Revista Veterinaria. 2024; 35(2): 16-21.
https://doi.org/10.30972/vet.3527858
Artículo Original

Revista Veterinaria
Facultad de Ciencias Veterinarias-UNNE
ISSN: 1668-4834 / ISSN-e: 1669-6840

Estimating attrition of Sprague Dawley rats for research involving primary neuronal cultures

Denis, B.[1,2,3]; De Jesus, R.[3,4]; Gittens, R.A.[1,2,3,5,6*]

[1]Center for Biodiversity and Drug Discovery, Instituto de Investigaciones Científicas y Servicios de Alta Tecnología (INDICASAT-AIP), City of Knowledge, Panama. Apartado 0843-01103, Panamá 5. [2]MD-PhD Program for Clinical and Biomedical Research, School of Medicine, Universidad de Panamá (UP), Panama. Apartado 3366, Panamá 4. [3]Sistema Nacional de Investigación (SNI-SENACYT). City of Knowledge, Panama. Apartado 0816-02852. [4]Animal Facility, Instituto de Investigaciones Científica y Servicios de Alta Tecnología (INDICASAT-AIP). City of Knowledge, Panama. Apartado 0843-01103, Panamá 5. [5]Deptartment of Research & Innovation, Centro de Investigación e Innoavación Educativa, Ciencia y Tecnología (CiiECYT-AIP). Tocumen, Panama. [6]School of Industrial Technology, Instituto Técnico Superior Especializado (ITSE). Tocumen, Panama. ✉ rgittens@itse.ac.pa

Abstract
The use of laboratory animals for biomedical research is strictly regulated to prevent their suffering and inadequate use. While experimentation is in process, attrition usually can increase the number of animals needed to achieve the objectives. Unfortunately, attrition is not commonly reported in literature. In the present study, we worked with primary neuronal cell cultures to establish the first *in vitro* brain ischemia model in Panama. We report the effects of attrition in the final number of animals. Primary cortical cell cultures were produced from Sprague Dawley rat embryos. Vaginal cytology was used to evaluate oestrus cycle. Pregnant rats had routine evaluation plus clinical/weight follow up. Sample size calculations estimated 26 rats for the study using an attrition rate of 25%. The final number of animals used empirically was 26; however, the measured attrition was 50% (13). These results highlight the importance of calculating attrition for studies requiring laboratory animals, taking in consideration the context and available data from local institutions to allow for the most efficient use of laboratory animals, especially for newly established laboratories in developing countries.

Key words: laboratory animals, rat embryos, sample size, statistical analysis.

Estimación de incidencias en ratas Sprague Dawley en investigaciones que involucren cultivos neuronales primarios

Resumen. El uso de animales de laboratorio para investigación biomédica esta estrictamente regulado para prevenir su sufrimiento y uso inadecuado. Mientras que la experimentación está en proceso, las incidencias usualmente pueden incrementar el número de animales necesarios para alcanzar los objetivos. Desafortunadamente, las incidencias no son reportadas comúnmente en la literatura. En el presente estudio, trabajamos con cultivos neuronales primarios para establecer el primer modelo de isquemia cerebral *in vitro* en Panamá, reportando los efectos de las incidencias en el número final de animales. Los cultivos neuronales primarios fueron producidos de embriones de ratas *Sprague Dawley*. La citología vaginal fue utilizada para evaluar el ciclo estral. Las ratas gestantes además de sus evaluaciones de rutina recibieron seguimiento clínico y de peso. El cálculo de la muestra estimó la necesidad de 26 ratas para el estudio, utilizando una tasa de incidencias del 25%. El número final de animales utilizado empíricamente fue 26; sin embargo, la incidencia observada fue de 50% (13). Estos resultados subrayan la importancia del cálculo de incidencias para estudios que requieren animales de laboratorio, debiendo tomar en consideración el contexto y la información disponible de instituciones locales para permitir el uso más eficiente de los animales, en especial para laboratorios recientemente establecidos de países en vías de desarrollo.

Palabras clave: animales de laboratorio, embriones de rata, cálculo de muestra, análisis estadístico

Recibido: 08 julio 2024. **Aceptado:** 05 septiembre2024

INTRODUCTION

The 3 R's principle proposed by Russell and Burch in 1959 (Russell and Burch 1959, Russell et al. 1992, Tannenbaum and Bennett 2015), established the framework embedded in most national and international legislation on the use of animals for scientific research, and states that all such research should be done respecting the concepts of *Replacement*, *Reduction* and *Refinement* (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011).

