as a basis for studying the current situation with the methods of diagnostics, prevention, and control of parasitic diseases in animals in other regions.

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ANIMAL PATHOLOGY, MORPHOLOGY, PHYSIOLOGY, PHARMACOLOGY AND TOXICOLOGY

ПАТОЛОГИЯ ЖИВОТНЫХ, МОРФОЛОГИЯ, ФИЗИОЛОГИЯ, ФАРМАКОЛОГИЯ И ТОКСИКОЛОГИЯ

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Original Empirical Research

H_2S-Dependent Mechanisms of Caspase-3 Expression and Localization in Brain Cells of Mice with Traumatic Brain Injury

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EDN: HPMRMX

Abstract

Introduction. Traumatic brain injury (TBI) is a neurotrauma widespread in animals. TBI causes a complex cascade of pathological processes: primary brain injury turns into secondary brain injury associated with inflammation, oxidative stress, excitotoxicity and apoptosis. Secondary injury aggravates the condition after injury. In this regard, the role of hydrogen sulfide (H_2S) as a gasotransmitter involved in neuromodulation, anti-inflammatory, antioxidant and anti-apoptotic processes in the central nervous system is of particular interest. Caspase-3 is an important element in TBI-induced apoptosis. H_2S has a potential to modulate the expression and activity of caspase-3 affecting the survival of nerve cells and brain recovery after TBI. However, H_2S-dependent mechanisms of caspase-3 regulation in traumatic injury are not fully investigated. The aim of the research is to study the role of H_2S in the expression and localization of caspase-3 in neurons and astrocytes of mice with TBI.

Materials and Methods. The research was conducted at the Bioengineering Department of DSTU (Rostov-on-Don) from April 20 to June 1, 2024 in conditions compliant with the international and national standards. The objects of the study were 36 adult male mice divided into three groups: control group and two experimental ones. TBI was simulated by dropping a weight (200 g) on the intact skulls of mice anesthetized with chloral hydrate. During 7 days after the TBI, the animals were daily administered the sodium sulfide (Na_2S), a donor of H_2S, which can efficiently release H_2S, or the aminooxyacetic acid (AOAA), an inhibitor of cystathionine β-synthase (CBS), an enzyme responsible for the endogenous synthesis of H_2S, until the animals were withdrawn from the experiment. The use of Na_2S and AOAA enabled efficient modulation of the level of endogenous H_2S in the brain. The control group was administered physiological saline solution. Brain sections fixed in 4% paraformaldehyde (PFA) solution were incubated with antibodies to caspase-3 and to the neuronal nuclear antigen (NeuN) or to the astrocytic marker (GFAP). Colocalization was assessed using the *ImageJ* software. Caspase-3 expression in the brain penumbra was analysed by Western blotting using primary antibodies against caspase-3 and β-actin and secondary antibodies IgG conjugated to horseradish peroxidase. For detection, the chemiluminescence method was used.

Results. The initial level of caspase-3 in the brain cells of mice under study was low. Seven days after injury, TBI had induced caspase-3 expression in neurons and glial cells of the ipsilateral injured hemisphere in animals of all groups. Administering the donor Na_2S led to decrease of caspase-3 level in neurons by 32%, whereas administering the inhibitor AOAA led to its increase by 31% compared to the injured nerve cells of animals from the control group, which were administered the physiological saline solution. This was confirmed by the values of the M1 colocalization coefficient demonstrating colocalization of caspase-3-positive cells with the neuronal nuclear antigen (NeuN). Similar effects were demonstrated in astrocytes, which were visualized using the astrocyte-specific marker GFAP. Western blot analysis confirmed these data and showed a significant decrease of caspase-3 level with administering Na_2S and its increase with that of AOAA 7 days after TBI.

Discussion and Conclusion. The results of the study demonstrate that TBI leads to significant activation of caspase-3 in neurons and astrocytes of the injured hemisphere of mice brain, which means development of apoptosis in response to traumatic injury. Administering Na_2S has efficiently decreased caspase-3 level, which indicates its neuroprotective and

anti-apoptotic effect. Whereas, administering the AOAA has induced an increase in caspase-3 expression, which confirms the important role of CBS and, therefore, of H_2S in regulation of cell death after TBI. The reliability of these findings was ascertained by both immunohistochemical and Western blot analysis. The obtained data contribute to better understanding the fundamental H_2S-dependent signaling mechanisms of survival and death of neurons and glial cells during traumatic injury of the nervous system. The CBS inhibitor and H_2S donor used in our study may serve a basis for development of the clinically efficient neuroprotective agents.

Keywords: traumatic brain injury, hydrogen sulfide, H_2S, sodium sulfide, Na_2S, aminooxyacetic acid, AOAA, cystathionine β-synthase, CBS, caspase-3, apoptosis

Declaration of Compliance with the Principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes: the authors declare that all research was conducted in compliance with the principles of Good Laboratory Practice.

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Оригинальное эмпирическое исследование

H_2S-зависимые механизмы экспрессии и локализации каспазы-3 в клетках головного мозга мышей при черепно-мозговой травме

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Аннотация

Введение. Черепно-мозговая травма (ЧМТ) является распространенной нейротравмой среди животных. ЧМТ вызывает сложный каскад патологических процессов: первичное повреждение мозга переходит во вторичное, связанное с воспалением, окислительным стрессом, эксайтотоксичностью и апоптозом. Вторичное повреждение усугубляет состояние после травмы. В этой связи особый интерес представляет роль газотрансмиттера сероводорода (H_2S), который участвует в нейромодуляции, противовоспалительных, антиоксидантных и антиапоптотических процессах в центральной нервной системе. Важным элементом апоптоза при ЧМТ является каспаза-3. H_2S потенциально может модулировать экспрессию и активность каспазы-3, влияя на выживание нервных клеток и восстановление мозга после ЧМТ. Однако H_2S-зависимые механизмы регулирования каспазы-3 при травматическом повреждении до конца не изучены. Цель статьи — исследовать роль H_2S в экспрессии и локализации каспазы-3 в нейронах и астроцитах головного мозга мышей при ЧМТ.

Материалы и методы. Исследования проводились на кафедре «Биоинженерия» ДГТУ (г. Ростов-на-Дону) в условиях, соответствующих международным и национальным стандартам, в период с 20 апреля по 1 июня 2024 г. Объект исследования — 36 взрослых самцов мышей, поделенных на три группы: контрольную и две экспериментальные. Моделирование ЧМТ проводилось путем сброса груза (200 г) на необнаженный череп под анестезией хлоралгидратом. Донор H_2S сульфид натрия (Na_2S), способный эффективно высвобождать H_2S, либо ингибитор (аминооксиуксусная кислота, AOAA) цистатионин-β-синтазы (CBS), фермента, ответственного за эндогенный синтез H_2S, — вводились ежедневно после ЧМТ в течение 7 дней до выведения животных из эксперимента. Использование Na_2S и AOAA позволяло эффективно модулировать уровень эндогенного H_2S в головном мозге. Контрольной группе вводили физиологический раствор. Срезы мозга, фиксированные в 4 % PFA, инкубировались с антителами к каспазе-3 и нейрональному ядерному маркеру NeuN либо к астроцитарному маркеру GFAP. Колокализация оценивалась с помощью программы *ImageJ*. Анализ экспрессии каспазы-3 проводился в пенумбре мозга методом вестерн-блота с использованием первичных антител к каспазе-3 и β-актину и вторичных антител IgG, конъюгированных с пероксидазой хрена, детекция — хемилюминесцентным методом.

Результаты исследования. Изначальный уровень каспазы-3 в клетках головного мозга исследуемых мышей был низким. ЧМТ индуцировала экспрессию каспазы-3 в нейронах и глиальных клетках ипсилатерального поврежденного полушария через 7 дней во всех группах после травмы. Использование донора Na_2S приводило к сни-

жению уровня каспазы-3 в нейронах на 32 %, тогда как ингибитор AOAA вызывал его увеличение на 31 % относительно травмированных нервных клеток контрольной группы, которой вводили физиологический раствор, об этом свидетельствуют показатели коэффициента M1 колокализации каспазы-3-положительных клеток с маркером нейрональных ядер NeuN. Аналогичные эффекты были продемонстрированы и на астроцитах, которые визуализировались с помощью специфичного астроцитарного маркера GFAP. Вестерн-блот анализ подтвердил эти данные, показывая значительное снижение уровня каспазы-3 при использовании Na_2S и его увеличение при введении AOAA через 7 дней после ЧМТ.

Обсуждение и заключение. Результаты исследования демонстрируют, что ЧМТ приводит к значительной активации каспазы-3 в нейронах и астроцитах поврежденного полушария головного мозга мышей, отражая развитие апоптоза в ответ на травматическое повреждение. Применение Na_2S эффективно снижало уровень каспазы-3, указывая на его нейропротекторное и антиапоптотическое действие. В то же время введение AOAA индуцировало увеличение экспрессии каспазы-3, подтверждая важную роль CBS и, соответственно, H_2S в регуляции клеточной гибели после ЧМТ. Надежность этих наблюдений была подтверждена как иммуногистохимически, так и методом вестерн-блот анализа. Полученные данные помогут лучше понять фундаментальные H_2S-зависимые сигнальные механизмы выживания и гибели нейронов и глиальных клеток при травматическом повреждении нервной системы, а ингибитор CBS и донор H_2S, использованные в нашем исследовании, могут лечь в основу разработки клинически эффективных нейропротекторных препаратов.

Ключевые слова: черепно-мозговая травма, сероводород, H_2S, сульфид натрия, Na_2S, аминооксиуксусная кислота, AOAA, цистатионин-β-синтаза, CBS, каспаза-3, апоптоз

Декларация о соблюдении принципов Европейской конвенции о защите позвоночных животных, используемых для экспериментов и других научных целей: авторы заявляют, что все проведенные исследования соответствовали принципам конвенции и правилам надлежащей лабораторной практики.

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Introduction. Traumatic brain injury (TBI) is one of the most widespread causes of deaths and disabilities in animals. TBI leads to a complex cascade of pathophysiological processes, including primary mechanical injury of brain tissue and subsequent secondary injury due to inflammation, oxidative stress, excitotoxicity and apoptosis. Secondary injury developing after trauma significantly affects the progression of the severity of condition, therefore, scientists worldwide stive to find efficient treatment strategies and neuroprotective agents [1–3].

In recent years, the role of gasotransmitters, such as hydrogen sulfide (H_2S), in the pathophysiology of TBI is becoming an object of growing interest. For a long time H_2S was deemed to be just a toxic gas but now it is recognized as an important bioactive molecule capable of participating in a number of physiological and pathological processes, including neuromodulation, vascular regulation, anti-inflammatory activity and antioxidant protection. In the central nervous system, H_2S acts as a neuromodulator and neuroprotector, affecting various signaling pathways, including pathways of apoptotic cell death [4, 5].

One of the key pro-apoptotic proteins is caspase-3 — a cysteine protease that cleaves proteins specifically after aspartate and is an important effector of apoptosis. It has been demonstrated that caspase-3 can be a potential target for H_2S and its active derivatives. However, information about the role of H_2S-dependent mechanisms in the regulation of caspase-3 is contradictory. Some studies show that H_2S can decrease the expression of this enzyme [6–8], while other scientific papers demonstrate H_2S-dependent expression of caspase-3 and a cytotoxic effect [9–11]. Thus, the mechanisms enabling H_2S to affect the expression and localization of caspase-3 in TBI are still poorly understood [9].

The research aims to investigate H_2S-dependent mechanisms of caspase-3 regulation during TBI. We expect H_2S to be capable of modulating caspase-3 expression and localization, thereby affecting the intensity and duration of the neuroinflammatory response during TBI. Study of these mechanisms can contribute to better understanding the complex pathogenesis of TBI and open new perspectives for developing therapeutic strategies aimed at modulating neuroinflammation and improving recovery processes in the brain.

Materials and Methods

Ethical Approval

All studies were approved by the DSTU Ethics Committee and were held in compliance with the international bioethics requirements.

Object of Research and Procedures

The research was conducted in the “MedTsifra” laboratory of the Bioengineering Department, Bioengineering

and Veterinary Medicine Faculty of DSTU, from April 20 to June 1, 2024. In total, 36 adult male CD-1 mice aged 14 to 15 weeks and weighing 20–25 g were included in the experiment. The mice were kept under standard vivarium conditions: in plastic cages with sawdust bedding, at a temperature of 22 ± 2 °C, relative humidity of 50–60%, and a 12-hour light-dark regime. They were fed standard granulated feed for laboratory rodents, with free access to water.

The mice were divided into three groups: the control group, which was injected with physiological saline solution, and two experimental groups, which were injected with the sodium sulfide (Na_2S, 0.1 mg/kg; Khimikon, Russia) — the donor of H_2S, or the aminooxyacetic acid (AOAA, 5.0 mg/kg; Tianjin Xidian Chemical Technology Co., Ltd., China) — the inhibitor of cystathionine-β-synthase (CBS), respectively. The use of Na_2S and AOAA made it possible to efficiently modulate the level of endogenous H_2S in the brain: Na_2S is capable of efficient release of H_2S; and AOAA is an inhibitor of CBS, the enzyme responsible for the endogenous synthesis of H_2S. After injury, the drugs were administered intraperitoneally daily during 7 days until the animals were withdrawn from the experiment.

One and the same version of the standard protocol for simulating TBI was used for all groups. Mice were anesthetized by intraperitoneal injection of chloral hydrate (300 mg/kg). A weight was then dropped from a height of 3 cm on the intact skull. The coordinates of the weight drop were set as follows: 2 mm dorsal to bregma, 1 mm lateral to the midline. A weight-drop device consisting of a metal rod (with a tip of 3 mm in diameter, 5 mm in length) weighing 200 g was used to perform a stroke. After awakening from anesthesia, the mice returned to their usual place of staying [12].

Immunofluorescence Analysis

The following protocol was used to identify caspase-3 localization 7 days after TBI. The brain area around the focus of necrosis resulting from the stroke by a weight and the area from the left undamaged hemisphere were excised. The excised piece of cerebral cortex of a mouse was fixed in 4% PFA for 12 hours at +4°C with constant stirring. Then, 20-μm-thick sections were obtained using a Leica VT1000 S automatic vibrating blade microtome (Leica Biosystems Nussloch, Germany). To ensure background blocking, the sections were incubated for 60 min at +24°C in a 5% BSA and 0.3% Triton X-100 solution.

Then the sections were incubated with primary antibodies anti-CASP3 (1:100, Rabbit, AF6311, Affinity, China, antibody against caspase-3) and anti-NeuN (1:1000, Mouse, FNab10266, Fine Test, China, antibody against the neuronal nuclear antigen NeuN) for 48 hours at +4 °C. After repeated washing in PBS, the sections were incubated with secondary antibodies anti-Rabbit IgG (H+L) Fluor 488-conjugated (1:500; S0018, Affinity Biosciences, China) and anti-Mouse IgG (H+L) Fluor 594-conjugated (1:500; S0005, Affinity Biosciences, China). The sections were then immersed into glycerol and used as the specimens for analysis carried out by means of the Altami LUM 1 Fluorescent Microscope (Ningbo Haishu Honyu Opto-Electro Co., LTD, China in collaboration with "Altami" Co., Russia) equipped with a high-resolution digital camera EXCCD01400KPA (Hangzhou Toup Tek Photonics Co., Ltd., China).

The analysis of colocalization of caspase-3 and NeuN was carried out using a special Image J software package supplemented with the JACoP plugin [13]. Mean Fluorescence Intensity of caspase-3 was evaluated based on 10 photographs of sections for each mouse (from experimental and control groups) according to the following formula:

$$I_{norm} = \frac{I_{mean} - I_{back}}{I_{back}},$$

where I_{mean} is the mean intensity in the studied area, I_{back} is the mean background intensity.

Immunoblotting (Western blotting)

The expression of caspase-3 in conditions of activation or inhibition of the H_2S signaling pathway in the penumbra of the cerebral cortex of mice after TBI was studied using the method of Western blotting. Seven days after injury, the animals were decapitated, the brain was removed, and the infarcted area was excised on ice using a hollow cutter. Next, a 2-mm-wide ring corresponding to the penumbra area was excised using another cutter. These rings were compared with control cortex specimens taken from the other side of brain of the same mouse. Specimens were homogenized using the Tissue Lysis Buffer solution with protease and phosphatase inhibitors added. The resulting homogenate was then centrifuged to obtain a supernatant. The protein concentration in the supernatant was determined using the Bradford method. Then, 25 μg of protein in 20 μl were added to the wells in a polyacrylamide gel and subjected to electrophoresis in the presence of sodium dodecyl sulfate. Mini-PROTEAN Tetra (Bio-Rad, USA) was the equipment used. After electrophoresis, proteins were transferred to a nitrocellulose membrane by the semi-dry transfer using the Trans-Blot Turbo Transfer System (Bio-Rad, USA). Then, primary antibodies against caspase-3 (1:100, AF6311, Affinity, China) and against β-actin and secondary antibody anti-Rabbit IgG conjugated to horseradish peroxidase (HRP) (1:1000; S0001, Affinity Biosciences, China) were used. Then, chemiluminescent protein detection was carried out. Chemiluminescence was detected using the SH-Advance523 gel documentation system (Shenhua Science Technology Co., Ltd., China).

Statistical analysis

For statistical processing and analysis of the obtained results, the method of single-factor analysis of variance was used. Pairwise multiple comparisons were made using the Tukey's Honest Significant Difference (HSD). The parametric tests were used if the rules of normality and homogeneity of variances were met, which were assessed by the Shapiro-Wilk and Brown-Forsythe tests, respectively. If the assumption about normality and homogeneity of variances was not confirmed, the nonparametric Kruskal-Wallis test was used. Statistically significant reliability was considered at $p<0.05$. The SigmaPlot software package, version 12.5 (Systat Software, Inc., USA), was used for statistical analysis.

Results. Immunofluorescence microscopy demonstrated that caspase-3 localized in nerve and glial cells. Their nucleoplasm was visualized using the fluorochrome Hoechst 33342. Also, the analysis revealed colocalization of caspase-3 and NeuN — a neuronal nuclear antigen, and GFAP — an astrocyte marker (Fig. 1 *a*).

