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ESTIMATION OF THE EFFICIENCY OF HORMONE-REGULATING SYNCHRONIZATION OF OVULATION IN FEMALE MICE

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The aim of the wok is to assess the efficiency of hormone-regulating synchronization of ovulation in female mice, to increase the number of simultaneously fertilized individuals and obtain their offspring in the planned time frame.
Materials and methods. The study was carried out on 180 female mice of three lines – CBA/lac, C57BL/6, BALB/c (n = 60), divided into three subgroups: intact (mating without confirmation of the estrous phase) (n = 20), cytological examination of vaginal secretions before mating with the determination of the estrous phase (n = 20), hormone-regulating synchronization of the estrous cycle with the introduction of progesterone (4.5 mg/100 g) on the 1st and prostaglandin F2α (0.083 mg/100 g) on the 7th day, once from the beginning of the experiment followed by immediate mating (n = 20). The planned date of delivery was considered the 22nd day from the moment of mating. The ovulation synchronization index (OSI) was assessed on the 14th day after mating.
Results. On the 14th day from the beginning of the experiment, the ovulation synchronization index in the intact groups of the CBA/lac, C57BL/6, BALB / c lines, was 25%, 25%, 40%, respectively. On the 14th day, the number of pregnant individuals admitted to mating after the established estrus by the method of cytological assessment of vaginal secretions according to OSI, was 65%, 60%, 75%, respectively. In the experimental groups, OSI was 80%, 75%, 100%, respectively. On the 22nd day, the number of delivered females of CBA/lac, C57BL/6, BALB/c lines in the intact group, was 3, 1, 3 individuals; in the control group – 10, 6, 9, and in the experimental group – 16, 15, 17, which is significantly higher than in the control and intact groups ($p<0.05$).
Conclusion. Hormone-regulating synchronization of ovulation in female mice significantly increases the number of delivered individuals on the 22nd day, relative to those synchronized by estrus by 53%, and to intact groups by 85.5%. It has been revealed that an additional effect of hormonal synchronization of ovulation is an increase in the number of offspring by 120% in comparison with the control groups and by 390% in comparison with the intact groups. This method of timing planning of the offspring birth of the experimental animals reduces the time spent on preclinical studies of drugs for the following types of assessment of toxic effects: reproductive toxicity, embryotoxicity, teratogenicity, effects on fertility.
Keywords: estrous cycle, estrus, progesterone, vaginal cytology, ovulation synchronization, prostaglandin F2α

ОЦЕНКА ЭФФЕКТИВНОСТИ ГРУППОВОЙ ГОРМОН-РЕГУЛИРУЮЩЕЙ СИНХРОНИЗАЦИИ ОВУЛЯЦИИ У САМОК МЫШЕЙ

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RESEARCH ARTICLE

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Цель. Оценить эффективность гормон-регулирующей синхронизации овуляции у самок мышей, для увеличения количества одновременно оплодотворенных особей и получения потомства в запланированные сроки.
Материалы и методы. Исследование было проведено на 180 самках мышей трех линий – CBA/lac, C57BL/6, BALB/c (n=60), разделенные на три подгруппы: интактные (спаривание без подтверждения фазы эструса) (n=20),цитологическое исследование вагинального секрета перед спариванием с определением фазы эструса(n=20), гормон-регулирующей синхронизации эстрального цикла с введением прогестерона (4,5 мг/100 г) на 1-е и простагландина F2α (0,083 мг/100 г) на 7-е сутки однократно от начала эксперимента с последующим немедленным спариванием(n=20). Запланированной датой родов считались 22 сутки с момента спаривания. Индекс синхронизации овуляции (ИСО) оценивался на 14 сутки с момента спаривания.
Результаты. На 14-й день с начала эксперимента индекс синхронизации овуляции в интактных группах линий CBA/lac, C57BL/6, BALB/c составил 25%, 25%, 40% соответственно. Количество беременных особей на 14 сутки, допущенных к спариванию после установленного эструса методом цитологической оценки вагинального секрета согласно ИСО, составило 65%, 60%, 75% соответственно. В экспериментальных группах ИСО составил 80%, 75%, 100% соответственно. На 22 сутки количество родивших самок линий CBA/lac, C57BL/6, BALB/c в интактных группе составило 3, 1, 3 особи, в контрольных 10, 6, 9, а в экспериментальной группе 16, 15, 17 что достоверно выше чем в контрольных и интактных группах ($p<0,05$).
Заключение. Гормон-регулирующая синхронизация овуляции у самок мышей достоверно увеличивает количество разродившихся особей на 22 сутки относительно синхронизированных по эструсу животных на 53% и интакта на 85,5%. Выявлено, что дополнительным эффектом гормональной синхронизации овуляции является увеличение количества приплода в 2,2 раза в сравнении с контрольными группами и в 3,9 раз в сравнении с интактными группами. Данный способ планирования сроков рождения потомства экспериментальных животных сокращает временные затраты проведения доклинических исследований лекарственных препаратов по следующим видам оценки токсических эффектов: репродуктивная токсичность, эмбриотоксичность, тератогенность, влияние на фертильность. Кроме того, данный способ расширяет возможности экспериментального моделирования патологий беременности и плода с последующей оценкой их фармакологической коррекции.
Ключевые слова. эстральный цикл, эструс, прогестерон, вагинальная цитология, синхронизация овуляции, простагландин F2α