Reduction can be achieved through a more precise sample size estimation (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011, Richter et al. 2018). The calculation of the number of sample size can be performed mainly by two methods (Arifin and Zahiruddin 2017, Ilyas et al. 2017): 1) *Power analysis*, which is the most favored method for the calculation of sample size when you have previous data; or 2) *Resource equation*, which is a simple method based on the law of diminishing return, useful when no previous data is available, when the experiment has multiple endpoints or when complex statistical analysis is required.

While experimentation is in process, attrition can increase the number of animals needed to achieve the objectives, which can affect the results and duration of the study (Holman et al. 2016, Percie du Sert et al. 2020). Some guidelines suggest adding anywhere from 10 to 20% to the total sample size (Charan and Kantharia 2013). However, one meta-analysis of attrition in preclinical studies for stroke and cancer, found that the few studies that reported or included detectable attrition had 25% or more animals lost (Holman et al. 2016). Unfortunately, it is common practice in the literature to not explicitly share information about the incident types considered for sample calculation, if the animal number approved by the Institutional Animal Care and Use Committee (IACUC) was met, or if more animals than calculated originally were needed to achieve the objectives of the experiments.

In the present study, we worked to establish the first *in vitro* brain ischemia model in Panama using primary cortical neurons. Here, we report the effects of attrition not considered during sample calculation in the final number of animals used for the development of primary cortical cell cultures for experimentation in an oxygen-glucose deprivation (OGD) model as a simulation of the conditions in brain ischemia. However, the calculations presented in this study can be helpful for the planning of other types of research projects involving primary neuronal cultures from Sprague Dawley rat embryos.

MATERIALS AND METHODS

Sample Calculation. Resource equation was chosen for sample calculation due to lack of previous studies related to stroke model in our country, the unknown impact of our infrastructure and experience in the attrition rate, and multiple endpoints in our experimental design (*i.e.*, neuronal vs mixed primary culture; and OGD model with endpoints of 1, 1.5 and 2 hours).

For the development of primary cell cultures, where our goal was primarily to compare cell number and other qualitative characterizations between mixed and neuronal cultures, the "group comparison one-way ANOVA" formula was used (Arifin and Zahiruddin 2017):

$$n = DFk + 1$$

where $\boldsymbol{n}$ is the number of wells per group, $\boldsymbol{k}$ is the number of groups (*i.e.*, two groups: neuronal and mixed), and $\boldsymbol{DF}$ is the degrees of freedom (*i.e.*, we compared results for 10 and 20 degrees of freedom, as recommended in the literature (Arifin and Zahiruddin 2017).

Meanwhile, for the establishment of the OGD model, the "group comparison repeated measures" formula was chosen (Arifin and Zahiruddin 2017):

$$n = DFk\ r + 1$$

where the additional variable $\boldsymbol{r}$ is the number of endpoints (*i.e.*, three endpoints: 1, 1.5 and 2 h).

For our calculations, we used the following conditions and assumptions. Each "sample" represented the cells cultured in one well of a 24-well tissue culture plate. Each "well" would use 1 million cells for culture. Primary cells would be isolated from the cortex of a Sprague Dawley 17-day old embryo (E17). Each pregnant rat could provide approximately 10 embryos. Each group would have a minimum of three samples (n = 3) and each experiment would be independently repeated three times (n = 3). Attrition was assumed to be 25% as suggested in the literature (Holman et al. 2016).

Animal Handling. The INDICASAT-AIP Institutional Animal Care and Use Committee approved the experimental procedures used in this study (approval no. CICUA-21-006) on August 20, 2021.

Sprague-Dawley rats were kept in "Specific Pathogen Free" conditions, which involved evaluation for *Mycoplama pulmonis, Clostridium piliforme, Bordetela bronchiseptica*, endoparasites, ectoparasites and enteric helminths. Animals were kept inside Blue Line ventilated racks (Tecniplast, Buguggiate, Italy), in standardized conditions. The cages were prepared with Bed-o'Cobs ¼" bedding (The Andersons, Maumee, OH, USA), and different types of enrichment, such as clean PVC or cardboard tubes, as well as paper towels. The microbiological status of the colony is verified every six months. The room was maintained at a temperature 20.2 °C and a relative humidity of 56.7%. The photoperiod was 12 hours of light and 12 hours of darkness; noise did not exceed 85 dB during normal operations. Laboratory Rodent Diet 5001 (LabDiet, Minneapolis-St. Paul, MN, USA) and filtered water were sterilized by autoclaving and offered ad libitum.