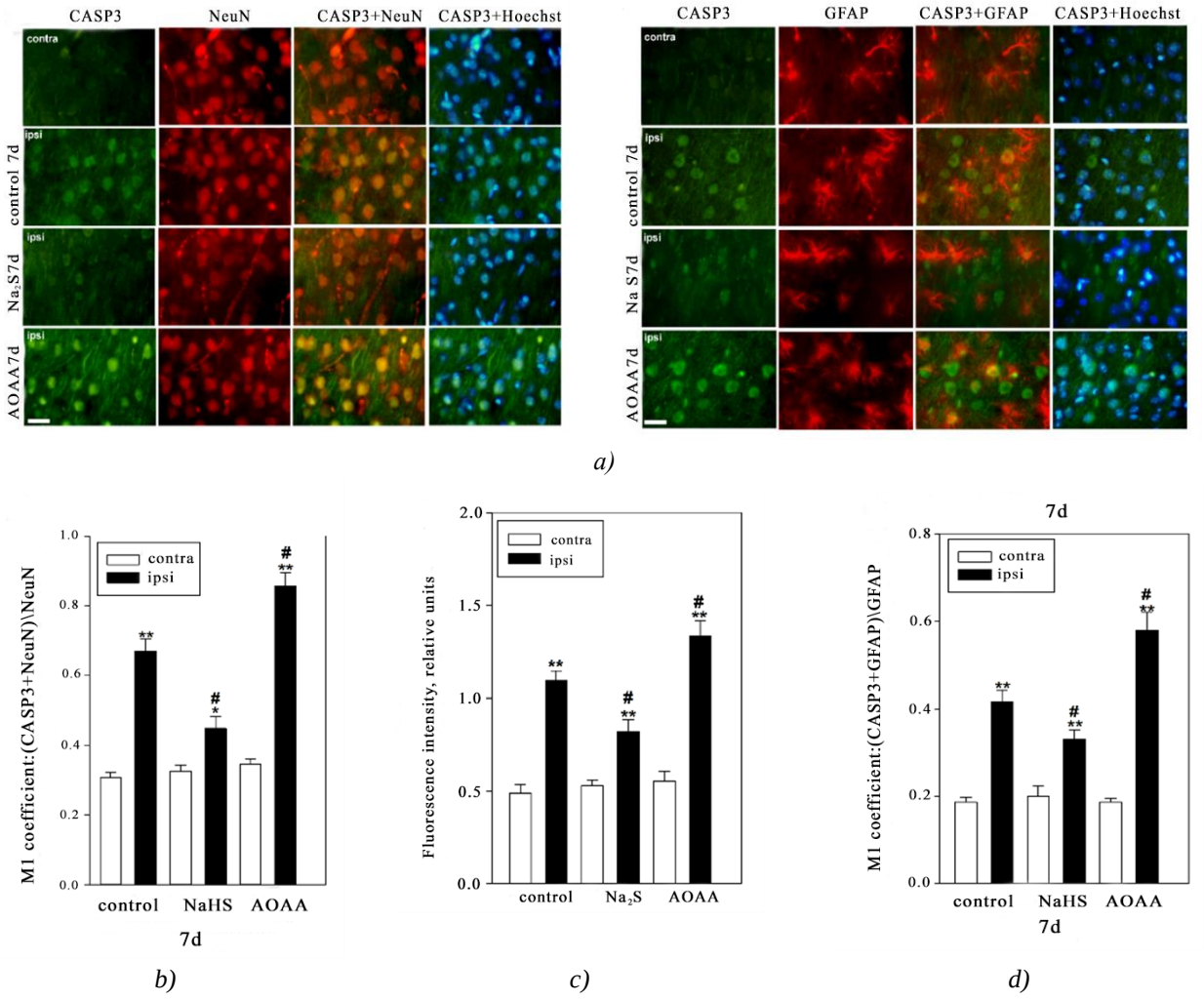

Fig. 1. Immunofluorescence microscopy:

a — expression of caspase-3 (Casp3, green fluorescence) in neurons and astrocytes in the brain of mice from the control group and experimental groups 7 days after injury. Scale bar: 20 μm. NeuN is a marker of neuronal nuclei (red fluorescence); NeuN+Casp3 and Hoechst+Casp3 — colocalization. GFAP is a marker of astrocytes (red fluorescence); GFAP+Casp3 and Hoechst+Casp3 — colocalization. Hoechst fluorescence is Hoechst 33342 (blue fluorescence) visualizing the nuclei of all cells

b — M1 colocalization coefficient of caspase-3 and NeuN in contralateral and ipsilateral neurons in mice from the control group and experimental groups 7 days after injury

c — correlation of the Mean Fluorescence Intensity of caspase-3 in the cytoplasm of neurons of the contralateral and ipsilateral cortex in mice from the control group and experimental groups 7 days after injury

d — M1 colocalization coefficient of caspase-3 and GFAP in astrocytes of contralateral and ipsilateral cortex in mice from the control group and experimental groups 7 days after TBI. Contra — contralateral cortex; Ipsi — ipsilateral cortex. One-way ANOVA. M±SEM. n=6

Note: **p<0.05, **p<0.01 — ipsilateral cortex relative to the contralateral cortex of one animal; #p<0.05 — ipsilateral cortex of mice from the experimental group compared to the ipsilateral cortex of mice from the control group during the same period of time after injury

Herewith, the expression of caspase-3 in neurons of the ipsilateral and contralateral cerebral cortex in mice from the control group and experimental groups differs significantly. This is confirmed by the M1 coefficient values reflecting the degree of colocalization of NeuN and the target protein, namely, caspase-3. In the contralateral cortex, the level of caspase-3 was insignificant in all groups throughout all time intervals after the simulation of TBI. However, traumatic impact led to the rapid increase of caspase-3 expression in neurons of the damaged brain area relative to the contralateral area by 2.2 times 7 days after TBI ($p<0.01$). The use of Na_2S led to a decrease of caspase-3 level by 32% in the ipsilateral cortex relative to the ipsilateral hemisphere of the animals from the control group ($p<0.05$) (Fig. 1 *b*).

The use of the inhibitor of CBS had the opposite effect. Thus, 7 days after the injury, in the damaged neurons in the brain of animals that were injected with AOAA the M1 coefficient value of caspase-3 increased by 31% ($p<0.05$) relative to the damaged cells in the control group. Also, the expression of caspase-3 increased by 2.5 times ($p<0.01$) in neurons of ipsilateral hemisphere in the group of animals injected with AOAA relative to the neurons of the opposite hemisphere in the same animal (Fig. 1 *b*).

Analysis of caspase-3 fluorescence in the cytoplasm of neurons also showed that the level of this enzyme increased in the damaged nerve cells. The fluorescence intensity in the cytoplasm of neurons in animals from the control group and in experimental animals administered Na_2S and AOAA increased by 2.7 times ($p<0.01$), by 56% ($p<0.01$), and by 2.4 times ($p<0.01$), respectively, relative to the contralateral cortex. At the same time, 7 days after TBI, the level of caspase-3 decreased in the ipsilateral cortex of animals administered Na_2S and increased in the group of animals administered AOAA relative to the ipsilateral cortex of animals from the control group by 26% ($p<0.05$) and 21% ($p<0.05$), respectively. (Fig. 1 *c*).

Next, we examined the level of caspase-3 in astrocytes, which were identified using the specific marker GFAP. The colocalization analysis allowed us to establish the expression of caspase-3 in this type of glial cells after TBI. Moreover, the donor Na_2S and the inhibitor AOAA had opposite effect on the dynamics of caspase-3 expression in astrocytes after TBI (Fig. 1 *d*). Thus, it was shown that the level of this enzyme increased in the ipsilateral cortex of animals from the control group and experimental groups administered Na_2S and AOAA relative to the contralateral cortex by 2.2 times ($p<0.01$), 65% ($p<0.01$), and by 3 times ($p<0.01$), respectively. Also, 7 days after TBI, the level of caspase-3 decreased in the ipsilateral cortex of animals administered Na_2S and increased in the group of animals administered AOAA, relative to the ipsilateral cortex of animals from the control group, by 19% ($p<0.05$) and by 41% ($p<0.05$), respectively. (Fig. 1 *c*).

The fluorescence microscopy data confirmed the results obtained during the Western blot analysis of the total fraction of the brain nervous tissue of animals from the control group and experimental groups administered Na_2S and AOAA (Fig. 2).

It was shown that 7 days after TBI, caspase-3 expression in the ipsilateral cortex of animals from the control group and experimental groups administered Na_2S and AOAA increased relative to the contralateral cortex by 2 times ($p<0.001$), by 36% ($p<0.05$), and almost by 2.6 times ($p<0.001$), respectively. At the same time, caspase-3 expression in the ipsilateral cortex of animals administered Na_2S and AOAA relative to the ipsilateral cortex of animals from the control group decreased by 24% ($p<0.05$) and increased by 44% ($p<0.05$), respectively (Fig. 2).

Summarizing the obtained results, based on the immunofluorescence microscopy, it can be concluded that caspase-3 colocalizes with NeuN (neuronal nuclear antigen) and GFAP (astrocyte marker) in neurons and glial cells. In the control group and experimental groups, differences in caspase-3 expression were observed between the ipsilateral (injured) and contralateral (uninjured) cerebral cortex. Injury led to a significant increase of caspase-3 expression in neurons and glial cells in the injured area 7 days after TBI, which indicated activation of apoptosis.

The use of the donor Na_2S decreased the level of caspase-3 in neurons of the ipsilateral cortex, and the inhibitor AOAA caused the opposite effect, increasing the expression of caspase-3. Similar results were obtained when studying caspase-3 in astrocytes, where increased expression was observed in the group administered AOAA and decreased — in the group administered Na_2S.

Western blot analysis of the total fraction of the brain nervous tissue of animals from the control group and experimental groups confirmed the results of immunofluorescence microscopy. Administering Na_2S decreased the expression of caspase-3 in the damaged area, while AOAA fostered its increase. These data indicate an important role of H_2S in the regulation of apoptosis in neurons and glial cells after TBI.

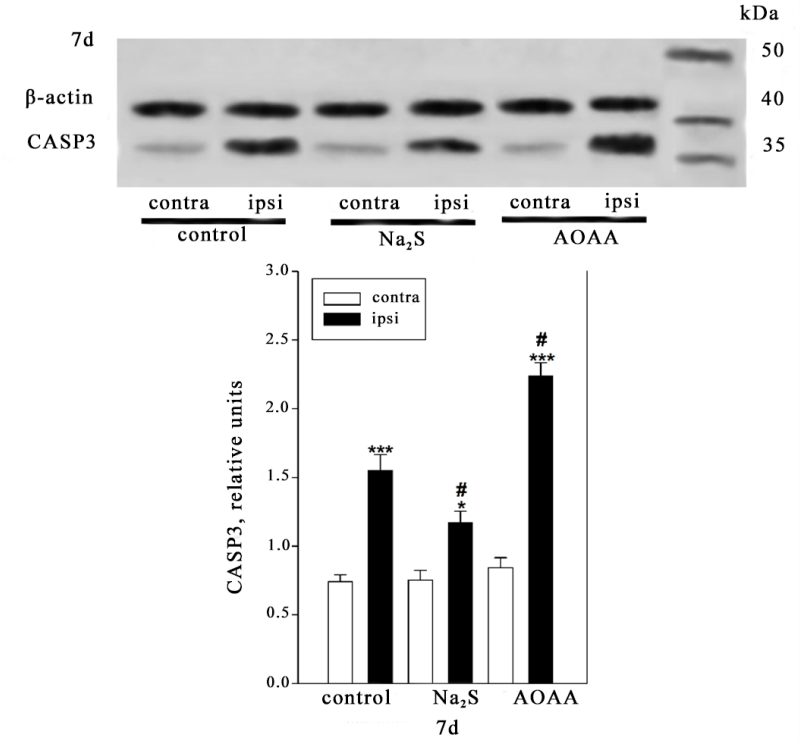

Fig. 2. Western blotting. Caspase-3 expression in the contralateral and ipsilateral cortex of animals from the control group and experimental groups 7 days after injury. One-way ANOVA. M±SEM. n=6.

Note: *p<0.05, ***p<0.001 — ipsilateral cortex relative to contralateral cortex of one animal; #p<0.05 — ipsilateral cortex of animals from the experimental group relative to ipsilateral cortex of animals from control group during the same period of time after injury

Discussion and Conclusion. Currently, H_2S is known as a powerful regulator of the apoptosis in neurotrauma. H_2S-dependent mechanisms of cell death regulation have been studied in many experimental models using different donors of H_2S and inhibitors of enzymes responsible for H_2S synthesis. However, the role of H_2S-dependent signaling mechanisms in regulating the expression and localization of caspase-3 in neurons and glial cells in TBI has not been sufficiently studied.

In our study, it was shown that H_2S is an important link in control of the caspase-3 level in brain cells in the settings of TBI-simulating induced mechanical injury. The intensity of cell death during TBI-induced secondary injury directly correlates with the level of pro-apoptotic proteins. Caspase-3 is one of the key apoptotic proteins. The increased level of this proteolytic enzyme indicates the initiation of processes leading to cell death. Na_2S — the fast donor of hydrogen sulfide, and AOAA — the traditional inhibitor of the key enzyme of H_2S synthesis in nervous tissue, used in our research, had opposite effects on the expression of this protein. The initial level of caspase-3 in the undamaged neurons and glial cells in the brain was low. However, 7 days after TBI, the level of caspase-3 significantly increased, especially in neurons, as well as in glial cells. The use of Na_2S made it possible to decrease the expression of this enzyme. The opposite effect was observed when CBS was inhibited by AOAA.

H_2S regulates caspase-3 level through a variety of molecular mechanisms, many of which are related to its ability to affect the anti-apoptotic and antioxidant systems in cells. One of the key pathways is increasing expression of Bcl-2 family proteins [14], which inhibit the activation of caspases, including caspase-3 [15]. Bcl-2 interacts with pro-apoptotic effector proteins such as Bax and Bak and prevents the release of cytochrome c from mitochondria. This is an important step in the apoptotic process, as Cyt c is involved in formation of the Apaf-1 apoptosome complex [16], which activates caspases and initiates cell death. H_2S, by increasing the level of Bcl-2, stabilizes mitochondrial membranes and prevents apoptosis

Additionally, H_2S acts as a potent antioxidant efficiently decreasing the level of reactive oxygen species (ROS), which play an important role in the induction of apoptosis through the activation of caspases [4, 5, 17, 18]. An increase in the level of ROS causes mitochondrial damage that leads to the activation of caspases and subsequent cell death. However, reducing properties of H_2S can directly neutralize ROS by decreasing their oxidative potential. This decreases the cellular stress, including stress on mitochondria, and prevents the initiation of the apoptotic cascade. Moreover, H_2S can activate a number of antioxidant enzymes, such as superoxide dismutase and catalase, protecting cells from excessive levels of ROS [4, 19].

H_2S can also directly interact with the caspase-3 enzyme. It should be noted that caspase-3 contains an essential cysteine residue that can become a target for modification via the persulfidation process, in which H_2S adds sulfur to the thiol group of cysteine. Persulfidation of cysteine 163 in the active site of caspase-3 leads to inhibition of its proteolytic activity, which prevents the cleavage of caspase-3 substrates and, accordingly, slows down or stops apoptosis [20]. This indicates that H_2S can have a dual effect on apoptosis: indirect — through the regulation of anti-apoptotic and antioxidant pathways, and direct — by inhibiting caspase activation.

Additionally, H_2S can modulate other signaling pathways related to cell death. For example, it can activate Nrf2 (nuclear factor erythroid type 2) signaling pathway, which is a key regulator of cellular antioxidant defence mechanism. Activation of Nrf2 leads to the transcription of multiple genes responsible for antioxidant and cytoprotective functions, thereby decreasing the oxidative stress and protecting cells from apoptotic damage [21, 22].

It should also be noted that H_2S can interact with other gasotransmitters, such as nitric oxide (NO) and carbon monoxide (CO), forming the complex signaling networks that regulate cell survival [23]. Synergistic or antagonistic interaction of H_2S with NO and CO can affect various stages of apoptosis, including activation or inhibition of caspases. Thus, in our study, we examined in detail the expression and localization of caspase-3 in neurons and astrocytes in the brain in TBI. The results obtained have both fundamental and practical importance. Data about the role of H_2S in regulation of the key pro-apoptotic protein caspase-3 expand our understanding of the intracellular signaling processes of nervous system cell survival and death, whereas AOAA and Na_2S can become the basis for the development of clinically efficient neuroprotective agents.

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Е.Ю. Кириченко: анализ полученных результатов, валидация, написание рукописи.

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ПАТОЛОГИЯ ЖИВОТНЫХ, МОРФОЛОГИЯ, ФИЗИОЛОГИЯ, ФАРМАКОЛОГИЯ И ТОКСИКОЛОГИЯ

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Main Indices of Laboratory Rabbit Cardiovascular System

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Abstract

Introduction. Rabbits, ferrets, dogs, miniature pigs and monkeys are considered to be the preferable test systems for assessing the efficacy of the developed medicinal products for the cardiovascular system. The heart rate and distribution of cardiac ion channels in the heart of these species is similar to that in the human heart. The present work focuses on rabbits as they are one of the most widely used species in preclinical research, however, the results of measurements carried out on a sufficient sample of intact animals can hardly be found in the open sources. Thus, the aim of the study is to determine the features of the electrocardiogram and to establish the reference intervals of the key cardiovascular indices as well as of the blood pressure in the sexually mature laboratory rabbits of different breeds, ages and sexes, and to compare the obtained data with that in the literature sources.

Materials and Methods. The study was conducted at JSC "Research-and-Manufacturing Company "Home of Pharmacy" (Leningrad Region) in the period from 2019 to 2024. 597 electrocardiograms and 863 values of systolic and diastolic blood pressure in rabbits of various breeds and sexes aged 4 to 8 months were analysed and reference intervals for them were calculated. To minimise possible variability of parameters caused by stress and motor activity of animals, they were anesthetized during physiological studies with a drug containing tiletamine and zolazepam (5 mg/kg) in combination with xylazine hydrochloride (1–2 mg/kg) administered intramuscularly or intravenously. The data were retrieved by recording the background values in all rabbits in the experiment (before administering the studied medicinal products), as well as in rabbits in the control and intact groups. Reference intervals were calculated using a nonparametric (number of values more than 120) or robust (number of values less than 120) methods.

Results. When studying the electrocardiograms of rabbits, the highest variability was noted in the P wave. Also, the absence of one of the Q, S, T waves in one or two leads and absence of S-T and T-P intervals were frequently observed. When comparing rabbit breeds and body weight ranges, no significant differences were found in the calculated reference intervals of the indices assessed. When comparing to the literature sources, the electrocardiogram parameters mainly matched the obtained reference intervals, except for the QRS interval. The obtained intervals of blood pressure indices in the state of anaesthesia were significantly lower than the values presented in the literature for the awaken animals.

Discussion and Conclusion. The calculated reference intervals of the most of electrocardiogram parameters in different rabbit breeds are comparable to the data available in literature and do not significantly vary in relation to age, weight and sex. The obtained reference intervals and the described features of the electrocardiogram of rabbits can be used in the analysis and interpretation of data received during preclinical research.

Keywords: reference intervals, rabbits, pharmacological safety, blood pressure (BP), electrocardiogram (ECG), cardiovascular system (CVS)

Declaration of Compliance with the Principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes: the authors declare that all research was conducted in compliance with the principles of Good Laboratory Practice.

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Оригинальное эмпирическое исследование

Основные показатели функционирования сердечно-сосудистой системы лабораторных кроликов

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Аннотация

Введение. Предпочтительными тест-системами в ходе оценки влияния разрабатываемых лекарственных препаратов на сердечно-сосудистую систему являются кролики, хорьки, собаки, карликовые свиньи и обезьяны. Сердце данных видов имеет сходство с сердцем человека в частоте сердечных сокращений и распределении сердечных ионных каналов. Данная работа сфокусирована на кроликах как одном из наиболее широко задействованных в доклинических исследованиях виде, при этом в открытых источниках практически не встречаются результаты измерений на достаточной выборке интактных животных. Таким образом, целью исследования было определение особенностей электрокардиограммы и референтных интервалов основных показателей сердечно-сосудистой системы и артериального давления у половозрелых лабораторных кроликов разных пород, возраста и пола, и сравнение полученных данных с литературными источниками.

Материалы и методы. Исследование проведено в АО «НПО «ДОМ ФАРМАЦИИ» (Ленинградская область) в период с 2019 по 2024 гг. Проанализированы 597 электрокардиограмм и 863 значения систолического и диастолического артериального давления кроликов различных пород и пола в возрасте от 4 до 8 месяцев и рассчитаны их референтные интервалы. Для нивелирования возможной вариативности параметров, обусловленной стрессом и двигательной активностью животных, при проведении физиологических исследований применяли анестезию: внутримышечно или внутривенно препарат, содержащий тилетамин и золазепам (5 мг/кг), в комбинации с ксилазина гидрохлоридом (1–2 мг/кг). Данные получены при регистрации фоновых значений у всех кроликов в эксперименте (до введения исследуемых препаратов), а также у кроликов, находившихся в контрольных и интактных группах. Расчет референтных интервалов проводился непараметрическим (количество значений больше 120) или робастным методом (количество значений менее 120).