INTRODUCTION

Currently, the productivity of laboratories with mice of various lines as their main model organisms, is increasing, This species has gained its great popularity relatively recently, and, as a result of the efforts of many scientists over the past decades, a large number of inbred lines of mice, have been created and maintained. These events influenced a further research, thereby, making a huge contribution to modern ideas about immunology, oncology, embryology and neurobiology [1].

To prepare pregnant female mice for the experiment, the group of authors initially tested the method of vaginal cytology. This technique is based on the identification of the phases of the estrous cycle, followed by sampling the individuals in the estrous phase, and their subsequent placing to males to copulate [2].

The estrus determination in the group of individuals is important in the sampling of the animals; the aim is their subsequent mating and obtaining the offspring for experimental purposes [3]. However, this requires a long-term screening of the entire animal population, the experimenter has special skills and knowledge, which implies making a mistake. Vaginal cytology is a non-invasive and inexpensive way to determine the phase of the cycle, requiring certain skills in interpreting the morphological picture of vaginal secretion cells. This method is tedious and time-consuming [4].

A group hormone-regulating synchronization of ovulation is very popular in livestock farming. The basis of this method is a pharmacological correction of the hormonal cycle in order to induce ovulation within necessary time limits.

The progesterone used in the proposed scheme, has a strong antigonadotropic effect. The increased progesterone levels alter the characteristics of the two outer layers of the endometrium.

There is thickening of the cervical mucus, which leads to desynchronization of the endometrial changes necessary for an egg cell implantation, and significantly suppresses the penetration of spermatozoa [5]. The progesterone levels peak in the middle of the secretory phase, reducing the level of the luteinizing hormone (LH) and the follicle stimulating hormone (FSH), so that the secondary oocyte does not leave the dominant follicle and does not pass into the lumen of the fallopian tube. These changes make it impossible to fertilize an egg cell with sperm.

Prostaglandin F2α (PGF) is a biologically superpotent substance that plays an important role in the control of reproduction. The use of the drug in cattle is based on its luteolytic properties [6].

In addition, the experimental data on cattle indicate that in the peri-ovulatory period, intrafollicular prostaglandin is necessary for the ovulation process [7].

THE AIM of the study was to estimate the number of pregnancies in the female mice subjected to the hormone-regulating synchronization of ovulation, compared with planning the timing of pregnancy and the birth dates in the animals using the determination of the estrus phase.

MATERIALS AND METHODS

The research protocol was reviewed and approved of, at the meeting of the commission for work with labo-

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ratory animals of the Research Institute of Pharmacology of Living Systems, Belgorod State University. The carried out work, met the requirements of the Law of the Russian Federation "On the Protection of Animals from Cruelty" dated 24 June, 1998, the rules of laboratory practice when conducting preclinical studies in the Russian Federation (GOST 3 51000.3-96 and GOST R 53434-2009), European Community directives (86/609 EU), and the Rules of Laboratory Practice adopted in the Russian Federation (Order of the Ministry of Health of the Russian Federation No. 708 dated 29.08.2010).