Oestrous Cycle Evaluation. Oestrous cycle was evaluated in 2- to 4-month-old female rats. In brief, normal saline solution was introduced in the vagina of the rats and immediately aspirated with a transfer pipette, then placed in a slide and covered with a coverslip. The samples were evaluated in a BA310LED / MOTO1371 light microscope (Motic, Kowloon, Hong Kong). Rats in oestrus were

candidates for pairing and were taken to their respective male rat cages. The following day, the oestrous cycle was evaluated again for copulation signs such as a mucous plug in vaginal introitus or anestrus/sperm in vaginal sample, following methods previously established in the literature (Long and Evans 1922, Sharp et al. 1998, Hamid and Zakaria 2013, Umamageswari et al. 2020). Female rats showing these signs were followed up.

Weight/Clinical Follow-Up. The weight of selected rats was monitored at 6, 13 and 16 days. Swollen abdomen and visible teats were signs for clinical pregnancy determination. Common evaluation was routinely performed. If all follow-up criteria were met, euthanasia to obtain embryos was done at day 17 after anesthesia with Terrell™ isoflurane, USP (Piramal Critical Care, Bethlehem, PA, USA).

Statistics. Qualitative data was analyzed using percentage and frequency distribution. For quantitative data, mean and standard deviation were calculated. Database development and statistical analysis was performed using the Jamovi computer software, version 2.4.0, retrieved from https://www.jamovi.org.

RESULTS

The sample size calculations for our experimental design resulted in requiring 3 pregnant rats for the development of the primary cell culture, plus an additional 18 rats for the establishment of the OGD model, which accounted for 21 rats. We considered a 25% attrition rate, which resulted in the calculation of 26 pregnant rats in total.

A total of 26 Sprague Dawley female rats, were mated during 12 months of experimentation. The day after mating, 18 (69%) showed signs of copulation and 8 (31%) presented no clear evidence and had to be removed from the study. We identified three different signs of copulation: 14 rats with spermatozoon (78%), 3 with mucous plug (17%) and 1 in anestrus (5%). At day 17, euthanasia was performed on 13 (72%) of the rats that were ready to use for experiments; 3 (16%) weren't use due to scheduling problems, so they were provided to other experiments; and 2 (11%) did not produce embryos (Figure 1).

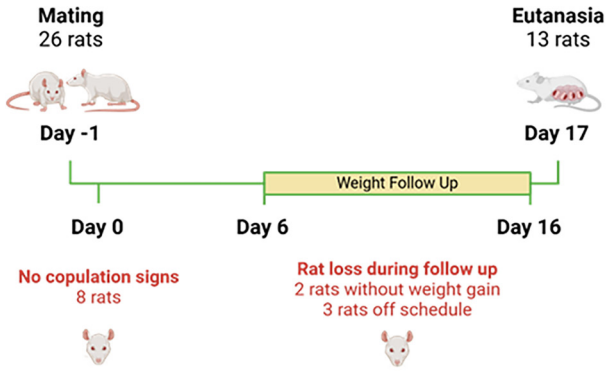

Figure 1. Flowchart of rat attrition during breeding and experimental use. 26 rats were used for the study, 8 rats didn't show copulation signs after mating, 2 rats had variable weight gain during follow up, 3 can´t be used by schedule situations but were used in other studies, finally 13 rats achieve the time for experimentation. Created with BioRender.com.

All rats with signs of copulation (18) were followed-up until the day before euthanasia, of which 88.9% (16) showed continuous weight gain and 11.1% (2) had variable weight gain. Rats with continuous weight gain had a mean increase of 34.2 g per week and an average increase of 99.9 g in 16 days, meanwhile the rats without continuous weight gain only increased a total of 18.5 g in the same period (Figure 2).