Результаты исследования. При изучении электрокардиограммы кроликов наибольшая вариативность отмечена у зубца P, также часто наблюдали отсутствие одного из зубцов Q, S, T в одном или двух отведениях и интервалов S-T и T-P. Значительных различий, рассчитанных референтных интервалов по оцениваемым показателям при сравнении между породами и диапазонами массы тела кроликов, не обнаружено. При сравнении с литературными источниками параметры электрокардиограммы преимущественно попадают в полученные референтные интервалы, за исключением интервала QRS. Полученные интервалы показателей артериального давления в состоянии наркотизации значительно ниже представленных в литературе значений у животных в сознании.

Обсуждение и заключение. Рассчитанные референтные интервалы большинства параметров электрокардиограммы кроликов различных пород сопоставимы с данными литературы и не имеют значительных колебаний в зависимости от возраста, массы и пола. Полученные референтные интервалы и описанные особенности электрокардиограммы кроликов могут быть использованы при анализе и интерпретации данных, полученных в доклинических исследованиях.

Ключевые слова: референтные интервалы, кролики, фармакологическая безопасность, артериальное давление (АД), электрокардиограмма (ЭКГ), сердечно-сосудистая система (ССС)

Декларация о соблюдении принципов Европейской конвенции о защите позвоночных животных, используемых для экспериментов и других научных целей: авторы заявляют, что все проведенные исследования соответствовали принципам конвенции и правилам надлежащей лабораторной практики.

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Introduction. Electrophysiological studies are an important tool for assessment of cardiovascular system (CVS) functioning. These studies are an integral part of research on the pharmacological safety of new medicinal products, thus, help to evaluate the pharmacological efficacy of potential therapeutic agents. The animal species most commonly used in experimental cardiac electrophysiology are mice, guinea pigs, rats, miniature pigs, ferrets, rabbits, dogs, and monkeys. The electrocardiograms (ECG) of mice and rats demonstrate significant differences from humans', including differences in the process of cardiac impulse generation, therefore, the mechanisms of arrhythmia may differ from those in humans. Moreover, the small size of rodent's heart complicates surgical interventions on this organ and prevents its use for testing cardiac devices such as valve prostheses or pacemakers [1]. The cardiac ion channel distribution in rabbits, ferrets, dogs and miniature pigs is similar to that in human heart, therefore, these animals proved to be suitable for studying ventricular repolarization or pro- and antiarrhythmic effects of medicinal products[3] [2, 3]. Moreover, the heart size and similar to human processes of cardiac impulse generation and lipoprotein metabolism in laboratory rabbits have led to their widespread use as test systems for studying human cardiovascular diseases [4–6]. Currently, laboratory rabbits are often used to study genetic cardiomyopathies in humans and long QT syndrome [7], as well as the mechanisms of cardiac conduction disorders and arrhythmias [1, 8].

It should be noted that one of the necessary approaches to studying the CVS is the ability of a medicinal product to slow ventricular repolarization (prolong the QT interval)[4]. In the past it was found that some medicinal products could cause severe life-threatening arrhythmias (such as torsades de pointes), which can be fatal. Such properties were, for example, found in cizapride and terfenadine, which were withdrawn from the market for this reason. The effect of medicinal products on ventricular repolarization and arrhythmogenic risk is actively evaluated at the preclinical and clinical stages of medicinal product development[5].

Most often, CVS indices are determined in the frame of establishing the pharmacological safety profile, when the laboratory rabbits are used as second animal species (non-rodents)[6] [9]. In the public domain, one can find mean values or intervals of ECG parameters, including heart rate (HR) and blood pressure (BP), obtained from a small number of rabbits in the control group, as well as from domestic rabbits. However, in open sources there are practically no summarised data on reference values of CVS indices for different breeds of rabbits, and ECG changes possible to encounter in healthy rabbits are also extremely poorly represented.

*The aim of the research i*s to determine the ECG features and reference intervals for the main CVS indices (ECG and BP) in mature laboratory rabbits of different breeds, ages and sexes, and then compare the obtained data with literature sources.

Materials and Methods. All studies were conducted at JSC "Research-and-Manufacturing Company "Home of Pharmacy" (Leningrad Region) in the period from 2019 to 2024 in compliance with the principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes[7], and rules of Good Laboratory Practice[8]. The effect of the medicinal products on the cardiovascular system was studied by assessing the blood pressure, heart rate, and electrocardiogram; additionally, *in vivo*, *in vitro*, and/or *ex vivo* obtained data were analysed, including the methods for assessing repolariza-

[3] On the Guidelines for the Study of Pharmacological Safety of the Medicinal Products for Medical Use. Recommendation of the Board of the Eurasian Economic Commission No. 18 of October 27, 2020. (In Russ.) URL: https://docs.eaeunion.org/upload/iblock/7f5/a2kjnu4hopvymlaefwp0caw97k3nv2us/err_30102020_18_doc.pdf (accessed: 02.06.2025).

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[5] On the Guidelines for the Study of Pharmacological Safety of the Medicinal Products for Medical Use. Recommendation of the Board of the Eurasian Economic Commission No. 18 of October 27, 2020. (In Russ.). URL: https://docs.eaeunion.org/upload/iblock/7f5/a2kjnu4hopvymlaefwp0caw97k3nv2us/err_30102020_18_doc.pdf (accessed: 02.06.2025).

[6] On Approval of the Guidelines for Preclinical Safety Studies for the Purpose of Conducting Clinical Trials and Drug Registration. The Decision of the Board of the Eurasian Economic Commission No. 202 of November 26, 2019. (In Russ.). URL: https://docs.eaeunion.org/upload/iblock/072/nzmd6i2fwbj90ndkm4riswwcz9ecgsa2/clcd_29112019_202_doc.pdf (accessed: 02.06.2025).

[7] European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. ETS No.123. Strasbourg: Council of Europe; 1986. URL: https://clck.ru/3MeTdd (accessed: 02.06.2025).

[8] On Guidelines for Working with Laboratory (Experimental) Animals when Conducting Preclinical (Non-Clinical) Studies. Recommendations of the Board of the Eurasian Economic Commission No. 33 November 14, 2023. (In Russ.) URL: https://docs.eaeunion.org/upload/iblock/46d/ccxcn6996c79u3xvgecp05qhx5g5e692/err_20112023_33_doc.pdf (accessed: 02.06.2025).

tion and conduction disorders in the myocardium[9,10,11,12,13,14]. Data for calculating reference intervals for BP and ECG parameters were obtained by recording the background values in all rabbits in the experiment (before administering the studied medicinal products), as well as in rabbits in the control and intact groups.

Regulatory documents for conducting preclinical studies describe the main set of pharmacological safety studies aimed at investigating the effect of the tested agent on the vital functions of a human body (cardiovascular, central nervous and respiratory systems)[15,16,17,18,19]. These studies are conducted prior to clinical development in compliance with the principles stipulated in the S7A and S7B Guidelines of ICH[20,21]. If necessary, additional and subsequent safety pharmacological studies may be conducted in the later stages of clinical development[15].

Thereby, additional studies on the cardiovascular system are aimed at deeper understanding of the obtained results of the vital functions, e.g.: measurement of cardiac output, ventricular contractility, vascular resistance[22,23,24]. To assess additional parameters, echocardiography is normally used as a clarifying examination [10, 11].

In all the experiments, rabbits from the nurseries, with the necessary veterinary documentation, were used of the Soviet Chinchilla, White Giant, and New Zealand breeds aged 4 to 8 months. The animals were kept in the equally standard conditions: air temperature 15–22°C, relative humidity > 45%, 12-hour daylight. Non-pregnant and nulliparous females were used. The rabbits were kept individually or in pairs (same sex) in standard plastic cages with wood bedding, or in groups of no more than 5 specimens of the same sex in isolated boxes with a removable panel floor. The floor area of the cage per animal complied with the Guide for the Care and Use of Laboratory Animals[25].

[9] S7A Safety Pharmacology Studies for Human Pharmaceuticals. Guidance for Industry U.S. Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). ICH; 2001. URL: https://www.fda.gov/media/72033/download (accessed: 02.06.2025) [GOST R 56700–2015. Medicines for Medical Applications. Safety Pharmacology Studies for Human Pharmaceuticals (In Russ.). URL: https://files.stroyinf.ru/Data2/1/4293758/4293758370.pdf (accessed: 02.06.2025).

[10] GOST R 56701–2015. Medicines for medical applications. Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals. (In Russ.). URL: https://clck.ru/3MeU4u (accessed: 02.06.2025).

[11] S7B Non-Clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals. Guidance for Industry. U.S. Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). ICH; 2005. URL: https://www.fda.gov/media/72043/download (accessed: 02.06.2025).

[12] On the Guidelines for the Study of Pharmacological Safety of the Medicinal Products for Medical Use. Recommendation of the Board of the Eurasian Economic Commission No. 18 of October 27, 2020. (In Russ.). URL: https://docs.eaeunion.org/upload/iblock/7f5/a2kjnu4hopvymlaefwp0caw97k3nv2us/err_30102020_18_doc.pdf (accessed: 02.06.2025).

[13] On Approval of the Guidelines for Preclinical Safety Studies for the Purpose of Conducting Clinical Trials and Drug Registration. The Decision of the Board of the Eurasian Economic Commission No. 202 of November 26, 2019. (In Russ.). URL: https://docs.eaeunion.org/upload/iblock/072/nzmd6i2fwbj90ndkm4riswwcz9ecgsa2/clcd_29112019_202_doc.pdf (accessed: 02.06.2025).

[14] On Rules for Registration and Examination of Medicinal Products for Medical Use. Decision of the Board of the Eurasian Economic Commission No. 78 of November 3, 2016. (as amended on May 29, 2024). (In Russ.) URL: https://docs.eaeunion.org/upload/iblock/cf0/9pi8f6zal9za9xhhcz1lt5aq6nxd7gvi/cncd_21112016_78_doc.pdf (accessed: 02.06.2025).

[15] GOST R 56701–2015. Medicines for medical applications. Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals. (In Russ.). URL: https://clck.ru/3MeU4u (accessed: 02.06.2025).

[16]On the Guidelines for the Study of Pharmacological Safety of the Medicinal Products for Medical Use. Recommendation of the Board of the Eurasian Economic Commission No. 18 of October 27, 2020. (In Russ.). URL: https://docs.eaeunion.org/upload/iblock/7f5/a2kjnu4hopvymlaefwp0caw97k3nv2us/err_30102020_18_doc.pdf (accessed: 02.06.2025).

[17] On Approval of the Guidelines for Preclinical Safety Studies for the Purpose of Conducting Clinical Trials and Drug Registration. The Decision of the Board of the Eurasian Economic Commission No. 202 of November 26, 2019. (In Russ.). URL: https://docs.eaeunion.org/upload/iblock/072/nzmd6i2fwbj90ndkm4riswwcz9ecgsa2/clcd_29112019_202_doc.pdf (accessed: 02.06.2025).

[18] On Circulation of Medicines. Federal Law of Russian Federation No. 61-FZ of April 12, 2019 (edition of April 1, 2024) (In Russ.). URL: https://roszdravnadzor.gov.ru/i/upload/images/2025/4/7/1744053375.88887-1-3771747.pdf?ysclid=mc34cwsi54623589355 (accessed: 02.06.2025).

[19] On Guidelines for Working with Laboratory (Experimental) Animals when Conducting Preclinical (Non-Clinical) Studies. Recommendations of the Board of the Eurasian Economic Commission No. 33 November 14, 2023. (In Russ.). URL: https://docs.eaeunion.org/upload/iblock/46d/ccxcn6996c79u3xvgecp05qhx5g5e692/err_20112023_33_doc.pdf (accessed: 02.06.2025).

[20] S7A Safety Pharmacology Studies for Human Pharmaceuticals. Guidance for Industry. U.S. Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). ICH; 2001. URL: https://www.fda.gov/media/72033/download (accessed: 02.06.2025) [GOST R 56700–2015. Medicines for Medical Applications. Safety Pharmacology Studies for Human Pharmaceuticals (In Russ.). URL: https://files.stroyinf.ru/Data2/1/4293758/4293758370.pdf (accessed: 2.06.2025)]

[21] S7B Non-Clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals. Guidance for Industry. U.S. Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). ICH; 2005. URL: https://www.fda.gov/media/72043/download (accessed: 02.06.2025).

[22] S7A Safety Pharmacology Studies for Human Pharmaceuticals. Guidance for Industry. U.S. Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). ICH; 2001. URL: https://www.fda.gov/media/72033/download [GOST R 56700–2015. Medicines for Medical Applications. Safety Pharmacology Studies for Human Pharmaceuticals (In Russ.). URL: https://files.stroyinf.ru/Data2/1/4293758/4293758370.pdf (accessed: 02.06.2025).

[23] GOST R 56701–2015. Medicines for medical applications. Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals. (In Russ.) URL: https://clck.ru/3MeU4u (accessed: 02.06.2025).

[24]On the Guidelines for the Study of Pharmacological Safety of the Medicinal Products for Medical Use. Recommendation of the Board of the Eurasian Economic Commission No. 18 of October 27, 2020. (In Russ.). URL: https://docs.eaeunion.org/upload/iblock/7f5/a2kjnu4hopvymlaefwp0caw97k3nv2us/err_30102020_18_doc.pdf (accessed: 02.06.2025).

[25] *Guide for the Care and Use of Laboratory Animals. Eight Edition. Translation to Russian.* Belozerceva IV, Blinov DV, Krasilshikova MS (Eds.). Moscow: IRBIS; 2017. 336 p. (In Russ.). URL: https://bioethics.msu.ru/knowledge/standarts/Guide_book_short.pdf (accessed: 02.06.2025).

Access to water was not restricted throughout the duration of each experiment. Animals were fed twice a day, in the morning and in the evening, in conformity with the Directive 2010/63/EU[26] и and the Guide for the Care and Use of Laboratory Animals[27]. The compound feed for rodents prepared in accordance with GOST 34566–2019[28] requirements was used as the feed.

Although it is preferable to conduct physiological studies in the awaken animals, the use of anesthesia is acceptable to minimise possible variability of parameters caused by stress and motor activity of animals[29]. In literature sources the use of anesthetics such as tiletamine/zolazepam in combination with xylazine in rabbits is reported to have relatively minor side effects with anesthesia deep enough for the study [12–14]. Animals were anesthetized with a drug containing tiletamine and zolazepam in combination with xylazine hydrochloride 5 mg/kg and 1–2 mg/kg, respectively, administered intramuscularly or intravenously.

The blood pressure and ECG were recorded at different time intervals during a day in anesthetized animals in the supine position. After confirming the absence of reflexes in the animal, the ECG was recorded using a computerized veterinary electrocardiograph “Poli-Spektr-8/E” (“Neurosoft” LLC, Russia). The ECG was recorded for 1 minute in three standard and six chest leads. The following parameters were assessed within the ECG analysis: heart rate (HR, beats/min), RR (ms), P (ms), PQ (ms), QRS (ms), QT (ms), and the amplitude of the R- and T-waves (mV). The duration of the intervals and the P-wave were assessed in three standard leads (I, II, and III), as the most frequently used and visualized in the assessment of CVS pathologies [15, 16], the amplitude of the waves was measured in lead II as a mean one. Duration of the intervals in the majority of cases coincided in the two leads; with rare exceptions. When the duration was different, the measurement was carried out in the lead with the longest duration (usually in lead II).

After electrocardiography, the animals’ blood pressure, including systolic and diastolic values, was measured non-invasively using a cuff and a Zoomed BPM-2 veterinary pressure monitor (“ZOOMED” LLC, Russia). The cuff was placed on the right or left thoracic limb, the measurement was taken three times with an interval of 1 to 3 minutes between each, then the mean value of the SBP and DBP readings was calculated. Incorrect outlier values of the device (change in numbers by more than 20 units relative to previously measured values) were not taken into account and immediately re-registered.

The values of the measured BP and ECG in the form of the lower and upper limits of the reference interval were presented in the tables separately for males and females for each of the breeds studied, as well as by body weight ranges, regardless of breed.

Statistical analysis of reference intervals was performed using the licensed software *Microsoft Excel* and free software *Reference Value Advisor v2.1* (*National Veterinary School*, Toulouse, France), compliant with the CLSI Guideline [30] for robust calculation method. To identify differences resulting from comparing data from different sexes, breeds, and body weight ranges by means of a paired T-test, the licensed software *Statistica 10.0* (*StatSoft*, USA) was used.

The currently approved CLSI Guideline on Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory recommends to use a nonparametric method for defining intervals in the sample sizes not less than 120 values and a robust method in the sample sizes between 40 and 120 values. As follows from the data in the literature, the parametric approach is also one of the most commonly used approaches, however, this method is overlooked in the CLSI Guideline despite its potential to demonstrate the best performance for normally distributed data [17]. The parametric method is considered unstable when analysing less than 150 values [18] and is reported in the literature as used, when there are more than 200 values.

Calculation of reference intervals for ECG parameters and blood pressure readings was conducted, depending on the number of animals, by a robust method (number of values less than 120), whereas compliance with the normal distribution law was determined according to Anderson-Darling test, and in the case of abnormal distribution, the Box-Cox transformation was used; otherwise (number of values more than 120), calculation was conducted by a nonparametric method.

Moreover, according to the CLSI Guideline[31], it is necessary to exclude from the data array the statistical outliers that lie outside the interval (Q1–1.5×IQR) – (Q3+1.5×IQR), where Q1 and Q3 are the limits of the 1st and 3rd quartiles;

[26] *Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010. On the Protection of Animals Used for Scientific Purposes. Translation to Russian.* Krasilshchikova MS, Belozertseva IV (Eds.). St. Petersburg; 2012. 48 p. URL: https://ruslasa.ru/wp-content/uploads/2017/06/Directive_201063_rus.pdf (accessed: 02.06.2025).

[27] *Guide for the Care and Use of Laboratory Animals. Eight Edition.* Translation to Russian. Belozerceva IV, Blinov DV, Krasilshikova MS (Eds.). Moscow: IRBIS; 2017. 336 p. (In Russ.). URL: https://bioethics.msu.ru/knowledge/standarts/Guide_book_short.pdf (accessed: 02.06.2025)

[28] *GOST 34566–2019. Complete Mixed Feeds for Laboratory Animals. Specifications*. (In Russ.) URL: https://files.stroyinf.ru/Data2/1/4293727/4293727880.pdf (accessed: 02.06.2025).

[29] *S7B Non-Clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals. Guidance for Industry. U.S. Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER).* ICH; 2005. URL: https://www.fda.gov/media/72043/download (accessed: 02.06.2025).

[30] *Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline – Third edition. C28-A3c. Vol. 28. No. 30.* CLSI; 2010. URL: https://webstore.ansi.org/preview-pages/CLSI/preview_CLSI+C28-A3.pdf (accessed: 02.06.2025).

[31] *Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline – Third edition. C28-A3c. Vol. 28. No. 30.* CLSI; 2010. URL: https://webstore.ansi.org/preview-pages/CLSI/preview_CLSI+C28-A3.pdf (accessed: 02.06.2025).

IQR is the interquartile interval [17, 19]. The proportion of detected outliers was determined using Tukey's method.

Research Results. The electrocardiogram of a rabbit has specific features that should be taken into account when assessing the cardiac function, such as the degree of expression of the waves [20]. Therefore, during the analysis, the specific features of ECGs of rabbits, not typical for humans, were recorded and presented in Table 1, which shows the percentage of changes relative to all the animals studied (597 electrocardiograms — 308 males and 289 females). It should be noted that during the analysis there were found the ECG recordings with the isoelectric line practically untrackable due to interference, represented as repeating positive and negative waves, which made measurement of the wave amplitude impossible, and measurement of the duration of the intervals — difficult.