Animals

The females, regardless of the group, were placed to the males in ratio of 2:1 to copulate. The planned birth date was considered 22 days later from the beginning of mating. Excluding the tribal selection, the following female mice were selected for the experiment: they were the same age and weight of CBA/lac, C57BL/6, BALB/ lines, each line was represented by 60 individuals, and males of the corresponding lines in the amount of 30 individuals from the laboratory mouse bank of "Stolbovaya" (Moscow region).

The choice of individuals of these lines was justified by their most frequent use in biomedical research [8].

The animals were kept in individual ventilated cages. Non-coniferous sawdust was used as a bedding material. The animals were given standard granulated complete feed for laboratory animals – extruded LBK-120 GOST R 50208-92. (ZAO "Tosno animal formula-feed plant"). Feeding of the animals was carried out according to the standards in accordance with the species of the animals. Purified tap water was given *adlibitum* in standard drinkers.

Study design

The animals of three lines were divided into three groups: intact (natural mating) (n=20), estrous synchronization (cytological examination of vaginal secretions before mating with the determination of the estrous phase) (n=20), hormonal synchronization of the estrous cycle (n=20). The individuals belonging to the control group, were selected if estrus had been established in them. The females of the intact and experimental groups had not been preselected. The females, regardless of the group, were placed to the males in ratio of 2:1 to copulate. The planned birth date was considered 22 days later from the beginning of mating.

In the control groups, the animals were admitted to mating after confirming their estrous phase by assessing the vaginal secretions.

To carry out the first stage of the hormonal synchronization of ovulation in the mice, progesterone (suspension for the injection, ZAO “Mosagrogen”, RF) was injected intramuscularly at the dose of 4.5 mg/100 g, regardless of the phase of the estrous cycle of the females. 7 days after the administration of progesterone, an intramuscular injection of prostaglandin F2α (ZAO “Mosagrogen”, RF) was carried out at the dose of 0.083 mg/100 g. The estimated time of the onset of ovulation was 34–72 hours after the administration of the second drug.

The pharmacological correction of the estrous cycle of the female mice was evaluated by examining the cytological picture of vaginal secretions.

The ovulation synchronization index (OSI) was calculated after the established fact of pregnancy on the 14th day from the beginning of mating. The birth rate index (BRI) was analyzed by the fact of birth.

$$OSI = \frac{\text{The number offertilized females}}{\text{The number of females placed to malestocopulate}} \times 100\%;$$

$$BRI = \frac{\text{The number of offspring}}{\text{The number offertilized females}} \times 100\%$$

Vaginal smear/cytology

The manipulation was carried out the next day after the progesterone injection, 3 days later, immediately before the injection of prostaglandin F2α and the next day after it.

Vaginal secretions were collected from a fixed female, for the purpose of cytological assessment of the estrous cycle phase. A small amount (20 µl) of distilled water was gently injected into the vagina using a pipette, followed by drawing the previously injected liquid into the pipette. This procedure was repeated 4–5 times. It is important to make sure that the pipette is placed at the entrance of the vaginal canal and does not penetrate the vaginal opening. The liquid containing a few drops of the cell suspension, is placed then on a slide, air-dried, and stained according to the Romanovsky-Giemse method [9, 10]. After that, the slide was covered with a cover glass, and the quantitative and qualitative composition of the secretion cells was immediately examined under a light microscope (Biomed 5) at 40× magnification (Fig. 1).

The vaginal secretions consist of three types of cells. These include leucocytes, keratinized epithelial cells, and nucleated epithelial cells. The estimation of the estrous cycle phase, is based on the proportion of these cells in the vaginal secretions [11].

Numerous rounded nucleated cells that are uniform in size and appearance, are the hallmark of the proestrus phase (A). The estrus phase shows abundant non-nuclear keratinized epithelial cells (B). Nucleated epithelial cells are present in the late metestrus (C). Diestrus is characterized by the presence of polymorphonuclear leukocytes and several epithelial and keratinized cells within the field of view [12].