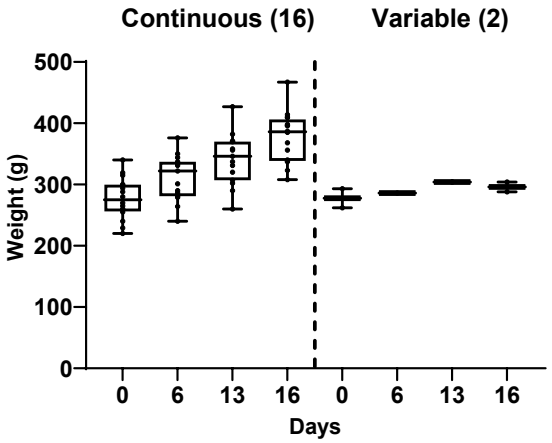

Figure 2. Box and whiskers graph (min and max) of the weight gain recorded on mated rats. Rats with continuous weight gain increase a mean of 99.9 g in 16 days, meanwhile rats with variable weight gain only increase a mean of 18.2 g.

Rats that exhibited continuous weight gain were considered pregnant and allowed to go for euthanasia. Indeed, all these rats with increased weight produced embryos, with a mean of 13.7 ± 3.54 (Figure 3). The majority (10 of 13) of rats produced 13 or more embryos; however, 3 of the rats produced less than 10 embryos, which was the minimum amount assumed in the sample size calculations.

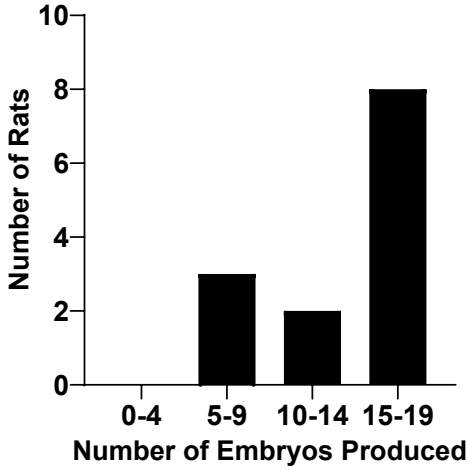

Figure 3. Frequency distribution of the number of embryos produced by pregnant rats. Pregnant rats produce a mean of 13.7 + 3.54 embryos, but three rats produce less than 10 embryos.

All 26 rats estimated in the initial sample size calculations and mated for the experiments were needed. A total of 13 rats (50%) were lost to attrition. The incidents that were considered attrition and that affected the final number of pregnant rats available for euthanasia and experimentation could be categorized into the following types: mating problems, pregnancy complications, reduced number of embryos produced and *in vitro* experimentation failures. Three of these situations were not considered in our initial attrition estimation.

DISCUSSION

In the present study we reported in detail all the incidents related to attrition that were encountered during experimentation while developing primary cortical cell cultures and establishing the first brain ischemia model in Panama. Most animal research literature does not provide enough details about the animal characteristics and handling protocols, final number of animals used, and the number of animals lost due to difficulties obtaining results or incidents (Sena et al. 2010, van der Worp et al. 2010, Holman et al. 2016). This can make it harder for different labs, including upcoming research institutions in developing countries, to reproduce studies already available in the literature.

This study used all the animals calculated in the sample size analysis, including attrition estimates. Four sources of attrition incidents were identified. The first source of incidents was related to mating problems with the timed-pregnancy procedure: 8 mated rats did not show signs of copulation and three rats with signs of copulation could not be used due to scheduling problems. These events were probably influenced by lack of expertise with the protocols, mating at inappropriate period of oestrous cycle (Ajayi and Akhigbe 2020), rat sexual behavior problems (Agmo 1997), or animals in stressful situations (Agmo 1997, Anthony et al. 2005, Gaskill and Garner 2017).

The second incident was associated to pregnancy complications that were captured during weight follow-up of the 18 rats with signs of copulation: 16 produced embryos, but 2 did not. Despite the presence of sperm or vaginal plug when evaluated after mating, the 2 rats that did not produce embryos, showed variable weight gain during follow-up. Pseudo pregnancy related to female rat fertility problems were considered as possible causes (Long and Evans 1922, Swingle et al. 1951).

The third incident involved the number of embryos produced by rats. Of the 13 rats that were pregnant and euthanized for experimentation, 3 produced less than the mean number of embryos expected and used for our sample size calculations, which was 10. Possible incidents during pregnancy for these 3 rats were detected: 1) the first rat was exposed to noise and vibrations during an infrastructure corrective maintenance event at the animal facility; 2) the second rat showed aggressive behavior during mating despite being in well-documented estrus; and 3) the third rat showed signs of preterm labor when euthanized. With this information we considered that all these rats were stressed at some point in their pregnancy. It is well documented that a stressful environment is not suitable for animal breeding (Agmo 1997, Anthony et al. 2005, National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011, Gaskill and Garner 2017) and can induce alterations in pregnant rat weights and their fetuses (Williams et al. 1998). Even if difficult to predict, possible environmental and infrastructural situations should be considered during incident sample calculation.