Table 1

ECG features of male and female rabbits, % of the total number of studied animals (n=597)

Index	Features
Males, n=308	
P-wave	42% peaked in leads I and II 11% slight shift in leads I and II 10% absent (3.5% in leads I and II, 6.5% in lead III) 4% bifid(notched) in lead II 3% poly-phasic in lead II 3% negative in lead III
Q-wave	24% absent (12% in lead II, 6% in lead III, 1% in lead I, and 5% in three leads) 15% amplitude greater than S-wave amplitude in lead II
R-wave	2% poly-phasic in lead II 1% bifid (notched) in lead II
S-wave	33% amplitude greater than Q-wave amplitude in lead II 22% absent (15% in three leads, 7% in lead II, 5% in lead I) 2% poly-phasic in lead II 2% positive J-wave present
T-wave	10% negative (7% in lead III, 3% in lead I) 5% absent in lead III
ST interval	2% absent in three leads
TP interval	9% absent or nearly absent in three leads (mainly at heart rate greater than 250 bpm)
Females, n=289	
P-wave	40% peaked in leads I and II 27% negative in lead III 11% slight shift in leads I and II 10% absent (3.5% in leads I and II, 6.5% in lead III) 4% poly-phasic in lead II 3% bifid (notched) in lead II
Q-wave	29% absent (11% in lead III, 9% in lead II, 2% in lead I, and 7% in three leads) 10% amplitude greater than S-wave amplitude in lead II
R-wave	1% poly-phasic in lead II 3% bifid (notched) in lead II
S-wave	31% absent (15% in lead I, 6% in lead III, 1% in lead II, and 9% in three leads) 29% amplitude greater than Q-wave amplitude in lead II 3% positive J-wave present
T-wave	21% negative (20% in lead III, 1% in lead I) 7% absent in lead III
ST interval	2% absent in three leads
TP interval	10% absent or nearly absent in three leads (mainly at heart rate greater than 250 bpm)

Also, in males and females, there were observed cases of strong dependence of the Q- and S-wave amplitude on the lead — the wave could be practically absent in one lead and exceed the values of the R-wave amplitude in another one (to negative values). In males, this phenomenon was registered in 3% of ECG recordings with S-wave and in 5.5% — with a Q-wave; in females — in 1.4% of ECG recordings with S-wave and in 1.7% — with a Q-wave. Due to the absence of a Q-wave in ECG recording, the PQ interval is generally replaced by the PR interval [21, 22].

The amplitude of the QRS complex in the leads depends on the projection of the electrical axis of the heart, this measurement was not carried out in rabbits due to the anatomical features of the heart and the ECG image, the difficulty of interpretation, as well as the lack of data in the literature on the correct body position for this analysis. However, during the analysis, the amplitude of the R-wave was measured and the correlation of the height of the R-wave in each of the three leads was determined. In 54% of the recordings in males and in 51% in females, the R-wave had the highest amplitude in lead I and an equivalent negative amplitude in lead III, consequently, in lead II the amplitude was almost the same as in lead I or lower, but remained positive. In 22% of cases in males and in 26% of cases in females, the R-wave amplitude in lead II was higher than in leads I and III. In 14% of the recordings in males and in 16% in females, the R-wave amplitude in lead III exceeded that in lead II, which, in turn, exceeded the amplitude in lead I. In the remaining ECG recordings (9% in males and 7% in females), measurements were not performed due to the difficulties in correct assessment of the R-wave amplitude.

Reference intervals for ECG parameters and blood pressure readings are presented separately for males and females for each rabbit breed in Tables 2–4. The duration of ECG intervals was calculated from the rise/depression relative to the isoelectric line of the wave indicated first in the interval (e.g. the Q-wave in the QT interval) until the return of the impulse to the isoelectric line (including in the case of its depression) after the wave indicated last in the interval (e.g. the T-wave in the QT interval) or at the top of the J-wave, if any. As observed earlier, the greatest number of features and the percentage of their occurrence were found in the P- and S-waves, as well as in the Q-wave (Table 1). Therefore, the amplitudes of these waves were not measured to avoid presenting incorrect data. The amplitude of the R-wave was measured relative to the segment of the electrical line located before the QRS complex (Q-wave), the amplitude of the T-wave was measured relative to the segment recorded immediately after the measured T-wave.

Table 2

Reference intervals for ECG parameters and Blood Pressure readings for Soviet Chinchilla rabbit breed (nonparametric method of calculation)

Index	*Males*	*Females*
ECG parameters		
Heart Rate, bpm	151–282 (n=134)	156–269 (n=151)
RR, ms	213–389 (n=133)	221–364 (n=148)
P, ms	29–45 (n=131)	33–45 (n=147)
PQ, ms	53–78 (n=131)	58–84 (n=150)
QRS, ms	38–50 (n=132)	36–50 (n=151)
QT, ms	126–177 (n=131)	133–179 (n=152)
R-wave, mV	0,032–0,394 (n=129)	0,057–0,441 (n=144)
T-wave, mV	0,022–0,166 (n=128)	0,033–0,206 (n=147)
Blood Pressure readings		
Systolic	69–144 (n=166)	66–133 (n=166)
Diastolic	33–88 (n=166)	33–84 (n=167)

Note: here and in Tables 3–5: n is the number of values after excluding outliers from the sample.

Table 3

Reference intervals for ECG parameters and Blood Pressure readings for New Zealand rabbit breed (robust method of calculation)

Index	*Males*	*Females*
ECG parameters		
Heart Rate, bpm	147–266 (n=76)	164–257 (n=71)
RR, ms	208–379 (n=76)	223–346 (n=70)
P, ms	28–51* (n=76)	29–47 (n=73)
PQ, ms	53–91 (n=76)	55–87 (n=73)
QRS, ms	41–53 (n=75)	40–54 (n=71)
QT, ms	135–180 (n=68)	132–179 (n=73)
R-wave, mV	0,010–0,294 (n=70)	0,0–0,411* (n=70)
T-wave, mV	0,016–0,216 (n=75)	0,012–0,222 (n=71)
Blood Pressure readings		
Systolic	70–136 (n=134)	69–130 (n=133)
Diastolic	34–84 (n=135)	31–75 (n=136)

Note: here and in Tables 4, 5: * — values calculated using the Box–Cox transformation.

Table 4

Reference intervals for ECG parameters and Blood Pressure readings for White Giant rabbit breed (robust method of calculation)

Index	*Males*	*Females*
ECG parameters		
Heart Rate, bpm	148–255 (n=95)	142–269 (n=64)
RR, ms	215–377 (n=96)	205–385 (n=64)
P, ms	32–47 (n=97)	30–49 (n=63)
PQ, ms	58–86 (n=98)	57–91* (n=64)
QRS, ms	40–57* (n=95)	40–59 (n=64)
QT, ms	128–169 (n=95)	133–172 (n=63)
R-wave, mV	0,024–0,432* (n=93)	0,023–0,420* (n=60)
T-wave, mV	0,021–0,248 (n=96)	0,036–0,310* (n=64)
Blood Pressure readings		
Systolic	70–147** (n=165)	59–139 (n=90)
Diastolic	35–92** (n=166)	25–75 (n=89)

Note: here and in Table 5: ** — calculation was performed using a nonparametric method (n>120).

When analysing the calculated reference values for three rabbit breeds, the absence of significant differences between the breeds can be found with regard to the heart rate, interval duration, and blood pressure values.

One of the important criteria potentially capable of influencing the ECG parameters, along with breed, age, and sex [23], is the size of animals [24], which, among other factors, depends on the breed and age. Therefore, it was

decided to compare and calculate reference intervals for the ranges of weights of male and female rabbits, regardless of breed. Such a calculation can potentially be applied to other rabbit breeds in the corresponding weight ange. When comparing ECG parameters using a paired T-test, differences were revealed for most of the measured ECG intervals between animals weighing under 3.5 kg and over 3.5 kg.

The reference intervals for ECG parameters, calculated separately for males and females of each weight range, are presented in Table 5.

Table 5

Reference intervals for ECG parameters in rabbits with regard to body weight (robust method of calculation)

Index	*Males weighing 2.0–3.5 kg***	*Males weighing 3.5–5.8 kg*	*Females weighing 1.8–3.5 kg***	*Females weighing 3.5–6.0 kg*
Heart Rate, bpm	149–281 (n=206)	152–273 (n=102)	163–267 (n=174)	146–267 (n=114)
RR, ms	213–402 (n=206)	216–384* (n=102)	222–358 (n=172)	211–370 (n=110)
P, ms	29–46 (n=206)	29–50 (n=102)	31–46 (n=172)	30–49* (n=114)
PQ, ms	54–82 (n=203)	56–89 (n=102)	57–83 (n=172)	59–89* (n=114)
QRS, ms	39–53 (n=197)	41–53 (n=97)	36–53 (n=174)	39–56 (n=113)
QT, ms	125–178 (n=202)	132–176 (n=95)	132–178 (n=175)	131–175 (n=113)
R-wave, mV	0,035–0,396 (n=194)	0,010–0,329 (n=99)	0,035–0,451 (n=161)	0,010–0,396* (n=109)
T-wave, mV	0,027–0,236 (n=197)	0,020–0,214 (n=101)	0,032–0,227 (n=163)	0,022–0,219 (n=109)

When comparing the HR data available in the literature, they turned to be within the range of the obtained reference intervals for the corresponding breed (216±18 bpm, M±SD [15]) or weight (264±33.9 bpm, M±SD [21] 261±13 bpm, M±SD [22], 260±1.8, M±SD [24]). Also, the calculated interval encompassed all the values presented in an article, in which the result of measuring the HR of each of the 90 rabbits was expressed as a percentage relative to the sample [25]. At the same time, when comparing the data of another study conducted on immature rabbits (2–2.5 months), HR proved to be above the reference intervals (240–300 bpm) [20].

In one of the literature sources, a calculation of mean values and reference intervals for a sample of 46 healthy domestic rabbits of different sexes was provided. The mean values of the ECG parameters, including the amplitudes of the R- and T-waves, corresponded to the body-weight-range-intervals, however, the reference intervals provided in the literature source were only partially comparable with the intervals calculated in the present article: the lower limit of almost all parameters was outside the reference values (of this article) [21]. The differences in the intervals might be due to the relatively small sample (46) studied in the literature source (this assumption could be confirmed by the fact that in that article the minimum and maximum values in animals' ECG practically coincided with the reference intervals calculated in it). Whereas, when comparing with the ECG parameters provided in the next literature source that presented a summary of the parameters based on the review of several articles [24], it could be said that P-intervals (0.03–0.04 s), PQ (0.057 s; 0.070 s) and QT (0.132 s; 0.140 s; 0.150 s) coincided with the reference intervals, the QRS interval was either at the lower limit of the interval (0.040 s) or went beyond it (0.031 s). The height of the R-wave in lead II was also within the reference intervals (0.07–0.25 mV and 0.10–0.15 mV). The duration of P (0.03–0.04 s), PQ (0.07–0.08 s), QT (0.13–0.15 s) in immature rabbits was also within the calculated ranges, whereas the QRS interval values were at the lower limit of the references (0.03–0.04 s) [20]. In the paper, which presented the ECG of a rabbit of New Zealand breed, recorded continuously for 24 hours, most of the HR and QT duration values were within the reference intervals, the presented QRS interval values were near the lower limit of the reference intervals [26]. In another study, the QRS and QT interval values were significantly higher than the results obtained in this work for New Zealand rabbits, while the amplitude of the R- and T-waves fell within the calculated reference values, as well as the blood pressure values [27].

In a study on domestic rabbits, the QRS interval was within the calculated reference intervals, while the heart rate values were significantly lower, and the QT interval values, proportionally, were significantly higher than the results obtained in this work for the corresponding weight [28]. These features could result both from the characteristics of domestic rabbits and the drugs used for anesthesia.

When comparing the obtained intervals of SBP and DBP with the values presented in the literature, it was found that the readings were significantly higher, if measured in awaken animals [29] than in anesthetized animals, therefore literature data were higher than the calculated intervals. This could be explained by the mental condition of an animal at the time of measurement. All mean values indicated in the source presenting the review of data summarised from many papers were within the limits of the reference intervals [24]. In another source, where a number of animals with the certain values of SBP were presented [25], it was observed that 92% of rabbits fell within the reference intervals, the remaining values were slightly higher.

The proportion of detected outliers for the studied indices for each sex and breed relative to the data array is presented in Tables 6–8. Table 9 presents the proportion of outliers separately for males and females of each weight range.

Table 6

Proportion of statistical outliers in the analysis of ECG parameters and Blood Pressure readings for Soviet Chinchilla rabbit breed

ECG parameters	*Males, n=134*	*Females, n=152*
Heart Rate, bpm	0,0%	0,7%
RR, ms	0,7%	2,6%
P, ms	2,2%	3,3%
PQ, ms	2,2%	1,3%
QRS, ms	1,5%	0,7%
QT, ms	2,2%	0,0%
R-wave, mV	0,8%	1,4%
T-wave, mV	1,5%	2,6%
Blood Pressure readings	*Males, n=166*	*Females, n=167*
Systolic	0,0%	0,6%
Diastolic	0,0%	0,0%

Table 7

Proportion of statistical outliers in the analysis of ECG parameters and Blood Pressure indicators for New Zealand rabbit breed

ECG parameters	*Males, n=76*	*Females, n=73*
Heart Rate, bpm	0,0%	2,7%
RR, ms	0,0%	4,1%
P, ms	0,0%	0,0%
PQ, ms	0,0%	0,0%
QRS, ms	1,3%	2,7%
QT, ms	10,5%	0,0%
R-wave, mV	1,4%	0,0%
T-wave, mV	0,0%	0,0%
Blood Pressure readings	*Males, n=136*	*Females, n=136*
Systolic	1,5%	2,2%
Diastolic	0,7%	0,0%

Table 8

Proportion of statistical outliers in the analysis of ECG parameters and Blood Pressure *readings* for White Giant rabbit breed

ECG parameters	*Males, n=98*	*Females, n=64*
Heart Rate, bpm	3,1%	0,0%
RR, ms	2,0%	0,0%
P, ms	1,0%	1,6%
PQ, ms	0,0%	0,0%
QRS, ms	3,1%	0,0%
QT, ms	3,1%	1,6%
R-wave, mV	1,1%	0,0%
T-wave, mV	1,0%	0,0%
Blood Pressure readings	*Males, n=166*	*Females, n=92*
Systolic	0,6%	2,2%
Diastolic	0,0%	3,3%

Table 9

Proportion of statistical outliers in the analysis of ECG parameters in rabbits

Index	*Males weighing 2.0–3.5 kg*	*Males weighing 3.5–5.8 kg*	*Females weighing 1.8–3.5 kg*	*Females weighing 3.5–6.0 kg*
Heart Rate, bpm	0,0%	0,0%	0,6%	0,0%
RR, ms	0,0%	0,0%	1,7%	3,5%
P, ms	0,0%	0,0%	1,7%	0,0%
PQ, ms	1,5%	0,0%	1,7%	0,0%
QRS, ms	4,4%	4,9%	0,6%	0,9%
QT, ms	1,9%	6,9%	0,0%	0,9%
R-wave, mV	0,5%	1,0%	1,8%	0,0%
T-wave, mV	2,0%	0,0%	3,0%	3,5%

The greatest number of outliers were observed in males of the New Zealand rabbit breed for the QT interval (10.5%), while outliers for other ECG parameters and blood pressure readings in this and other breeds did not exceed 4.1% in females and 3.1% in males.

The greatest number of outliers were observed in males weighing 3.5–5.8 kg for the QRS (4.9%) and QT (6.9%) intervals. In males weighing 2.0–3.5 kg, the outliers did not exceed 4.4%, in females weighing 2.0–3.5 kg — 3.0%, in females weighing 3.5–5.8 kg — 3.5%.

Discussion and Conclusion. The described features of the ECG of rabbits can have a significant impact on the interpretation of data and correcting the results that are being obtained. For example, the absence of the Q-wave in the QRS complex was reported in the literature sources. However, apart from the images of ECG recordings, a more detailed description of the ECG image features in rabbits was practically unavailable in the literature.

The ranges of the reference intervals for the ECG parameters and blood pressure readings practically do not differ when analysed in different rabbit breeds, different weights and sexes. Nevertheless, the data were presented depending on the sex, breed and weight of the rabbits to optimize the process of analysing the results.

The established reference intervals for the ECG parameters coincide with some of the values presented in the literature, the most variable are the QRS interval and the amplitudes of the R- and T-waves, which may be induced by the quality of the recording and interpretation of the results by a certain researcher. The results of HR, SBP and DBP measurements mostly coincide with the data found in the literature, with the exception of the source in which the measurement of BP was performed on awaken animals, which significantly increased the range of values.

The obtained reference intervals and the described features of the rabbit electrocardiogram can be used in the analysis and interpretation of data received during preclinical studies.

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ANIMAL PATHOLOGY, MORPHOLOGY, PHYSIOLOGY, PHARMACOLOGY AND TOXICOLOGY

ПАТОЛОГИЯ ЖИВОТНЫХ, МОРФОЛОГИЯ, ФИЗИОЛОГИЯ, ФАРМАКОЛОГИЯ И ТОКСИКОЛОГИЯ

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Literature Review

Using the Vertebral Heart Scale Method of Measurement during X-Ray Diagnostics of Thoracic Cavity in Dogs and Cats: A Literature Review

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Abstract

Introduction. In veterinary medicine, X-ray diagnostics is one of the most popular and affordable methods of visual diagnostics of pathologies not only of the musculoskeletal system, but also of some parenchymatous organs in the thoracic and abdominal cavities. Identification of cardiac pathologies most often begins with X-ray examination, however, the use of special techniques is sometimes required for reliable determination of the presence or absence of cardiomegaly in animals under examination. One of the most reliable methods for assessing the condition of the heart is the vertebral heart scale (VHS) method. The aim of the present review is to summarise the data available in literature sources on the use of the vertebral heart scale method during X-ray diagnostics of the feline and canine thoracic cavity, and to assess the relevance and prospects for development of this technique.

Materials and Methods. The search for the articles for the scientific review was conducted in the PubMed and eLIBRARY.RU databases. The search covered only publications in Russian and English, published between January 1, 1995 and December 31, 2024. According to the criteria of including a scientific paper in a review, the article should have met the following selection criteria: 1) contain information about using the vertebral heart scale method of measurement in dogs or cats, 2) have a full-text version. The results were presented in the form of PRISMA flow chart and a table.

Results. The review encompassed 31 articles. In the papers, the following aspects were in the focus of study: vertebral heart scale (VHS) measurements in dogs and cats in the normal and pathological condition; requirements to the technique of taking X-rays intended for vertebral heart scale measurement; the use of artificial intelligence technologies to measure the vertebral heart scale in dogs and cats. The VHS values for different dog breeds were presented in a form of a table indicating the year of the study.

Discussion and Conclusion. According to the available scientific data, the vertebral heart scale method of measurement has proved to be an efficient alternative to other more labor-consuming and expensive methods of feline and canine cardiomegaly diagnostics. However, it is necessary to remember that this method has limitations. Further research in this area could aim at obtaining new data on species-, breed-, age-specific, physiological and pathological features of VHS measurements in dogs and cats. The use of artificial intelligence technologies for measuring the vertebral heart scale in cats and dogs is a highly potential trend.