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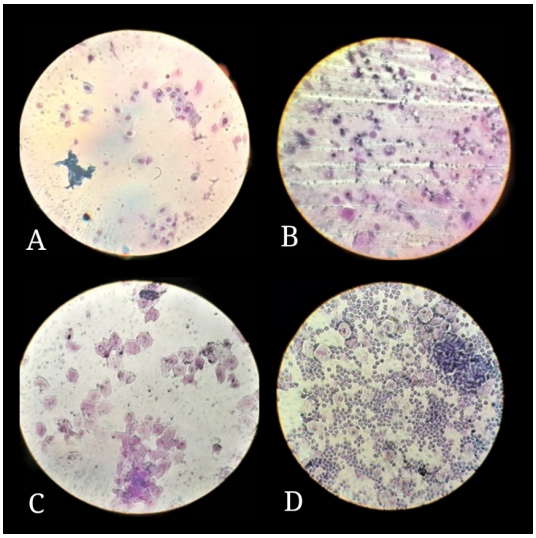

Figure 1 – Microscopic assessment of cell suspension drops of vaginal secretions in animals without hormone-regulating ovulation synchronization

Note: A – proestrus, B – estrus, C – metestrus, D – diestrus

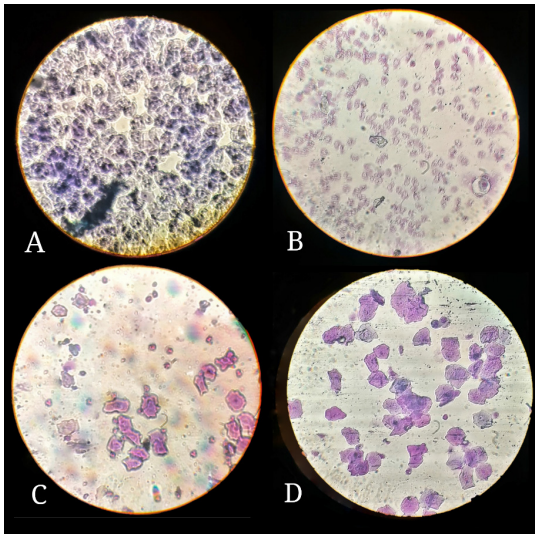

Figure 2 – Microscopic assessment of cell suspension drops of vaginal secretions in females after hormone-regulating ovulation synchronization

Note: A – cytological picture of the contents of the vaginal secretion on the next day after the administration of progesterone; B – on the 3rd day; C – on the 7th day before the administration of prostaglandin F2α; D – on the next day after the administration of prostaglandin F2α

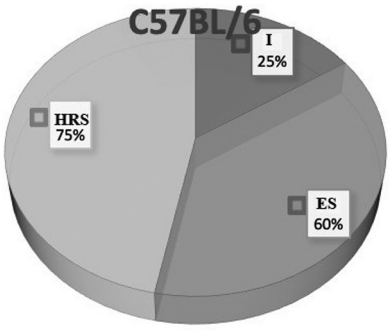

Figure 3 – The ratio of the ovulation synchronization index of individuals of the C57BL/6 line experimental groups

Note: I – intact group; ES – estrous synchronization; HRS – hormone-regulating synchronization

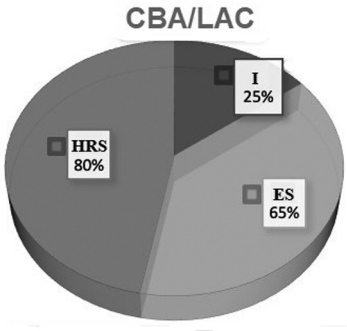

Figure 4 – The ratio of the ovulation synchronization index in individuals of the CBA/lac line experimental groups

Note: I – intact group; ES – estrous synchronization; HRS – hormone-regulating synchronization