The fourth, and last, incident had to do with cell culture contamination, which can be a common problem during primary cell culture (Lincoln and Gabridge 1998). This problem did not involve the animals directly, but it can increase the number of animals needed for the experiments and this must be considered during sample calculation if the goal of the animal sample size calculation involves primary cell culture.

The total number of animals calculated for this study was 26 rats, which included an estimated attrition of 25% of the 21 calculated experiments (5 rats, or 19% of the total 26 rats), chosen after the attrition reported in the literature (Holman et al. 2016). Empirically, the total number of rats used was exactly 26, just as calculated; however, we only used 13 rats for experimentation, which resulted in empirical incidents of attrition accounting for 50% of the total animals used.

The situations that lead to the final attrition incidents were also different to the ones reported in literature (Holman et al. 2016). The justifications for the difference between the number of rats calculated and those actually used for experimentation had to do primarily with the following: 1) litter size, which was 37% more than what was assumed in the theoretical calculations (*i.e.*, mean of 13.7 embryos compared to 10 embryos used for calculations); 2) cell culture seeding density, which was optimized to use only 500,000 cells per well (a decrease of 50% cells per experiment); and 3) experiment reproducibility, which allowed to reach the OGD model mortality goals in fewer experiments than anticipated.

Attrition incidents are largely known and expected in animal research, but they are not commonly reported in the literature, affecting the reproducibility of the experiments and the sample size analysis in new laboratories. During the establishment of our OGD model, we found different situations that increased the number of rats needed to achieve our goals, which we were able to offset by optimizing other parameters during our experimentation.

Sample size analysis is one of the main tools to ensure reliable and reproducible results for an efficient use of laboratory animals (Dell et al. 2002, Sena et al. 2010, Fitts 2011). However, the resource equation may be better suited for the calculation of animals needed during the formation of new labs in developing countries that are establishing models for the first time and that may have types of attrition incidents that could still be unknown. This highlights the importance of generating, publishing and using data from local institutions to allow a correct sample size calculation, improving the efficient use of laboratory animals to comply with the 3R's guidance.

Acknowledgements. We would like to thank all the staff of INDICASAT AIP animal facility for their great commitment to the care and management of laboratory animals (Dr. Lizzi Herrera, Vet. Alanna Madrid, Kevin Mata and Rene Rivera). This project was funded by SENACYT "New Researchers grant" No-101-2021. The results presented in this study were also supported in part with funds from NIH/NINDS Fogarty International Center (grant number 1R21NS098896-02. We are also thankful for the support of the Panamanian National System of Researchers (SNI 12-2020 & SNI 051-2023) and the Embassy of the Korean Republic in Panama for their infrastructure funding grant (2018). **Financial and non-financial competing interests.** The authors declare that there is no conflict of interest. **Funding.** The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by SENACYT [grant number No-101-2021]; NIH/NINDS Fogarty International Center [grant number 1R21NS098896-02]; SENACYT-SNI [grant number SNI-12-2020 & SNI-051-2023]; and the Embassy of the Korean Republic in Panama.

Data availability statement. The data that support our findings are available on request from the corresponding author.

ORCID

Denis B. bdenis@lisa-indicasat.org; https://orcid.org/0000-0003-1862-6207
De Jesus R. rdejesus@indicasat.org.pa; https://orcid.org/0000-0001-9670-291X
Gittens R.A. rgittens@itse.ac.pa; https://orcid.org/0000-0002-0286-3956