Keywords: literature review, X-ray diagnostics, dogs, cats, vertebral heart scale, VHS, cardiomegaly

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Обзор научной литературы

Применение метода определения кардиовертебрального индекса у собак и кошек при рентгенодиагностике грудной полости: обзор научной литературы

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Аннотация

Введение. Рентгенодиагностика является в ветеринарии одним из самых популярных и наиболее доступных методов визуальной диагностики патологий не только опорно-двигательного аппарата, но и некоторых паренхиматозных органов грудной и брюшной полостей. Выявление кардиологических патологий чаще всего начинают именно с проведения рентгенологического исследования, однако без применения специальных методик не всегда удается визуально достоверно определить наличие или отсутствие кардиомегалии у исследуемых животных. Одним из наиболее надежных способов оценки состояния сердца является метод определения кардиовертебрального индекса (VHS). Цель данного обзора — обобщение литературных данных по применению расчета кардиовертебрального индекса при рентгенодиагностике грудной полости у кошек и собак, оценка целесообразности и перспективы развития данной методики.

Материалы и методы. Поиск статей для научного обзора проводился в базах данных PubMed и eLIBRARY.RU. В поиск включались только публикации на русском и английском языках, размещенные за период с 1 января 1995 г. по 31 декабря 2024 г. Согласно критериям включения научной работы в обзор, статья должна: 1) содержать информацию о применении расчета кардиовертебрального индекса у собак или кошек, 2) иметь полнотекстовую версию. Результаты оформлены в виде блок-схемы PRISMA и таблицы.

Результаты исследования. В обзор была включена 31 статья. В работах рассматривалось следующее: определение показателей VHS у собак и кошек в норме и при патологии; требования к технике получения рентгенограмм для расчета кардиовертебрального индекса; применение технологии искусственного интеллекта для расчета кардиовертебрального индекса у собак и кошек. Значения VHS для разных пород собак структурированы в таблице с указанием года проведения исследований.

Обсуждение и заключение. Методика расчета кардиовертебрального индекса зарекомендовала себя, согласно имеющимся научным данным, как действующая альтернатива другим, более трудоемким и дорогостоящим методам диагностики кардиомегалий у кошек и собак. Однако необходимо помнить, что данный метод имеет свои ограничения. Дальнейшие исследования в этой области могут быть направлены на получение новых данных относительно видовых, породных, возрастных, физиологических и патологических особенностей показателя VHS у собак и кошек. Весьма перспективное направление — применение технологии искусственного интеллекта для расчета кардиовертебрального индекса у собак и кошек.

Ключевые слова: обзор научной литературы, рентгенодиагностика, собаки, кошки, кардиовертебральный индекс, КВИ, VHS, кардиомегалия

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Introduction. Nowadays, X-ray examination is one of the most affordable and widespread methods of visual diagnostics of pathologies of internal organs and systems in veterinary medicine. This method is particularly important for the studies of the thoracic cavity as it allows evaluating the lung parenchyma, condition of the great and pulmonary vessels, as well as the shape, size and location of the cardiac silhouette [1]. A chest X-ray can be used as a screening examination to determine the cardiac and non-cardiac status of a patient, which is especially relevant for older animals with ambiguous symptoms [1]. For more accurate and standardize assessment of the cardiac silhouette size, some methods of human medicine have been introduced into the veterinary practices, such as determining the cardiothoracic ratio (the ratio of the cardiac silhouette to the intercostal spaces), however, when applied to animals, these methods produce serious errors.

Currently, in veterinary medicine, one of the most reliable approaches for assessing the size of the cardiac silhouette on X-rays is Vertebral Heart Scale (VHS) measurement — calculating the ratio of the sum of the lengths of the short and long axes of the heart silhouette to the length

of the thoracic vertebral bodies. Fig. 1 shows a lateral radiograph of a canine thorax, which presents the principle of measuring the cardiac axes [1].

The long axis of the heart is measured from the ventral border of the left main bronchus to the most remote ventral point of the apex of the heart. The short axis is measured along a line perpendicular to the long axis, at the level of the caudal vena cava. The sum of the lengths of the two axes is compared to the length of the vertebrae, starting from the cranial edge of T4 (the fourth thoracic vertebra) [2]. This technique has been proposed as one of the methods for assessing cardiomegaly in dogs and cats.

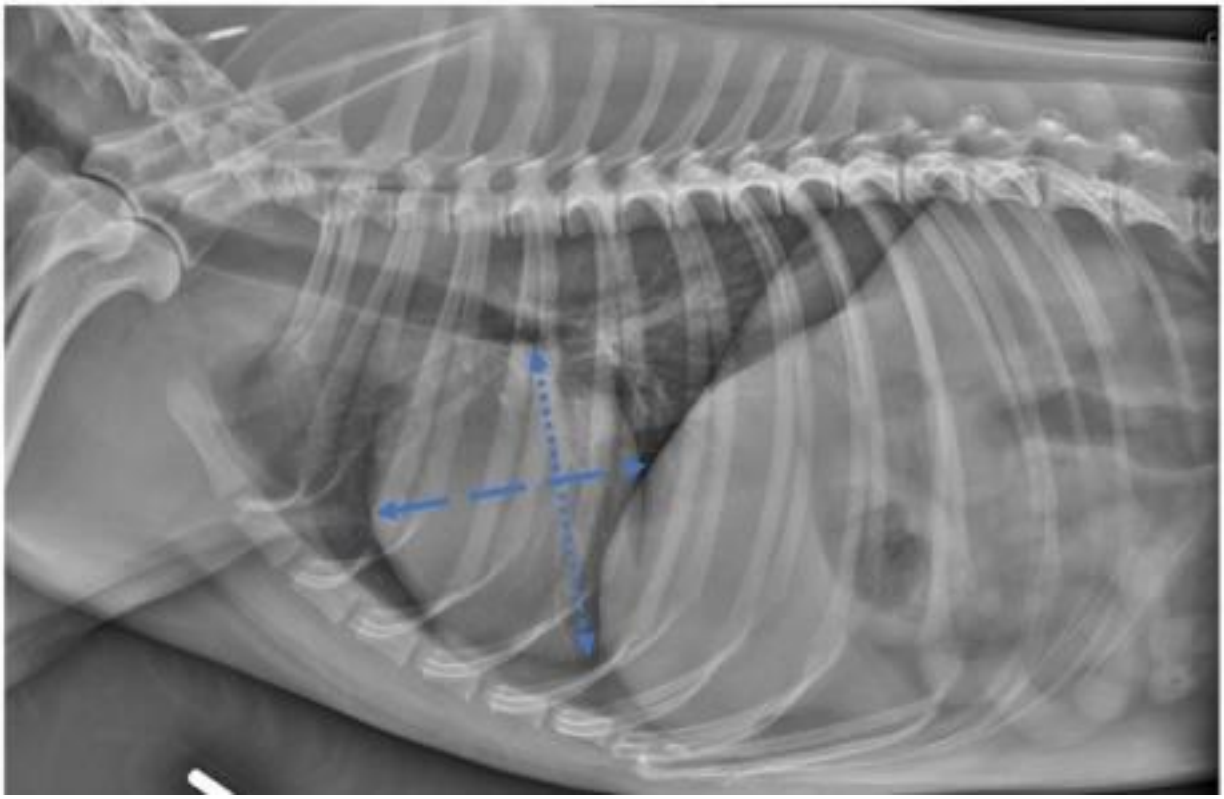

Fig. 1. Principle of vertebral heart scale measurement [1]

In 2001, H. Nakayama et al. conducted a study to determine the extent the echocardiography and electrocardiography results correlate with VHS. The experiment involved dogs with cardiomegaly of different severity. They were monitored using chest radiography, echocardiography, and electrocardiography in dynamics. Then, echocardiographic and electrocardiographic parameters were compared to VHS. The results of the study showed that the Left Atrial to Aorta ratio, left ventricular end-diastolic volume and end-systolic volume, P-wave duration and QRS interval duration correlated consistently with VHS [3].

However, the use of the vertebral heart scale (VHS) measurement method has its limitations. Firstly, although it enables quite objective determination of the presence of cardiomegaly in animals, it does not provide an opportunity to assess the entire area of the heart: VHS is a one-dimensional method and has functional limitation, as it uses only two linear measurements to determine the heart size, and not the entire heart circumference. Secondly, significant limitations are related to the interpretation of images, which is subjective in nature, since it largely depends on the education and experience of a radiologist (common causes of misinterpretation are perception errors, omissions resulting from the lack of attention, cognitive distortions, fatigue, distraction). Certain steps are currently taken to integrate the artificial intelligence (AI) technologies into X-ray diagnostics: this might provide a number of advantages, including the possibility of computerized interpreting complex images and calculating certain parameter. An experiment on measuring vertebral heart scale in dogs and cats by means of AI was held in 2021[1].

The aim of the present review is to summarise and analyse data available in the literature on the use of vertebral heart scale measurement during chest radiography in cats and dogs, to assess the relevance of this technique and prospects of development.

Materials and Methods. The search for scientific articles for the review was carried out in the PubMed (www.pubmed.ncbi.nlm.nih.gov/) and eLIBRARY.RU (www.elibrary.ru/defaultx.asp) databases. Only works in Russian and English published between January 1, 1995 and December 31, 2024 were included in the search. In PubMed, the search was conducted using the keywords: ((radiomics

or X-ray) and (Vertebral Heart Scale or VHS) and (radiographs or diagnostic imaging) and (cats) and (dogs)). The search for scientific publications in eLIBRARY.RU was carried out using the option of "advanced search" was used by entering the following combination of words into the dialog box: ((X-ray or X-ray diagnostics) and (Vertebral Heart Scale or VHS) and (cats and dogs or small domestic animals)). In the "Where to search?" box, the search was specified by the criteria: "in the title", "in the abstract", "in keywords". "Publication type" was indicated as "journal articles". Additional search criteria were the parameters "search taking into account morphology" and "search in publications that have full text on eLIBRARY.RU".

A scientific paper was included in the review based on the following criteria: 1) the article had to contain the information on the use of vertebral heart scale method of measurement in dogs or cats, 2) the article had to have a full-text version. Non-conformity of a literature source with the criteria for inclusion in the review was the reason for its exclusion. The results were presented in the form of a PRISMA flow chart and a table.

Research Results. In total, 63 sources were found as a result of the database search. On the whole, 13 duplicating sources were excluded, and 50 articles were selected for further analysis. After screening the titles and abstracts, 5 sources were removed. Potentially eligible full texts (n = 45) were uploaded to cloud storage and reviewed by all the experts to ensure their relevance. After this step, 14 more publications were excluded. The final number of articles selected for inclusion in the review was 31. A summary of the screening process is shown in the PRISMA flow chart (Figure 2).

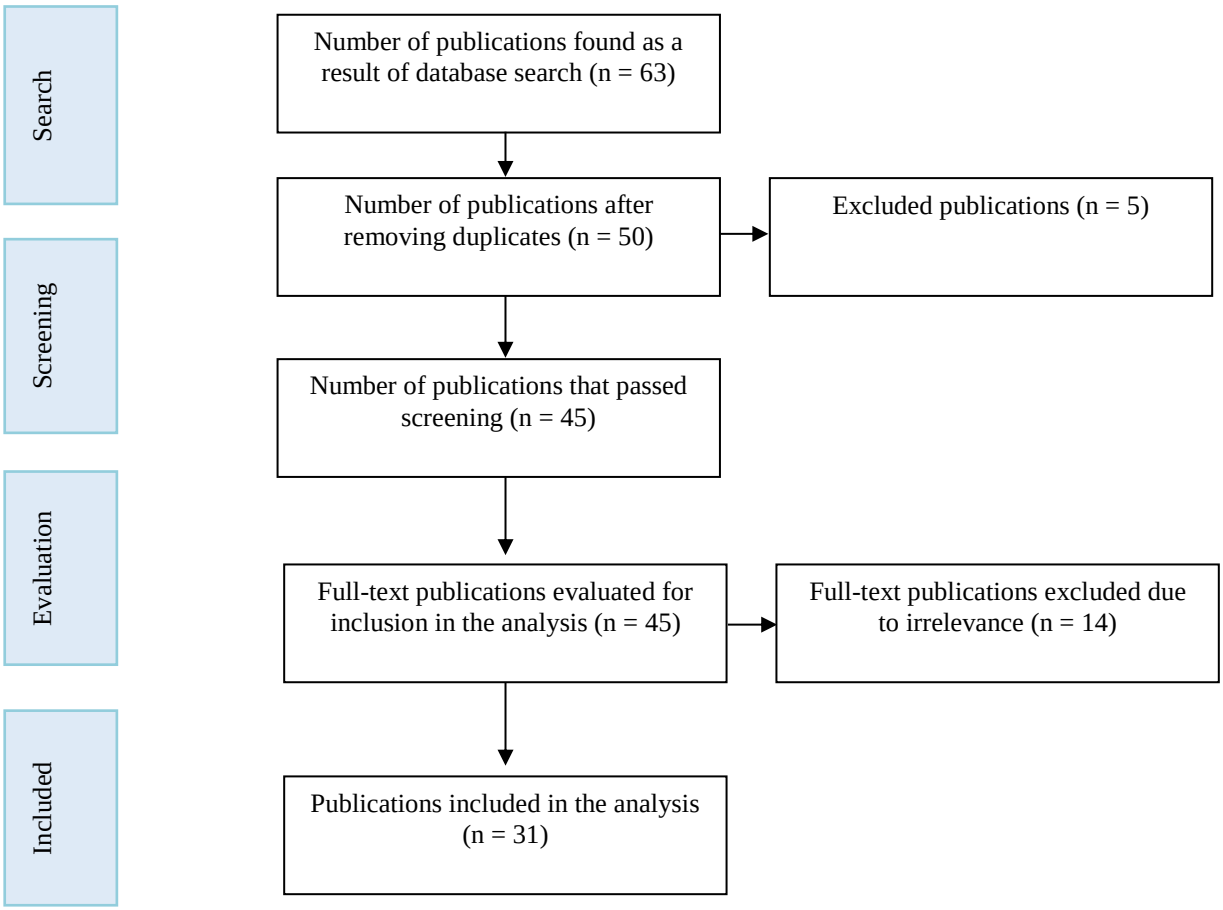

Fig. 2. PRISMA flow chart with the results of publication selection process

The majority of scientific publications (28 articles) referred to vertebral heart scale measurement in dogs and cats in the normal and pathological condition; 2 articles investigated the requirements to the technique of acquiring X-ray images intended for measuring VHS value; 1 article studied the use of artificial intelligence technology for measuring vertebral heart scale in dogs and cats.

The use of vertebral heart scale method of measurement for assessing the size of the cardiac silhouette during radiodiagnostics of dogs was first mentioned in 1995 [2]. J.W. Buchanan and J. Bücheler developed this method based on the fact that there was a good correlation between the size of the heart and the length of vertebral bodies regardless of the shape of canine thorax. 100 clinically healthy dogs at the age over one year (regardless of sex) were selected for

the study. On the obtained radiographs of the cardiac silhouette, the sizes of the long and short axes were determined using a beam caliper, summed up and compared with the length of the vertebrae, starting from T4: the ratio was 9.7 ± 0.5 vertebra. Differences between the values in dogs with a wide or deep chest, males and females, as well as between the right or left lateral views were insignificant and were not taken into account. Afterwards, the obtained VHS value was for a long time considered the norm for the dogs of various breeds [2].

Later, in 2000, a similar study involved 100 clinically healthy cats. In the lateral X-rays, the average heart size relative to the vertebrae was 7.5 ± 0.3 vertebrae. The data obtained made it possible to conclude that the method for determining the size of the heart relative to the vertebrae is easy to use. It enables objective assessment of the heart size, thus, can be helpful in determining the presence of cardiomegaly as well as in comparing the sizes of the cardiac silhouette on a series of consecutive radiographs. In addition, this study showed that the VHS method of measurement is effective not only in dogs, but also in cats [4].

The earliest experiments of VHS measurement involved only healthy animals. Therefore, to evaluate the capacity of vertebral heart scale measurement for the description of chest X-rays in animals with cardiac pathology, another study was conducted in 2000: chest X-rays of 50 dogs with established cardiac pathology, 26 X-rays of dogs with respiratory pathology, and 50 X-rays of dogs with no clinical signs of cardiovascular or respiratory diseases were mixed and examined by three veterinarians. In the first examination of the X-rays, VHS was not measured, and the diagnosis was made based on other radiographic features. The accuracy of cardiac disease diagnosis by each expert based on the subjective evaluation of radiographs without VHS measurement was moderately accurate (ranging from 76% to 83% of correct diagnoses). Afterwards, the experts re-examined the radiographs and measured VHS on both lateral and dorsoventral or ventrodorsal radiographs. It is worth noting that measuring vertebral heart scale on the examined radiographs did not almost influence the decision about correctness of the previously made diagnosis. However, all veterinarians got a higher mean VHS value in dogs with heart pathology than in dogs with other pathologies or without them. Based on the results of the experiment, it was concluded that VHS value more than 10.7 on the lateral X-rays can be considered a fairly consistent sign of heart pathology [5].

In 2001, the vertebral heart scale was measured in 11 puppies without signs of pathology. It was found that the standards for determining the indicators of enlarged heart in puppies and adult dogs were similar [6].

Later on, due to the large variability of breed characteristics in dogs, the studies on measuring the vertebral heart scale were carried out individually for each breed. In 2005, the VHS was measured for 44 dogs of Whippet breed having no heart and lung diseases. The mean vertebral heart scale value for this breed upon evaluation of the right lateral X-rays was 11.3 ± 0.5 vertebrae [7].

In 2007, the measurement was carried out in Greyhounds. It aimed to compare VHS in normal Greyhounds to that in Rottweilers and a group of dogs of other breeds. As a result, it was found that VHS was significantly higher in Greyhounds compared to Rottweilers and other breeds. For Greyhounds, the mean VHS on lateral radiographs was 10.5 ± 0.1, for Rottweilers — 9.8 ± 0.1, and for mixed breed dogs — 10.1 ± 0.2. This study also confirmed that relative cardiomegaly, subsequently detected at necropsy and ECG in Greyhounds, could be easily detected by plain radiography [8].

In 2007, radiographs of 50 adult stray cats were examined. All cats were short-haired, non-obese, and considered healthy based on clinical examination and electrocardiography. Long and short axes of heart were measured in millimetres. Length of vertebrae was measured caudally from T4. The sum of the long and short axes of the heart expressed as VHS was 7.3 ± 0.49 vertebrae in the right lateral view and 7.3 ± 0.55 vertebrae in the left lateral view. Differences between the right and left lateral views were minor [9].

In 2008, 19 Beagle dogs were examined using echocardiography, electrocardiography, noninvasive blood pressure measurement, complete blood count, and serum chemistry profile. Then, all studied dogs underwent the right and left lateral chest radiography, and vertebral heart scale measurement. The VHS in Beagles was 10.3 ± 0.4 vertebrae, which differed from the mean VHS value of 9.7 ± 0.5 as determined in JW Buchanan's original study for dogs of different breeds [2]. The data obtained had once again confirmed the fact that the results should be interpreted taking into account breed-specific differences, especially for those values that were significantly different from the reference range [10].

In 2009, the efficiency of VHS measurement was evaluated in dogs with cough and established degenerative mitral valve disease. Chest X-rays of 90 dogs with coughing in history and echocardiographic evidence of mitral valve disease were examined by two independent specialists. They were first asked to determine the cause of cough (cardiogenic, non-cardiogenic, or mixed) and then to measure VHS. Dogs with non-cardiogenic cough had significantly lower VHS

(mean value 11.0 ± 0.9) compared to the dogs with cardiogenic or mixed cough (12.8 ± 1 and 12.9 ± 0.9, respectively). Analysis of the results showed that VHS value less than or equal to 11.4 is a precise enough indication to exclude cough of cardiac origin in dogs with mitral valve disease. The results of the study suggest that VHS measurement may be an additional tool for differentiating the cause of coughing in dogs with cardiac diseases [11].