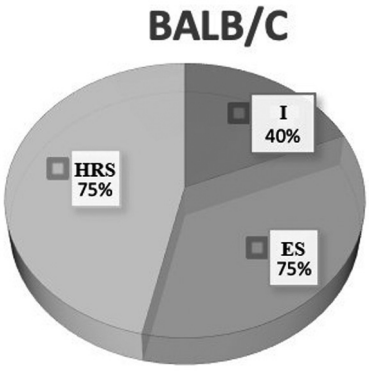

Figure 5 – The ratio of the ovulation synchronization index of individuals of the line BALB/C experimental groups

Note: I – intact group; ES – estrous synchronization; HRS – hormone-regulating synchronization

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Table 1 – Ovulation synchronization index

Line name / Groups	CBA/lac			C57BL/6			BALB/c		
	(I)	(ES)	(HRS)	(I)	(ES)	(HRS)	(I)	(ES)	(HRS)
Number of pregnant females on the 14th day	5	13	16	5	12	15	8	15	20
Number of females placed to males to copulate	20	20	20	20	20	20	20	20	20
OSI, %	25%	65%	80%	25%	60%	75%	40%	75%	100%

Note: I – intact group; ES – estrous synchronization; HRS – hormone-regulating synchronization.

Table 2 – The number of females which gave birth on the 22nd day

Line name / Intact group	CBA/lac (n=20)	C57BL/6 (n=20)	BALB/c (n=20)
	3	1	3
Estrous synchronization	10*	6*	9*
Hormone-regulating synchronization	16*	15*	17*

Note: * – p<0.05 compared to control and experimental groups

Table 3 – Birth rate index

Line name / Groups	CBA/lac			C57BL/6			BALB/c		
	(I)	(ES)	(HRS)	(I)	(ES)	(HRS)	(I)	(ES)	(HRS)
Number of pregnant females on the 14th day	5	13	16	5	12	15	8	15	20
Number of offspring	34	77	145	28	67	126	74	123	193
BRI, %	6,8	5,9	9,0	5,6	5,5	8,4	9,25	8,2	9,65

Hormonal regulation of the estrous cycle

To implement the first stage of the hormonal synchronization of ovulation in mice, progesterone was intramuscularly administered (the suspension for injection, ZAO “Mosagrogen”, RF “Progestomag”, Reg. 32-3-4.15-2649 No. PVR-3-4.15/03139 dated 27.06.2018) at the dose of 4.5 mg/100 g, regardless of the estrous cycle phase of females. 7 days after the administration of progesterone, the intramuscular injection of prostaglandin F2α (ZAO "Mosagrogen", RF "Magestrofan", Reg. 32-3-4.15-2649 No. PVR-3-4.15/03139 dated 06/11/15) was carried out at the dose of 0.083 mg/100 g. The probable time of the ovulation onset is 34–72 hours after the administration of the second drug. The regimen for the use of drugs is presented in the instructions for the veterinary use of drugs.

Statistical analysis

A statistical analysis comparing the number of births on day 22 in the groups, was carried out using *Pearson's Chi-square test.* Differences were identified at a significance level of 0.05. Statistical analysis was performed using Statistica 10.0 software. The differences were determined at the significance level of 0.05. The statistical analysis was performed using the Statistica 10.0 software.

RESULTS

Vaginal smear/Cytology

The next day after the progesterone injection to the female mouse (Fig. 2), a mixed cytological picture takes place; it does not make it possible to attribute the visible result to a certain cycle phase (A). Polymorphonuclear leukocytes and a small number of keratinized cells are observed 3 days after the administration of progesterone, which corresponds to the diestrus phase (B). On the seventh day before the injection of prostaglandin F2α, there is a predominance of rounded nucleated cells with a small impregnation of keratinized epithelial cells and polymorphonuclear leukocytes between them (C). In a vaginal smear taken the next day after the injection of prostaglandin F2α, there is a predominance of abundant non-nuclear keratinized epithelial cells with cells of irregular shape and granular cytoplasm (D).

Hormone-regulating estrous cycle synchronization

During the study it was found out that the hormonal synchronization of a group of females, increases the number of fertilized individuals by 55% relative to the intact groups, and by 18.3% relative to the control groups. The selection of individuals based on the cytological examination of vaginal secretions, increases the number of pregnant females by 36% (p <0.05) (Table 1). On day 22, the hormone-regulating synchronization increases the probability of giving birth by 53% (p<0.05) in comparison with the control group, and by 85.5% (p <0.05) in comparison with the intact group (Table 2). The ratio of the ovulation synchronization index is shown in Fig. 3, 4, 5.