REFERENCES

1. Agmo A. Male rat sexual behavior. *Brain Res Brain Res Protoc.* 1997; 1: 203-209.
2. Ajayi AF, Akhigbe RE. Staging of the estrous cycle and induction of estrus in experimental rodents: an update. *Fertil Res Pract.* 2020; 6: 5.
3. Anthony A, Ackerman E, Lloyd JA. Noise Stress in Laboratory Rodents. I. Behavioral and Endocrine Response of Mice, Rats, and Guinea Pigs. *J Acoust Soc Am.* 2005; 31: 1430-1437.
4. Arifin WN, Zahiruddin WM. Sample Size Calculation in Animal Studies Using Resource Equation Approach. *Malays J Med Sci.* 2017; 24: 101-105.
5. Charan J, Kantharia ND. How to calculate sample size in animal studies? *J Pharmacol Pharmacother.* 2013; 4: 303-306.
6. Dell RB, Holleran S, Ramakrishnan R. Sample size determination. *ILAR J.* 2002; 43: 207-213.
7. Fitts DA. Ethics and animal numbers: informal analyses, uncertain sample sizes, inefficient replications, and type I errors. *J Am Assoc Lab Anim Sci.* 2011; 50: 445-453.
8. Gaskill BN, Garner JP. Stressed out: providing laboratory animals with behavioral control to reduce the physiological effects of stress. *Lab Anim (NY).* 2017; 46: 142-145.
9. Hamid HY, Zakaria ZAB. Reproductive characteristics of the female laboratory rat. *Afr J Biotechnol.* 2013; 12: 2510-2514.
10. Holman C, Piper SK, Grittner U, Diamantaras AA, Kimmelman J, Siegerink B, Dirnagl U. Where Have All the Rodents Gone? The Effects of Attrition in Experimental Research on Cancer and Stroke. *PLoS Biol.* 2016; 14: e1002331.
11. Ilyas M, Adzim M, Simbak N, Atif A. Sample Size Calculation for Animal Studies Using Degree of Freedom (E); an Easy and Statistically Defined Approach for Metabolomics and Genetic Research. *Curr Trends Biomed Eng Biosci.* 2017; 10: 47-48.
12. Lincoln CK, Gabridge MG. Cell culture contamination: sources, consequences, prevention, and elimination. *Methods Cell Biol.* 1998; 57: 49-65.
13. Long JA, Evans HM. *The oestrous cycle in the rat and its associated phenomena.* Berkeley, Calif: University of California Press; 1922.
14. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011. *Guide for the Care and Use of Laboratory Animals.* 8th ed. Washington (DC).
15. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, Emerson M, Garner P, Holgate ST, Howells DW, Karp NA, Lazic SE, Lidster K, MacCallum CJ, Macleod M, Pearl EJ, Petersen OH, Rawle F, Reynolds P, Rooney K, Sena ES, Silberberg SD, Steckler T, Wurbel H. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *PLoS Biol.* 2020; 18: e3000410.
16. Richter V, Muche R, Mayer B. How much confidence do we need in animal experiments? Statistical assumptions in sample size estimation. *J Appl Anim Welf Sci.* 2018; 21: 325-333.
17. Russell WMS, Burch RL. The principles of humane experimental technique. London: Methuen; 1959.
18. Russell WMS, Burch RL, Universities Federation for Animal W. The principles of humane experimental technique. Special. Wheathampstead: Universities Federation for Animal Welfare Wheathampstead; 1992. XIV, 238 p.: gràf.; 21 cm.
19. Sena ES, van der Worp HB, Bath PM, Howells DW, Macleod MR. Publication bias in reports of animal stroke studies leads to major overstatement of efficacy. *PLoS Biol.* 2010; 8: e1000344.
20. Sharp PE, La Regina MC, Suckow MA. *The laboratory rat.* Boca Raton: CRC Press Boca Raton; 1998. 214 pages: illustrations; 24 cm.
21. Swingle WW, Seay P, Perlmutt J, Collins EJ, Barlow GJr., Fedor EJ. An experimental study of pseudopregnancy in rat. *Am J Physiol.* 1951; 167: 586-592.
22. Tannenbaum J, Bennett BT. Russell and Burch's 3Rs then and now: the need for clarity in definition and purpose. *J Am Assoc Lab Anim Sci.* 2015; 54: 120-132.

23. Umamageswari J, Balasubramanian S, Krishnakumar K, Preetha SP, Vijayarani K. A simple and rapid staining technique to confirm mating in Wistar rats. *J Entomol Zool Stud.* 2020; 8: 820-823.
24. van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O'Collins V, Macleod MR. Can animal models of disease reliably inform human studies? *PLoS Med.* 2010; 7: e1000245.
25. Williams MT, Hennessy MB, Davis HN. Stress during pregnancy alters rat offspring morphology and ultrasonic vocalizations. *Physiol Behav.* 1998; 63: 337-343.