In 2013, a study was published describing the measurement of VHS in dogs of the following breeds: Pug, Pomeranian, Yorkshire Terrier, Dachshund, Bulldog, Shih Tzu, Lhasa Apso, and Boston Terrier. The study included healthy animals with no subjective radiographic signs of cardiomegaly, without murmurs, or abnormal heart rhythms during clinical examination. It was found that in groups of dogs that included Pugs, Pomeranians, Bulldogs, and Boston Terriers, mean VHS was 10.7 ± 0.5 [12].

The aim of another experiment in the same 2013 was to evaluate the efficiency of VHS method in differentiating congestive heart failure from other causes of dyspnea in cats. To be included in the study, cats were examined using echocardiography and chest radiography within 12 hours after admission to the clinic. The study revealed that VHS > 9.3 vertebrae was a differentiating feature for detection of cardiac diseases. Measurements, which resulted in the range of 8.0 to 9.3 suggested that the cause of dyspnea might be equivocal (i.e. secondary to congestive heart failure or respiratory disease), in this case echocardiography would be the most useful method in providing additional diagnostic information. The results of the study had revealed that VHS method of measurement may be a useful tool facilitating differentiation of cardiogenic from non-cardiogenic causes of respiratory failure in cats in the emergency situation when echocardiography is unavailable or not warranted in an unstable patient [13].

In 2014, echocardiographic and radiographic examination of thorax was carried out in 150 cats aiming to evaluate the diagnostic accuracy of VHS and other related radiographic indices upon detecting cardiomegaly caused by various feline cardiac diseases. Eighty-three cats had various cardiac diseases, and 22 cats had no cardiovascular anomalies. The measurement yielded the following values: VHS in the lateral view in healthy cats was 7.56 ± 0.54; in cats with left-sided heart diseases — 8.62 ± 1.04; in cats with left-sided heart diseases but without left atrial enlargement or with moderate left atrial enlargement — 8.29 ± 0.83; in cats with left-sided heart diseases and with moderate to severe left atrial enlargement — 8.95 ± 1.13 [14].

In 2016, a study was conducted to establish reference values for VHS in Indian Spitz, Labrador Retrievers, and outbred dogs. In total, 60 animals (20 Indian Spitz, 20 Labrador Retrievers, and 20 outbred dogs) with no radiographic or clinical signs of cardiovascular or pulmonary diseases were included in the study. As a result of the study, the following VHS values in the right lateral view were established: 10.21 ± 0.13 — for Spitz breed, 10.39 ± 0.19 — for Labrador Retrievers, and 9.8 ± 0.21 — for outbred dogs [15].

In 2017, an experiment aimed at measuring the vertebral heart scale in Dachshund dog breed and comparing the results with the established reference range of 9.7 ± 0.5 was conducted. It involved 51 dogs of Dachshund breed, which underwent radiography and echocardiography. As a result of the study, it was found that mean VHS in the right lateral X-ray was 10.3 vertebrae (the range 9.25–11.55). Whereas VHS in females was higher than in males: 10.8 (9.50–11.55) versus 9.99 (9.25–10.8). The results revealed that in healthy Dachshunds, the mean VHS value exceeded the published standard range for dogs [16].

In 2019, vertebral heart scale was measured in 75 dogs with preclinical stage of atrioventricular valve endocardiosis. The study revealed that, with this pathology, increase of vertebral heart scale value confirmed the presence of cardiomegaly, but did not specify the parts of the heart with pathological changes [17].

In another study of 2019 VHS was measured in 61 dogs of Norwich Terrier breed without clinical signs of cardiovascular disease. It was found that VHS value in dogs of this breed was 10.6 ± 0.6, which is higher than the reference value for dogs (9.7 ± 0.5) [18].

Also in 2019, the VHS was measured in the Australian Cattle dog breed. The study involved 20 dogs having no heart or lung diseases. The mean VHS value in the Australian Cattle Dog turned to be also higher (10.5 ± 0.4) compared to the mean VHS originally indicated for 100 healthy dogs of different breeds (9.7 ± 0.5) [19].

In the study held in 2021, mean reference VHS value was established in Persian cats equalling to 8.16 vertebrae (higher than the value of 7.5 reported in earlier studies for other cat breeds). It was found that gender did not affect these values [20].

In the same year, 2021, VHS was measured in Maltese dog breed. It involved 81 clinically healthy dogs that had undergone a complete cardiac examination. The mean VHS was 9.53 ± 0.46 vertebrae [21].

At the end of 2021, VHS was measured in Brittany Spaniel dog breed. The study included 28 healthy dogs and 15 dogs with myxomatous mitral valve disease. Measurements were taken by examining the right lateral X-rays. The mean VHS ± SD (standard deviation)

was 10.6 ± 0.2 in healthy animals and 11.9 ± 1.1 in animals with identified pathology [22].

Another paper of 2021 encompassed VHS measurement in 30 dogs of Chihuahua breed. The aim of the study was to assess VHS value in healthy adult dogs, since this breed is predisposed to both congenital and acquired heart diseases. The VHS value in the sample population was 10.0 ± 0.6 [23].

In 2022, VHS was established for Pembroke Welsh Corgi dog breed and the influence of the thoracic vertebrae features on this measurement was assessed. As a result, it was possible to measure the vertebral heart scale in these animals, which equalled to 9.36 ± 0.27 (below the reference value). The authors supposed that it might be due to the slight difference of the features of thoracic vertebrae in Corgi from other breeds [24].

In 2022, a study involving dogs of Cavalier King Charles Spaniel breed was conducted. This breed is predisposed to developing myxomatous mitral valve disease, and radiographs are often used to detect signs of left-sided cardiomegaly secondary to this disease. The study was conducted in 30 clinically healthy adult dogs (22 females and 8 males), aged 1 to 6 years. Inclusion criteria were: absence of pathologies during physical examination, normal echocardiography, and chest radiographs without signs of anomaly. The mean VHS value in the sample was 10.08 ± 0.56. This value exceeded the previously published general reference value for dogs equal to 9.7 ± 0.5 vertebrae [25].

In the earlier experiments of 2021, VHS was measured for healthy dogs of Chihuahua breed. The aim of the study conducted in 2022 was to evaluate the influence of cardiac enlargement on VHS in Chihuahuas with myxomatous mitral valve disease. The experiment included 10 clinically normal Chihuahuas and 97 Chihuahuas with the disease. The sick dogs were divided into 3 groups depending on the severity of the disease: mild, moderate, severe. Mean VHS in healthy dogs was 9.66 ± 0.36; in those with mild myxomatous mitral valve disease — 10.13 ± 0.64; with moderate — 10.87 ± 0.71; with severe — 11.71 ± 0.78. Conclusion: VHS in dogs of this breed increased depending on the cardiac enlargement caused by myxomatous mitral valve disease [26].

In 2023, a chest X-ray examination was performed in 10 dogs diagnosed with dilated cardiomyopathy (DCM). Measurement of vertebral heart scale showed that, on average, its value increased by 0.5–1.0, which was a consistent indication of the presence of cardiomegaly confirmed by other methods of visual diagnostics as well [27].

In the same 2023, by measuring VHS in 29 clinically healthy dogs it was possible to establish its value for American Staffordshire Terrier dog breed. Each dog underwent ECG, chest X-ray and echocardiography. All dogs had no cardiac or pulmonary pathologies. The mean VHS value for this breed was 10.9 ± 0.6. This value did not differ significantly between males and females, and did not have consistent correlation with age or body weight of an animal [28].

In late 2023, the expediency of measuring VHS for predicting left-sided congestive heart failure in dogs with respiratory signs was determined. The study included 114 dogs with respiratory signs and radiographic evidences of lung disorders. Animals underwent chest echocardiography and radiography. The diagnosis of left-sided congestive heart failure was confirmed based on the presence of respiratory signs, left ventricular hypertrophy, and cardiogenic pulmonary edema. In half of the animals participating in the study this pathology was established, and in the other half — it was not. According to the results of VHS measurement in animals without pathology, its mean value was 11.1 (10.1–11.8), and in dogs diagnosed with this pathology, it was 12.9 (11.9–14.1). These results may help to exclude cardiogenic pathology in dogs with respiratory signs [29].

VHS values for different dog breeds are presented in Table 1, indicating the year of the study.

The review also included literature sources studying the requirements to the technique of acquiring X-ray images and the prospects of using artificial intelligence technology to measure VHS.

In 2008, the paper describing the effect of using the left or right lateral view in dogs for measuring VHS was published. The study was conducted on 63 healthy dogs. VHS was slightly higher in the right lateral decubitus position (9.8 ± 0.6) compared to the left position (9.5 ± 0.8). The sex and size of a dog did not significantly affect VHS values [30].

In 2015 the study was conducted aimed to evaluate the variability of VHS values caused by cardiac and respiratory cycles in Beagle dog breed. The experiment involved 14 dogs that underwent X-ray examination, VHS was measured during the inhalation phase and exhalation phase, and during systole and diastole in the left and right lateral decubitus positions. The mean VHS value was compared within and between cardiac and respiratory phases. The mean VHS for each phase of the respiratory and cardiac cycle was higher on images obtained in the right lateral decubitus position compared to the left lateral decubitus position. The largest differences were observed between VHS in the diastolic phase of inhalation (10.59 ± 0.49 and 10.35 ± 0.50 for the right and left lateral views, respectively) and systolic phase of exhalation (10.11 ± 0.37 and 9.92 ± 0.50 for the right and left lateral views, respectively). The results of the study emphasise the need for the clinicians to be aware of the potential influence of these factors when assessing VHS in dogs [31]

Table 1

Vertebral heart scale values for different dog breeds

Dog breed	Year of the study	Established VHS Value
Whippet	2005	11,3 ± 0,5
Greyhound	2007	10,5 ± 0,1
Rottweiler	2007	9,8 ± 0,1
Beagle	2008	10,3 ± 0,4
	2015	10,59 ± 0,49 (right) and 10,35 ± 0,50 (left) during the inhalation phase. 10,11 ± 0,37 (right) и 9,92 ± 0,50 (left) during the inhalation phase
Pug, Pomeranian, Shih Tzu, Yorkshire Terrier, Lhasa Apso, English Bulldog, Boston Terrier	2013	10,7 ± 0,5
Indian Spitz	2016	10,21 ± 0,13
Labrador Retriever	2016	10,39 ± 0,19
Dachshund	2017	10,3
Norwich Terrier	2019	10,6 ± 0,6
Australian Cattle Dog	2019	10,5 ± 0,4
Maltese	2021	9,53 ± 0,46
Brittany Spaniel	2021	10,6 ± 0,2
Chihuahua	2021 2022	10,0 ± 0,6 9,66 ± 0,36
Pembroke Welsh Corgi	2022	9,36 ± 0,27
Cavalier King Charles Spaniel	2022	10,08 ± 0,56
American Staffordshire Terrier	2023	10,9 ± 0,6

A forward-looking direction for X-ray diagnostics in veterinary medicine is the use of artificial intelligence (AI) technology for measuring vertebral heart scale. In the study of 2021, scientists compared the results of VHS measurement in cats and dogs performed by artificial intelligence and two certified specialists [1]. Thirty canine lateral chest X-rays and thirty feline lateral chest X-rays were used. The images were assessed by each specialist using two different methods for measuring the short axis of heart on canine radiographs: the original method published by Buchanan J.W. and a modified method. Whereas feline radiographs, were assessed using only Buchanan J.W. method. On the whole, VHS values measured by artificial intelligence, a radiologist, and a cardiologist had a high degree of agreement in both dogs and cats (intraclass correlation coefficient (ICC) = 0.998). The ability of the AI trained to find VHS reference values was consistent with the manual measurements carried out by the human specialists for both cats and dogs. The present study demonstrates an important benefit of such a computerized method for general veterinarians as it limits variability in the interpretation of results and provides more comparable VHS values over time [1].

Discussion and Conclusion. A review and analysis of scientific literature on application of vertebral heart scale

measurement method in dogs and cats during X-ray diagnostics of thoracic cavity enables to draw the following conclusions:

1. Research in the field of measuring vertebral heart scale in dogs and cats is conducted in three main areas: measuring VHS values in animals in normal and pathological condition; requirements to the technique of acquiring X-ray images for measuring the vertebral heart scale value; the use of artificial intelligence technology for measuring VHS in dogs and cats.

2. In veterinary medicine, the vertebral heart scale method of measurement has proved to be an efficient alternative to other, more labour-intensive and expensive methods of diagnostics of feline and canine cardiomegaly. It can be used in veterinary clinics to determine the cardiogenic or non-cardiogenic status of a patient.

3. Further research can aim at obtaining new data on species-, breed-, age-specific, physiological and pathological features of VHS measurement in dogs and cats.

4. Practices of using artificial intelligence in veterinary X-ray diagnostics shows its potential for measuring VHS in animals.

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INFECTIOUS DISEASES AND ANIMAL IMMUNOLOGY
ИНФЕКЦИОННЫЕ БОЛЕЗНИ И ИММУНОЛОГИЯ ЖИВОТНЫХ

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Monitoring Chicken Infectious Diseases in the Krasnodar Territory and the Republic of Adygea

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Abstract

Introduction. One of the major goals set before the agro-industrial complex is to supply the population of the Russian Federation with the high-quality raw materials and products of animal husbandry. Therefore, poultry farms are implementing modern technologies of intensive poultry farming and carry out regular prophylactic work to ensure epizootological safety of poultry rearing. To make these measures effective, it is necessary to specify the diseases typical for poultry in different regions of the country. The aim of the research is to study and analyse the pathogens isolated from pathological material from chickens reared in the Krasnodar Territory and the Republic of Adygea for planning the effective anti-epizootic, preventive and therapeutic measures.

Materials and Methods. The objects of the study were chickens from the poultry farms of the Krasnodar Territory and the Republic of Adygea and biological material from dead birds (pathological material). In total, 2018 samples were examined during the period of 2019–2023. The research was carried out using a range of methods (epizootological, clinical, pathoanatomical, bacteriological, serological) based on the commonly accepted techniques.

Results. During a five-year research period, 5 pathogens of infectious diseases were isolated from the pathological material obtained from chickens reared in the Krasnodar Territory and the Republic of Adygea (*E. coli, E. faecalis, St. aureus, Str. pneumoniae, S. pullorum*), both pure culture and mixed culture. 26 serotypes of *E. coli* pathogen were isolated from chickens. Whereas, percentage of escherichiosis detected in chickens in the Krasnodar Territory and the Republic of Adygea equaled to 41.43%; enterococcosis — to 26.51%; staphylococcal infection — to 18.11%; streptococcosis — to 12.29 %; salmonellosis — to 0.1%; mixed (escherichiosis and enterococcosis) infection — to 1.64%.

Discussion and Conclusion. Based on the conducted monitoring, it can be concluded that at poultry farms of the Krasnodar Territory and the Republic of Adygea the current situation with the infectious diseases in chickens has improved compared to that five years ago, however, the percentage of escherichiosis, streptococcosis, staphylococcosis and enterococcosis morbidity in poultry is still quite high. Therefore, we recommend the management of the farms to plan the anti-epizootic measures aimed at timely prevention of the above mentioned infectious diseases.

Keywords: poultry, chickens, bacteriological research, infectious diseases, monitoring, pathological material, Krasnodar Territory, Republic of Adygea

Declaration of Compliance with the Principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes: the authors declare that all research was conducted in compliance with the principles of Good Laboratory Practice.

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Оригинальное эмпирическое исследование

Мониторинг инфекционных болезней кур в Краснодарском крае и в Республике Адыгея

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Аннотация

Введение. Одной из основных задач агропромышленного комплекса является обеспечение населения Российской Федерации доброкачественным сырьем и продуктами животноводства. Для этого на птицеводческих предприятиях внедряют современные технологии ведения интенсивного птицеводства, проводят регулярные профилактические работы по обеспечению эпизоотологической безопасности выращивания птицы. Чтобы эти мероприятия были эффективными, необходимо знать, какие заболевания характерны для птицы в разных регионах страны. Целью данной работы является изучение и анализ выделенных патогенов из патологического материала от кур, выращенных в Краснодарском крае и в Республике Адыгея, для планирования эффективных противоэпизоотических, профилактических и лечебных мероприятий.

Материалы и методы. Объектом исследования были куры птицеводческих предприятий Краснодарского края и Республики Адыгея и биологический материал от павшей птицы (патматериал). Всего в период 2019–2023 гг. было исследовано 2018 проб. При проведении исследований использовали комплекс методов (эпизоотологические, клинические, патологоанатомические, бактериологические, серологические), которые осуществляли по общепринятым методикам.

Результаты исследования. Из поступившего патматериала от кур, выращенных в Краснодарском крае и в Республике Адыгея, за пятилетний период исследования было выделено 5 патогенов инфекционных болезней (*E. coli*, *E. faecalis, St. aureus, Str. pneumoniae, S. pullorum*), как в чистом виде, так и в виде ассоциации с другими микробами. Из патогенных *E. coli* у птицы выделено 26 серотипов. При этом выявленный эшерихиоз у кур в Краснодарском крае и в Республике Адыгея составил 41,43 %; энтерококкоз — 26,51 %; стафилококковая инфекция — 18,04 %; стрептококкоз — 12,29 %, сальмонеллез — 0,1 %, смешанная инфекция (эшерихиоз и энтерококкоз) — 1,64 %.

Обсуждение и заключение. На основании проведенного мониторинга можно заключить, что в настоящее время ситуация с инфекционными заболеваниями кур на птицеводческих предприятиях Краснодарского края и Республики Адыгея улучшилась по сравнению с предыдущими годами, однако процент заболевания птицы эшерихиозом, стрептококкозом, стафилококкозом и энтерококкозом остается весьма высоким. В связи с этим рекомендуем руководству хозяйств запланировать противоэпизоотические мероприятия, направленные на своевременную профилактику и лечение указанных инфекционных болезней.

Ключевые слова: птица сельскохозяйственная, куры, бактериологические исследования, инфекционные болезни, мониторинг, патматериал, Краснодарский край, Республика Адыгея

Декларация о соблюдении принципов Европейской конвенции о защите позвоночных животных, используемых для экспериментов и других научных целей: авторы заявляют, что все проведенные исследования соответствовали принципам конвенции и правилам надлежащей лабораторной практики.

Для цитирования. Шевченко А.А., Конов Б.Р., Черных О.Ю., Шевченко Л.В. Мониторинг инфекционных болезней кур в Краснодарском крае и в Республике Адыгея. *Ветеринарная патология*. 2025;24(2):55–62. https://doi.org/10.23947/2949-4826-2025-24-2-55-62

Introduction. One of the main objectives of the agro-industrial complex is supplying the population of the Russian Federation with the high-quality raw materials and products of animal husbandry. In the frame of this activity, poultry farms are implementing innovative technologies of intensive poultry farming, as well as carry out regular prophylactic work to ensure epizootological safety of poultry rearing. The most dangerous infectious diseases for chickens are tuberculosis, Newcastle disease, leucosis, escherichiosis, avian influenza, infectious laryngotracheitis, Marek's disease and others. In case of outbreak, these diseases cause mass poultry mortality inducing major economic damage to the poultry industry.

In recent years, the interest of the researchers to animal diseases caused by opportunistic microflora has raised [1, 2, 3]. In the context of decreased resistance of the organism to infection, violation of veterinary, animal hygiene, sanitary rules for rearing animals, there occur

diseases caused by concomitant microflora — streptococcosis, staphylococcosis, escherichiosis, enterococcosis, salmonellosis [4, 5]. According to the data obtained by many researchers, agricultural poultry can infect other species of animals, as well as humans, therefore, it is necessary to conduct timely monitoring of microbes and detect pathogenic species to prevent infected products from reaching animals and people [6, 7, 8]. The use of antibiotics for treatment of sick animals without checking their efficacy against the isolated microorganisms, violation of the dosage and course of therapy lead to rapid adaptation of microbes to unfavourable factors and emergence of the new variants of pathogens, especially of *E. coli*. Therefore, to prevent infectious diseases, the mandatory action plan of each poultry farm should include quick high-quality diagnostics and various veterinary and sanitary procedures to prevent contamination of animal husbandry raw materials and infecting susceptible animals and people [9, 10]. *The aim of the article* is to study and analyse the pathogens isolated from pathological material from chickens reared in the Krasnodar Territory and the Republic of Adygea to ensure efficient organisation of the anti-epizootic, preventive and therapeutic measures.