On the 14th day after mating, the pregnancy was confirmed in 25% of females of the C57BL/6 line in the intact group. The estrous synchronization of the cycle increased the number of pregnant individuals relative to the control ones, by 35%. The hormone-regulating synchronization of the ovulatory cycle increased the number of fertilized individuals by 50% relative to the intact

ones, and by 10% relative to the estrous synchronization ($p<0.05$).

On the 14th day after mating, the pregnancy was confirmed in 25% of females of the CBA/lac line in the intact group. The estrous synchronization of the cycle increased the number of pregnant individuals relative to the control ones, by 40%. The hormone-regulating synchronization of the ovulatory cycle increased the number of fertilized individuals by 55% relative to the intact ones, and by 15% relative to the estrous synchronization ($p<0.05$).

On the 14th day after mating, the pregnancy was confirmed in 25% of females in the CBA/lac line intact group. The estrous synchronization of the cycle increased the number of pregnant individuals relative to control ones by 35%. The hormone-regulating synchronization of the ovulatory cycle increased the number of fertilized individuals by 35% relative to intact ones and did not change in the estrous synchronization ($p<0.05$).

Assessment of the birth rate index

The number of offspring in the experimental animals of different groups is not the same. In the groups of the hormone-regulating stimulation, the average number of the offspring is higher in comparison with intact and estrous synchronization groups (Table 3).

DISCUSSION

The results of the study confirmed the hypothesis that the hormone-regulating correction of the ovulatory cycle in female mice, makes it possible for the experimenter to obtain a larger number of fertilized individuals within necessary time limits, with a minimum error in the date of birth.

According to the instructions for the veterinary use of the drugs "Progestomag" and "Magestrofan", progesterone inhibits the hypothalamic-pituitary system. As a result, there is no release of gonadotropic hormones – follicle-stimulating (FSH) and luteinizing (LH), hence, follicle maturation and ovulation do not occur.

This leads to the induction of the synthesis of cervical mucus by the epithelial cells of the cervix, its edema as a result of an increase in its blood supply. It also leads to the proliferation of the endometrium and an increase in the extensibility of the myometrium, reduces the release of gonadoliberin, thereby inhibiting new ovulations, preventing the maturation of follicles in the ovaries, and makes the ovulation impossible. From the sixth to seventh day, there is a decrease in the concentration of progesterone and estrogen, causing a natural increase in LH and FSH, as well as an increase in the content of estrogen in the blood plasma.

All the follicles growing in a cohort, have specific receptors for FSH and require gonadotropin, which is necessary for their growth. At this stage, the growing follicles do not have enough LH receptors to respond to stimulation, that is why this growth stage is often referred to as FSH-dependent.

The second stage of the hormone-regulating synchronization of ovulation, begins with the administration of prostaglandin F2α on the seventh day. The main effect of this biologically active substance is to stimulate the transition of the estrous cycle from the diestrus phase to estrus by overcoming the progesterone blockade of the cycle. In addition, prostaglandin F2α promotes the development of folliculogenesis, estrogen synthesis and, as a consequence, the onset of estrus. Prostaglandin F2α supports luteinolysis caused by the upsurge in LH, which leads to the ovulation and the release of egg cells from the dominant follicle within 16–32 hours.

During the ovulation, which occurs approximately 34–36 hours after the LH upsurge, the secondary oocyte in metaphase II leaves the dominant follicle and enters the lumen of the fallopian tube, where it can be fertilized [12].

The upsurge in luteinizing hormone (LH) stimulates the preovulatory follicles to form local autocrine and paracrine mediators, which coordinate complex intra- and extracellular molecular mechanisms, subsequently causing ovulation and luteinization. The key local mediators include progesterone and its nuclear receptor (PGR), prostaglandins (PTG) (PGE2 and PGF2α).