Materials and Methods. The research was conducted at the Department of Microbiology, Epizootology and Virology Department, at Territory-level Veterinary Laboratory of Kropotkin and at various poultry farms of the Krasnodar Territory and the Republic of Adygea in the period of 2019–2023. The objects of the study were agricultural poultry (chickens) and isolates of microorganisms (pathological material) from sick and dead birds. A total of 2018 samples were examined. A comprehensive examination was carried out taking into account the epizootological indicators, clinical signs in poultry, pathological changes and the results of laboratory tests as per the isolated pathogen.

Laboratory diagnostics was based on the use of bacteriological tests that enable the isolation of a pathogen, its identification, determination of the type, serotype, pathogenicity of a microorganism [11–14]. The diagnostics was carried out in compliance with the current regulatory documents adopted by the Veterinary Authorities of the Russian Federation on bacteriological methods of examining the pathological material (from the heart, liver, spleen, intestinal contents, bone marrow) obtained from fallen and forcedly killed birds.

The study on the isolation of the pathogen of colibacillosis (escherichiosis) was carried out in compliance with the "Methodological Guidelines for the Bacteriological Diagnostics of Colibacillosis (Escherichiosis) in Animals", approved on July 27, 2000 by the Deputy Head of the Veterinary Medicine Department of the Ministry of Agriculture and Food of the Russian Federation.

The study on enterococcosis and mixed intestinal infection was carried out in compliance with the "Methodological Guidelines for the Bacteriological Diagnostics of Mixed Intestinal Infection in Young Animals Caused by Pathogenic Enterobacteria" No. 13-7-2/1759 of October 11, 1999 adopted by the Veterinary Medicine Department of the Ministry of Agriculture and Food of the Russian Federation.

The study on staphylococcosis was carried out in compliance with the "Methodological Guidelines for Laboratory Diagnostics of Staphylococcosis in Animals" approved on June 30, 1987 by the Head of the Main Veterinary Directorate of the State Agro-Industrial Committee of the USSR.

The study on the isolation of pathogens of streptococcosis was carried out in compliance with the "Methodological Guidelines for Laboratory Diagnostics of Streptococcosis in Animals" approved on September 25, 1990 by the Deputy Head of the Main Veterinary Directorate with the State Veterinary Inspectorate under the State Commission of the USSR Council of Ministers for Food and Procurement.

For the study on salmonellosis, "Methodological Guidelines MU 4.2.2723–10 for Laboratory Diagnostics of Salmonellosis, Detection of Salmonella in Food Products and Environmental Objects" published by the Federal Centre for Hygiene and Epidemiology of Rospotrebnadzor in 2011 was used.

Standard laboratory equipment was used to conduct the research: a thermostat at 37 °C, and a microscope ("Biolam", Russia) [15, 16]. Bacteriological tests included determination of the morphological, tinctorial, bacteria culture, and serological indicators in the isolated microorganisms [17–20].

The epizootic situation was assessed based on the analysis of disease outbreaks registered in the record books of the farms.

Research Results. During laboratory study of the pathological material from poultry from the various farms of the Krasnodar Territory and the Republic of Adygea in the period of 2019–2023, 5 pathogens of infectious diseases were isolated: escherichiosis (*E. coli*), enterococcosis (*E. faecalis*), staphylococcosis (*St. aureus*), streptococcosis (*Str. pneumoniae*), mixed microflora (*E. coli, E. faecalis*), salmonellosis (*S. pullorum*) (Fig. 1, Table 1).

A significant number of different *E. coli* serotypes are known. Intestinal bacterium (E. coli) has different types of antigens: O-antigen (somatic), K-antigen (capsular), H-antigen (flagellar), some of them are pathogenic, others are non-pathogenic. Of the pathogenic *E. coli* from chickens, 26 serotypes of somatic O-antigen were isolated, the most common were serotypes 1, 2, 9, 15, 20, 27, 33, 45, 55, 78, 139 (Fig. 2, Table 2).

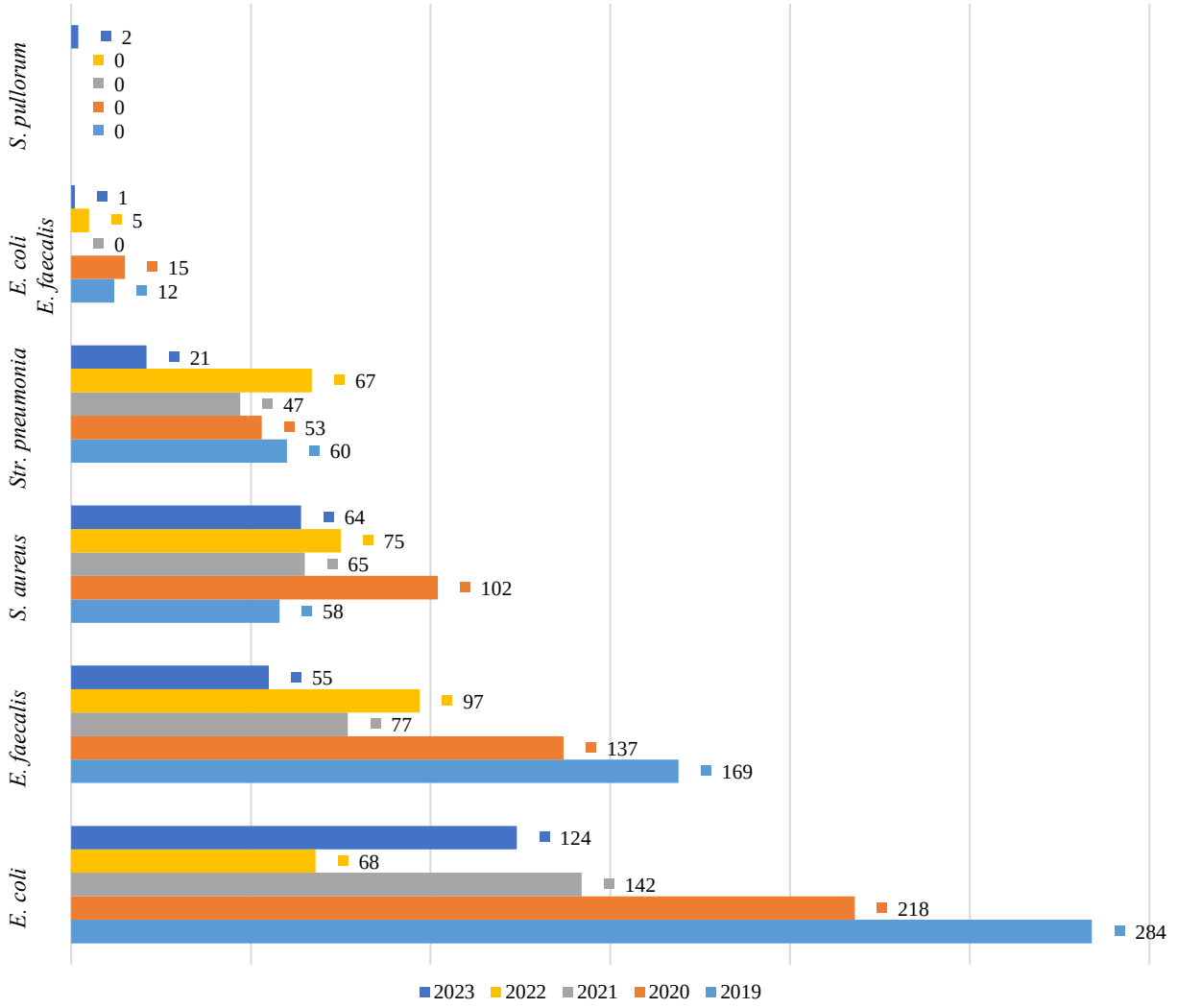

Fig. 1. Dynamics of pathogen isolation from chickens reared in the Krasnodar Territory and the Republic of Adygea in the period of 2019–2023

Table 1

Monitoring cases of infectious diseases in chickens in the Krasnodar Territory and the Republic of Adygea in the period of 2019–2023

No	Title	2019		2020		2021		2022		2023		Total	
		number	%*	number	%*	number	%*	number	%*	number	%*	number	%*
1.	Escherichiosis	284	48,54	218	41,52	142	42,90	68	21,79	124	46,79	836	41,43
2.	Enterococcosis	169	28,88	137	26,09	77	23,26	97	31,08	55	20,75	535	26,51
3.	Staphylococcosis	58	9,91	102	19,42	65	19,63	75	24,03	64	24,15	364	18,04
4.	Streptococcosis	60	10,25	53	10,09	47	14,19	67	21,47	21	7,92	248	12,29
5.	Escherichiosis + enterococcosis	12	2,05	15	2,85	0	0	5	1,6	1	0,37	33	1,64
6	Salmonellosis	2	0,34	0	0	0	0	0	0	0	0	2	0,1
Total		585	18,28	525	26,01	331	16,40	312	15,46	265	13,13	2018	100,0

Note: * — percentage of the total number of samples tested (n=2018)

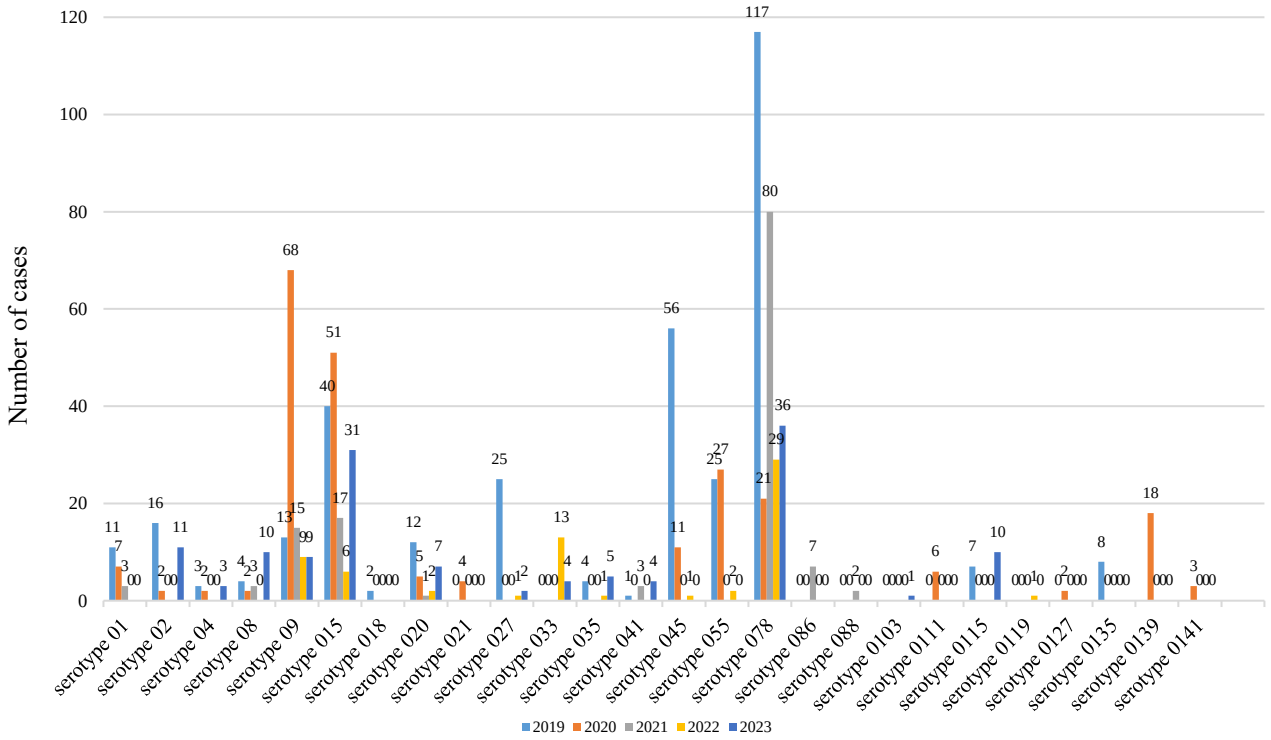

Fig. 2. Dynamics of isolated *E. coli* serotypes from pathological material from chickens reared in the Krasnodar Territory and the Republic of Adygea in the period of 2019–2023.

Table 2

Isolated *E. coli* serotypes from pathological material from chickens reared in the Krasnodar Territory and the Republic of Adygea in the period of 2019–2023

	E. coli Serotypes	2019		2020		2021		2022		2023		Total	
		Number	%*	Number	%**	Number	%***	Number	%****	Number	%*****	Number	%
1.	01	11	3,87	7	3,21	3	2,01	0	0	0	0	21	2,22
2.	02	16	5,63	2	0,91	0	0	0	0	11	9,01	18	1,9
3.	04	2	0,70	0	0	0	0	0	0	3	2,45	10	1,05
4.	08	4	1,40	0	0	3	2,01	0	0	10	8,19	22	2,32
5.	09	13	4,57	68	31,19	15	10,06	9	13,23	9	7,37	114	12,05
6.	015	40	14,08	51	23,39	17	11,40	6	8,82	31	25,40	145	15,32
7.	018	2	0,70	0	0	0	0	0	0	0	0	2	0,21
8.	020	12	4,22	5	2,29	1	0,67	2	2,94	7	5,73	27	2,85
9.	021	0	0	4	1,83	0	0	0	0	0	0	4	0,42
10.	027	5	1,76	0	0	0	0	1	1,47	2	1,63	28	2,96
11.	033	0	0	0	0	0	0	13	19,11	4	3,27	17	1,8
12.	035	4	1,40	0	0	0	0	1	1,47	0	0	5	0,53
13.	041	1	0,35	0	0	0	0	3	4,41	0	0	4	0,42
14.	045	26	9,15	0	0	11	7,38	1	1,47	0	0	68	7,19
15.	055	25	8,80	27	12,38	0	0	2	2,94	0	0	54	5,7
16.	078	115	40,49	21	9,63	80	53,69	29	42,64	36	29,50	342	36,15
17.	086	0	0	0	0	7	4,69	0	0	0	0	7	0,73
18.	088	0	0	0	0	2	1,34	0	0	0	0	2	0,21
19.	0103	0	0	0	0	0	0	0	0	1	0,81	1	0,10
20.	0111	0	0	6	2,75	0	0	0	0	0	0	6	0,63
21.	0115	0	0	7	3,21	0	0	0	0	10	8,19	17	1,8
22.	0119	0	0	0	0	0	0	1	1,47	0	0	1	0,10
23.	0127	0	0	2	0,91	0	0	0	0	0	0	2	0,21
24.	0135	8	2,81	0	0	0	0	0	0	0	0	8	0,84

	E. coli Serotypes	2019		2020		2021		2022		2023		Total	
		Number	%*	Number	%**	Number	%***	Number	%****	Number	%*****	Number	%
25.	0139	0	0	18	8,25	0	0	0	0	0	0	18	1,9
26.	0141	0	0	0	0	3	2,01	0	0	0	0	3	0,31
Total		284	30,02	218	23,04	149	15,75	68	7,18	122	12,89	946	100

Note: * — percentage of the total number of cases of escherichiosis in 2019 (n=284)
** — percentage of the total number of cases of escherichiosis in 2020 (n=218)
*** — percentage of the total number of cases of escherichiosis 2021 (n=149)
**** — percentage of the total number of cases of escherichiosis in 2022 (n=68)
***** — percentage of the total number of cases of escherichiosis in 2023 (n=122)

The pathogenic microorganisms *E. coli*, *E. faecalis*, *St. aureus*, *Str. pneumoniae*, *S. pullorum* detected as a result of monitoring, both pure culture and mixed culture with other microbes, caused the following infectious diseases in chickens: escherichiosis (41.43%), enterococcosis (26.51%), staphylococcosis (18.04%), streptococcosis (12.29%), mixed intestinal infection (escherichiosis and enterococcosis) — 1.64%, salmonellosis (0.1%).

Discussion and Conclusion. Based on the results of the conducted monitoring, a tendency towards improvement of the epizootic situation at poultry farms of the Krasnodar Territory and the Republic of Adygea can be noted compared to the situation five years ago. Nevertheless, the percentage of chickens infected with escherichiosis, streptococcosis, staphylococcosis and enterococcosis remains quite high. Due to high variability of the pathogen causing escherichiosis (*E. Coli*) (26 serotypes have been isolated over the past 5 years), it can infect humans through raw materials and products of animal husbandry. In this regard, the management of poultry farms is strongly recommended to take timely measures on diagnostics and prophylaxis of the above infectious diseases, as well as to treat poultry located in the unfavorable places after mandatory determination of the isolated pathogenic microorganisms' sensitivity to antibiotics. At the same time, the biological features of these types of microbes must be taken into account when developing the efficient technology for rearing poultry and animals at poultry and husbandry farms.

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HISTORY OF VETERINARY MEDICINE
ИСТОРИЯ ВЕТЕРИНАРИИ

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Original Theoretical Research

Photodynamic Therapy: The History of Formation and Development of the Neoplasm Treatment Method

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Abstract

At present, there exist many examples of successful implementation of photodynamic therapy in treatment of tumours of various localizations, as well as skin neoplasms and infectious diseases in both humans and animals. The article traces formation and development of the photodynamic therapy, shows the influence of the type of photosensitizer and the dose of radiation applied on the efficiency of treatment, it emphasizes the potential of this method due to its low invasiveness and ability to minimize side effects.

Keywords: photodynamic therapy, photosensitizer, irradiation, tumour, neoplasm, laser

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Оригинальное теоретическое исследование

Фотодинамическая терапия: история становления и развития метода лечения новообразований

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Аннотация

На сегодняшний день фотодинамическая терапия насчитывает большое количество примеров успешного применения при лечении раковых опухолей различной локализации, а также кожных новообразований и инфекционных заболеваний как людей, так и животных. В статье прослежен путь становления и развития фотодинамической терапии, показано влияние вида фотосенсибилизатора и дозы применяемого излучения на эффективность лечения, подчеркнута перспективность метода вследствие его низкой инвазивности и способности минимизировать побочные эффекты.

Ключевые слова: фотодинамическая терапия, фотосенсибилизатор, облучение, опухоль, новообразование, лазер

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Introduction. Development of the innovative efficient treatment of neoplasms is an important objective both for human and veterinary medicine, since cancer remains an acute problem despite significant scientific advances in the field of tumour diagnostics and treatment. One of the advanced and forward-looking approaches to treatment of oncological, as well as skin and infectious diseases, is photodynamic therapy (PDT), a method characterized by safety,

non-toxicity, minimal immune suppression, and low invasiveness [1]. From the molecular perspective, the efficiency of PDT depends on three pivotal factors necessary for a chemical reaction [2]:

1. Photosensitizer — a chemical substance capable of becoming active and generating reactive oxygen species that act on cells, when exposed to light of a certain wavelength. Photosensitizers can be organic or inorganic chemical compounds.

2. Light of the appropriate wavelength — to activate the photosensitizer, it is necessary to use light of a certain wavelength that corresponds to the absorption spectrum of the photosensitizer. The most commonly used light sources are a laser or a LED.

3. Dissolved oxygen in cells — the presence of oxygen in cells is essential for PDT efficiency, since the process involves activation of the photosensitizer by light resulting in conversion of the oxygen in the cells into singlet oxygen, which then attacks and destroys cancer cells or pathogens.

The aim of the present article is to track the history of photodynamic therapy development by illustrating data on discoveries in this area and successful application of the method in treatment of humans and animals; to show the efficiency of PDT in comparison to traditional methods of treatment and to evaluate expediency of further research in this area.

History of photodynamic therapy formation and development. Treatment of diseases using photochemotherapy was first mentioned in the literature of Ancient Egypt, China and India. Even then, doctors used natural photosensitizers (without knowing this word) — psoralens contained in parsnip, parsley, St. John's wort, black seeds of Psoralena corylifolia — to treat vitiligo and psoriasis, and noticed that the compounds of these herbs could be activated when exposed to sunlight [3, 4]. Historical references to treatment of various diseases using the effect of sunlight on the human body (heliotherapy) can be found in the works of famous scientists of Ancient Greece, such as Herodotus [5].

A qualitative leap in the development of photodynamic therapy occurred at the end of the 19th century thanks to the Danish physiotherapist N. Finsen, who studied the effect of sunlight on living organisms. In 1893, Finsen published the results of his research on the use of red light in treatment of chickenpox that helped to prevent pus formation in blisters. In 1903, the scientist was awarded the Nobel Prize for his achievements in the field of photodynamics [6].

In 1900, in the laboratory of the Austrian pharmacologist H. von Tappeiner, the medical student O. Raab first discovered an oxygen-dependent photochemical reaction. He experimentally established that certain dyes in the presence of oxygen and sunlight are capable of causing rapid death of Paramecia cells: the paramecium died when exposed to a combination of acridine dye solution and daylight, and survived in the absence of the dye or in its presence in the dark [3, 4, 7].

In 1903, Tappeiner and A. Jezionek developed their own PDT technology and conducted the first treatment sessions for diseases such as lupus, psoriasis, and skin cancer. They used a 1% eosin solution as a photosensitizer, and performed the prolonged insolation or artificial irradiation by an arc lamp [3, 4, 7]. At the same time, Tappeiner introduced the terms “photosensitizers” and “photodynamic action” into the scientific vocabulary, which made it possible to separate this effect from the photochemical reactions observed in photography [8].

Over the following years, the photodynamic therapy method underwent significant changes and improvements related to the development of the first porphyrin-based sensitizers. In 1911, W. Gausmann conducted an experiment that showed that Paramecia cells die when exposed to the lamp light in growth medium composed of hematoporphyrin [9]. In 1912, this substance was first tested in the human organism: the German doctor F. Meyer-Betz injected himself with 200 mg of hematoporphyrin intravenously, which resulted in solar photosensitivity of the body expressed in the form of edema and hyperpigmentation lasted for about two months.

In 1924, the French researcher A. Policard conducted research on animals with malignant tumours, injecting them with hematoporphyrin and then irradiating the tumour area with ultraviolet light: malignant neoplasms fluoresced in the orange-red region of the spectrum — this effect could be explained by the presence of porphyrin proteins in tumour cells [11, 12].

In 1942, two scientists from Berlin, G. Banzer and H. Auler, decided to study and evaluate the process of accumulation and fluorescence of porphyrins administered exogenously into the body. They found red fluorescence after subcutaneous and intramuscular administration of hematoporphyrin in the primary tumour and in metastases in rats [13, 14], which indicated the accumulation of

hematoporphyrin both in primary tumours and metastases, as well as in the lymph nodes. Exposure to light gave positive results [15].

In 1948, the American scientist W. Figg and his colleagues conducted an experiment involving 240 mice with transplanted tumours, who were injected with various porphyrins: hematoporphyrin, protoporphyrin, coproporphyrin and zinc hematoporphyrin. Observations showed that within 24–48 hours all of the above substances accumulated in tumours. After this period, fluorescence persisted for 10–14 days [16]. However, the use of these porphyrins as photosensitizers for PDT was hindered by their severe cutaneous phototoxicity.

In the 1960s, R. Lipson conducted a significant study aimed at enhancing the action of hematoporphyrin in tumour tissue. The experiment implied treating hematoporphyrin with sulfuric and acetic acids, followed by alkaline hydrolysis. As a result, a hematoporphyrin derivative was obtained, which subsequently started to be applied in fluorescent diagnostics of various oncological diseases, including lung, cervical and gastric cancer [17]. In 1966, Lipson and his team successfully used this derivative to treat a female patient with a recurrent ulcerating tumour of the mammary gland, which recurred just a few weeks after the completion of a course of radiation therapy. This study was an important step in the development of methods of diagnostics and treatment of oncological diseases, opening up new horizons for the use of photodynamic therapy.

In 1966-1967, studies were conducted aimed at assessing the efficacy of various sensitizing agents, including methylene blue, which stood out of the rest. The results of the experiments demonstrated its superiority over previously used reagents. In particular, significant destruction of tumour tissues was noted, as well as successful healing of wounds caused by unwanted damage [18].

In 1974, the first drug for photodynamic therapy called “Photofrin I” was registered. The drug was obtained from a mixture of hematoporphyrins using the membrane filtration technique. In 1975, Professor T. Doherty, head of the Photodynamic Therapy Centre at the Cancer Research Institute in Buffalo (USA), successfully treated 50% of mammary tumours in mice and Walker256 carcinosarcomas in rats. In his experiment, he used "Photofrin I", which got accumulated in target tissues, and then was activated by the red light of xenon lamp using optical filters [16, 17].

Since 1978, photodynamic therapy has been widely used clinically in the field of oncology. T. Doherty published the results of successful application of PDT in 25 patients suffering from Basal and Squamous cell carcinomas, melanoma, as well as recurrent and metastatic tumours of the skin and mammary gland. Irradiation of neoplasms was carried out using a xenon lamp 24–160 hours after the administration of the photosensitizer [17]. Subsequently, this method of treatment began to be actively developed in Japan, Germany, China, France, Great Britain and other countries.

In the 1980s, Doherty and colleagues isolated an active fraction of hematoporphyrin, called "Photofrin II", which differed from "Photofrin I" in its better selectivity for accumulation in tumour tissues and a pronounced antitumour effect. For a long time "Photofrin II" remained the only photosensitizer approved for treatment of oncological diseases of the skin and mucous membranes in many countries [19, 20].

In 1985, a female scientist B. Henderson described damage to blood vessels that fed tumour cells during their growth and progression induced by PDT. This mechanism became one of the key aspects of tumour destruction when using this treatment method [21].

It should be noted that the development of the photodynamic therapy and the increase of its efficiency was significantly related to the development, implementation and use of lasers. In 1964, scientists N.G. Basov, A.M. Prokhorov and C.H. Townes were awarded the Nobel Prize in Physics for outstanding achievements in the field of quantum electronics. Their fundamental research became the basis for the creation of generators and amplifiers based on the laser principle. This research not only opened new horizons in physics, but also served as a catalyst for the development of various types of lasers, which subsequently found wide application in various fields of science, including medicine.

In 1986, the Research Institute for Laser Surgery of the USSR Ministry of Health was established in the Soviet Union, headed by Professor O.K. Skobelkin, a surgeon, Honoured Scientist of the RSFSR and the founder of laser medicine in the country. In his scientific activity, the professor focused on the research aimed at creating a domestic photosensitizer and developing a laser device for photodynamic therapy [22].

In the 1980s, at the Institute of Biophysics of the USSR Ministry of Health, under the supervision of Professor G.V. Ponomarev, the drug “Dimegin” was developed based on natural protohemin [23]. In 1990, at Moscow State Academy of Fine Chemical Technology named after M.V. Lomonosov, under the supervision of Professor A.F. Mironov, the first domestic photosensitizer “Photogem” was synthesized from the group of hematoporphyrin derivatives [24].

A quite well-known representative of porphyrinic photosensitizers is 5-aminolevulinic acid (5-ALA), a natural precursor of the endogenous photosensitizer protoporphyrin IX (PP-IX). It was first used in 1990 by the Canadian scientist J. Kennedy. The mechanism of action was based on the ability of tumour cells to accumulate photoactive protoporphyrin IX in the presence of exogenous 5-ALA [8].

In 1994, clinical trials of the second-generation photosensitizer “Photosens”, representing a sulfonated aluminum phthalocyanine, were completed at the State Research Center "NIOPIK" under the supervision of Professors E.A. Lukyanets and G.N. Vorozhtsov. Today, “Photosens” is successfully used to treat malignant tumours of various localizations due to its high biological activity: the drug demonstrates a strong absorption band in the red region of the spectrum with a wavelength of 670–675 nm. Later, under the same supervision, the drug “Alasens” was developed, based on 5-ALA and since then used in treatment of keratoses, Basal cell carcinoma, bladder cancer, brain tumours, and stomach tumours [13].

Due to the reason that first-generation photosensitizers based on hematoporphyrin derivatives had a number of disadvantages (they got slowly accumulated in tumour tissues, had a fairly low therapeutic efficacy, and at the same time a long period of after-treatment cutaneous phototoxicity, which significantly deteriorated the quality of life of patients), further research in this area was aimed at developing second-generation photosensitizers that would have a broad absorption band in the red region of the spectrum [25]. Currently, drugs based on 5-ALA are registered in Norway — Metvix (PhotoCure ASA), Canada — Levulan (DUSA Pharma), the Republic of Belarus - Alamin (Institute of Bioorganic Chemistry of the National Academy of Sciences of Belarus) [16].

In the period of 1994–2001, research was conducted in Russia to develop technologies aimed at extracting a complex of biologically active chlorins from herbal raw material, among which the main component was E6 [26]. In 2002, the company “Rada-Pharma” LLC, under the supervision of Candidate of Chemical Sciences A.V. Reshetnikov, synthesized the domestic photosensitizer “Radachlorin” based on chlorin E6. And in 2004, “Veta-Grand” LLC, under the supervision of Professor G.V. Ponomarev, synthesized “Fotoditazin” based on E6.

Today, the following chlorine derivatives are widely used in clinical practice: “Foscan” (Biolitec AG, Germany), “Verteporfin” (Novartis Pharma, Switzerland), “Taloporfin” or “Laserphyrin” (Japan) [26]. Using light waves of a certain length, they are capable of targeted impact on tumour cells and their destruction. The development of such drugs is of great importance for medicine and veterinary science and opens up new possibilities in treatment of complex diseases.

In 2001, a third-generation photosensitizer called “Photolon” was developed in Belarus at the Republican Unitary Enterprise “Belmedpreparaty”. The main distinction of drugs of this generation is the technology of mixing photosensitizer molecules with molecules of other substances, such as nanoparticles or liposomes, which enables to significantly enhance selectivity for accumulation of photosensitizer in tumour tissue. The drug “Photolon” is a molecular complex consisting of chlorine E6 salt and polyvinylpyrrolidone. Due to its unique structure and properties, “Photolon” is widely used in oncology, in particular, in treatment of skin cancer, mucosal cancer and precancerous conditions of the cervix. Studies show that the use of this drug helps to increase the efficciency of photodynamic therapy due to improved accumulation of the active substance in tumour cells and minimization of the impact on healthy tissues [16].

On December 29, 2012, by Order No. 1629 of the Ministry of Health of the Russian Federation, photodynamic therapy was included in the high-tech medical care list, thus, emphasizing the importance of this advanced method of treatment, which continues to be developed and integrated into clinical practice. Currently, the development of PDT is aimed at creating new drugs that efficiently absorb light in the spectral region known as the “therapeutic window” (700–900 nm). In this region, the absorption of light by biological tissues is minimal, which allows for deep penetration of light into the body tissues, significantly reducing potential harm from radiation and increasing the efficiency of therapy.

Experimental studies of exogenous photosensitizers of various classes, such as naphthalocyanines and phthalocyanines, chlorins, pheophorbides and their derivatives, as

well as benzoporphyrin derivatives and purpurins, are being actively conducted. These compounds have unique optical properties, which makes their use in PDT prospective [27]. Research is focused on assessing the efficacy of these photosensitizers in the context of their ability to cause photochemical reactions when irradiated with light in therapeutic window. The main attention is paid to improving the selectivity of the action of drugs on tumour cells and minimizing side effects for healthy tissues.

Significant progress in the field of photodynamic therapy is explained not only by the development of photosensitizers, but also by improvement of light sources used in the treatment process. In particular, laser diodes have gained wide popularity due to their unique physico-chemical properties, which enable more precise and efficient action on tumour tissues [28]. Various schemes and modes of using light sources for efficient PDT are being studied and improved. In parallel with this, photodynamic diagnostics (PDD) is developing, which makes possible more precise detection of cancer and planning its treatment using the fluorescence of activated photosensitizers [5].

In Russia, photodynamic therapy is practiced in various medical institutions, including specialized oncology centres, clinics and research institutes, where it is used to diagnose and treat oncological diseases. In addition, PDT is used in dermatology, dentistry, ophthalmology, cosmetology, gynaecology, as well as in treatment of inflammatory skin diseases and vascular disorders. Innovations in the field of PDT include its integration into other types of therapy, such as chemotherapy, radiation therapy and targeted therapy, which increases the efficacy of treatment and minimizes side effects [5, 23].

PDT demonstrates high efficiency in treatment of various types of cancer. For example, when using the photosensitizer “Photolon” in combination with cisplatin in rats with M-1 sarcoma, complete tumour regression was achieved in 89% of animals and its growth was inhibited almost in 100% of animals [29]. In studies on mice with Ca-755 mammary adenocarcinoma, laser irradiation with a wavelength of 687 nm showed an improvement in the therapeutic effect, while at a wavelength of 678 nm the efficiency decreased to 23% [30]. PDT also showed good efficiency in treatment of pharyngeal cancer, laryngeal cancer and early forms of oral cancer achieving high cure rates. In addition, the use of nanobody-photosensitizer conjgates induced significant regression of orthotopic tumours with high HER2 expression in mice [31].

In veterinary medicine, photodynamic therapy is especially useful when surgical intervention or chemotherapy are contraindicated due to the animal’s condition. In 2009, O.A. Kuleshova and S.A. Yagnikov conducted a study in which they used “Fotoditazin” to treat oral mucosa spontaneous tumours in domestic dogs and cats. A positive effect was achieved with a photosensitizer dosage of 1–2 mg/kg and a light energy dose of 300-600 J/cm² [32].

In 2012, at the Centre for Biology and Veterinary Medicine of RUDN and the Department of Experimental Therapy and Diagnostics of the N.N. Blokhin Russian Cancer Research Center of RAMS, the accumulation and distribution of the photosensitizer was studied to determine the therapeutic dose for diagnostic fluorescence and photodynamic therapy in dogs and cats with tumour lesions of the abdominal organs, oral cavity, lungs and brain. It was found that the therapeutic dose of the photosensitizer was 1 mg/kg of the animal’s body weight. The accumulation time varied: 90 minutes for tumours of the chest and abdominal regions and the brain; 150 minutes for tumours of the oral mucosa. At a light energy density of 200 J/cm², PDT has an anti-inflammatory activity, and at 300–400 J/cm² — therapeutic [33].

In 2013, E.V. Davydov used two types of photosensitizers in the combined treatment of tumours in small domestic animals: chlorin E6 intravenously and externally in the form of a gel, and then “Dimegin” in the form of a gel. The study proved high efficiency of treatment with complete tumour regression [34].

In 2016, V.I. Telpukhov and E.V. Davydov, using PDT before surgery in cats with soft tissue sarcoma, found that with this variant of treatment, the tumour became more operable [35]. Also in 2016, when treating spontaneous mammary tumours in cats using photodynamic therapy, a good result was obtained: “Fotoditazin” was used as a photosensitizer, and the radiation source had a wavelength of 660±2 nm and a power of 1.5W. Within 6–10 days the tumour necrosis and rejection was observed, and upon further observation, complete regression was noted in the animals [36].

The use of PDT as a preoperative or intraoperative treatment method has become a future-oriented direction of research. In 2017, E.V. Davydov and colleagues used PDT in the preoperative mode, followed by radical mastectomy in small domestic animals with spontaneous mammary tumours. In the experimental group, suture healing time was twice as fast, the process of lymphorrhea decreased and no complications were observed [37].

In 2022, R. Shrestha and colleagues investigated the efficacy of photodynamic therapy using pure chlorin E6 (Ce6) for treatment of tumours in dogs and in mouse tumour models. Five dogs with different cancer types were treated with Ce6-PDT one to two times, and two dogs also underwent Ce6 fluorescence guided imaging by means of photodynamic diagnostics (PDD). In two patients with mammary carcinoma and histiocytic sarcoma, tumours significantly decreased in size and health improved. The additional use of Ce6-PDD made it possible to reveal tumour lesions that were not visible under white light. In mouse models of melanoma and pancreatic cancer, Ce6-PDT also showed significant tumour reduction [38].

In 2023, Professor D. Lo and colleagues used dogs to evaluate the efficacy of gold nanoparticles targeting prostate-specific membrane antigen as a method for fluorescence imaging and PDT of prostate cancer. After administration of nanoagents (AuNPs-FS158) and PDT activation with laser radiation (672 nm), it was found that photoluminescence of prostate tumours significantly increased compared to normal tissue. PDT caused damage in irradiated areas to a depth of 1–2 mm, including necrosis and inflammation, while non-irradiated areas remained without visible damage [39].

Also in 2023, a case of treatment of Squamous cell carcinoma of the corneal limbus in a cow using surgical excision and PDT was described. After surgery, EmunDo® (infracyanine green) was applied to the operation site and the cornea was irradiated with a laser diode with a wavelength of 810 nm and power of 500 mW to a total energy of 167 J. Eleven months after surgery, only slight corneal fibrosis was observed without signs of tumour recurrence [40].

In 2024, L. Sebbag and O. Peter described a clinical case of treatment of two cats with Squamous cell carcinoma of the eyelid using marginal resection and photodynamic therapy. After surgery, intracyanine dye was injected into the wound and PDT was performed using a laser diode (810 nm) in two stages: 1) six cycles of 500 mW for 30 sec per cycle in rapid movement; 2) one (case 1) or two cycles (case 2) for 30 sec at 2000 mW. Subsequent examinations showed that the eyelid in both cats had healed, with no evidence of tumour regrowth or ocular irritation [41].

Discussion and Conclusion. The presented historical review of the formation and development of photodynamic therapy shows that this method has come a long way from the first experiments using natural photosensitizers and sunlight to creation of the advanced highly effective drugs and high-precision laser diodes. PDT is used in human and veterinary medicine for treatment of a wide range of oncological diseases, including skin, esophageal, laryngeal, oral cavity, mammary gland cancers, etc. The method demonstrates efficiency both as monotherapy and in combination with other types of treatment, such as chemotherapy, radiation therapy and surgery. Photodynamic diagnostics is also being implemented for precision tumour imaging and therapeutic response monitoring. Innovative approaches based on the use of nanobodies are being developed for targeted delivery of photosensitizers to tumour cells, which increases selectivity and reduces systemic toxicity. PDT proves its potential in treatment of spontaneous tumours in animals, especially in the cases where there are contraindications to traditional treatment methods. The possibility of using PDT as a preoperative treatment to improve tumour resectability and reduce the risk of complications has been demonstrated. Research on optimizing PDT modes and developing new photosensitizers for veterinary use is underway.

However, despite the successes achieved, PDT has certain limitations, such as, for example, relatively shallow penetration of light, possibility of phototoxicity and dependence of this method's efficiency on tissue oxygenation. Therefore, further research should be aimed at:

– development of new generations of photosensitizers with improved pharmacological and photophysical properties;

– optimization of irradiation modes taking into account the absorption spectrum of photosensitizers and the depth of light penetration into tissues;

– studying the molecular mechanisms of PDT action and developing strategies to increase its efficiency by modulating signaling pathways and the immune response;

– development of new methods for delivering photosensitizers to tumour cells;

– conducting clinical trials to assess the efficiency and safety of PDT in deep-seated oncological diseases in humans and animals.

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