An increase in LH levels, increases progesterone production and PGR expression in periovulatory follicles, which is necessary for successful ovulation in various animal models [13]. For example, blocking progesterone biosynthesis [14], the inhibition of PGR activity by chemical inhibitors [15, 16] or knockout of genes encoding PGR synthesis [17, 18], led to anovulation in various experimental animal models.

All the above listed changes, lead to the onset of ovulation on the 8th–9th days after the first stage of the hormone-regulating ovulation synchronization.

It was confirmed that the selection of animals for mating, based on the cytological picture of the estrous cycle phase, increases the number of fertilized individuals. However, the range in the birth dates was 2–4 days, which complicates planning of the experimental protocol for studying the pharmacological correction of pregnancy pathology. There are known models in which the drug is administered at different periods of pregnancy, studying its effect on different periods of developing the offspring: from the 1st to the 6th days (the pre-implantation period), from the 6th to the 16th days (organogenesis), and from the 16th to the 19th days of pregnancy (fetogenesis) [19]. In this case, the range of the due birth dates of 2–4 days is a problem that should be minimized. According to the authors' observations, the date of placing females to males to copulate immediately after the injection of prostaglandin F2α, can be considered the first day of pregnancy, since 21–22 days after the two-stage hormone-regulating estrus synchronization, 80% of females gave birth, whereas in the estrus synchronization group, only 42% females gave birth.

In addition, the number of fetuses born to females

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whose ovulation induction was artificial, is 30% higher in comparison with natural. The authors hypothesize that this is due to an increased release of hormones from the hypothalamic-pituitary system into the bloodstream, which stimulates the release of more egg cells from the ovaries into ovulation. [20].

According to the degree of impact on the body, the drugs used in this scheme, are classified as low-hazard substances (hazard class 4 according to GOST 12.1.007).

In the subcutaneous or intramuscular administration of the drug Progestamag, the required exogenous level of progesterone in the blood for the manifestation of the therapeutic effect, is maintained for 6–7 days, but it does not exceed the physiological content in the body of the animals. In accordance with the instructions, there are no side effects and complications in farm animals, caused by the drug Magestrofan; as a rule, they are not observed.

Thus, the administration of progesterone to a female mouse at the dose of 4.5 mg/100 g, stimulates the transition of the ovulatory cycle phase to the secretory phase of the female's ovulatory cycle. The subsequent administration of prostaglandin F2α at the dose of 0.083 mg/100 g after 34–36 hours, provides the release of LH, which stimulates the release of the secondary oocyte into the lumen of the fallopian tube, where it can be fertilized.

In the authors'opinion, the proposed scheme has the following advantages:

The use of a combined hormone-regulating synchronization of ovulation is justified when conducting preclinical studies of embryotoxicity of drugs due to the absence of toxic effects of the components used on the fetus.

An accurate prediction of the birth date minimizes the risks of delayed research, makes it possiblet to plan the experiment rationally.

A sequential administration of two drugs with a fairly long interval, minimizes labor costs and greatly facilitates planning of further experiments.

Taking into account the fact of adopting the first day of pregnancy of an individual since the moment of mating, prospects for the study of pathologies of the pre-implantation period, organogenesis, fetogenesis, and intrauterine pathologies open up.

CONCLUSION

Hormonal ovulation stimulation in female mice, significantly increases a number of births on the 22nd day relative to the control group with a cytological confirmation of the estrous phase and the intact group. This method of planning the birth timing of the offspring of the experimental animals, reduces the time spent on preclinical studies of drugs for the following types of assessment of toxic effects: reproductive toxicity, embryotoxicity, teratogenicity, effects on fertility. In addition, this method expands the possibilities of experimental modeling of pathologies of pregnancy and fetus, with the subsequent assessment of their pharmacological correction.

Thus, the administration of progesterone to a female mouse at the dose of 4.5 mg/100 g, stimulates the transition of the ovulatory cycle phase to the secretory phase of the female's ovulatory cycle. The subsequent administration of Prostaglandin F2α at the dose of 0.083 mg/100 g after 34–36 hours, provides the release of LH, which stimulates the release of the secondary oocyte into the lumen of the fallopian tube, where it can be fertilized.

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AUTHORS' CONTRIBUTION

All authors have contributed equally to the research work.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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