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EFFICIENCY OF BUSULFAN USE FOR ELIMINATING PRIMORDIAL GERM CELLS IN THE GONADS OF CHICKEN EMBRYOS

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Abstract

The use of primordial germ cells to produce genetically modified and chimeric poultry is one of the promising areas of biotechnology. It is also considered as an alternative to traditional methods of selection and genome modification. The production of transgenic and chimeric animals using this technology provides the introduction of donor primordial germ cells into the dorsal aorta of recipient embryos during the migration of this cell type from the blood to the gonads. In the case of the colonization by donor germ cells of the recipient embryos gonads, further differentiation of donor cells to mature germ cells (both male and female) is possible. One of the main factors that determine the effectiveness of conducted manipulations is eliminating own primordial germ cells in a recipient embryos. In this regard, it remains urgent to develop effective methodical techniques for removing this type of cells. The aim of our study was to optimize methodological approaches for eliminating primordial germ cells in the gonads of chicken embryos. Busulfan was used as an alkylating agent in concentrations from 10 to 250 µg/embryo. Dimethyl sulfoxide (DMSO) was used to dilute busulfan, as well as its combination with DMEM medium in various ratios. The busulfan solution was injected into the chicken embryos prior to incubation or after 24 hour incubation. The efficiency of elimination of primordial germ cells in embryonic gonads was assessed on day 7 of incubation based on histological studies using immunohistochemistry on the expression of the SSEA-1 gene (stage-specific embryonic antigen-1). It has been established that the method of preparation and introduction of busulfan into embryos, as well as the dose of this drug determine the effectiveness of elimination primordial germ cells. Reduction of the negative effect on the development of chicken embryos when using DMSO for the dilution of busulfan was shown when the DMEM medium was included in the solution formulation at a concentration of 10 %. The high efficiency of eliminating primordial germ cells was observed when busulfan was used at a concentration of 100 µg per embryo. In this case, the number of germ cells in the gonads declined by 92 % compared to the control. The use of busulfan at a higher concentration while maintaining the effectiveness of this drug was characterized by an increase in embryonic mortality. At the same time, higher effectiveness of eliminating primordial germ cells was achieved with the introduction of busulfan after 24-hour incubation of embryos: the number of germ cells in the gonads of 7-day-old chicken embryos was 12.5 % less than in the case of injection of this drug before incubation.

Keywords: primordial germ cells, chicken, embryos, busulfan

The use of donor primordial germ cells to produce transgenic [1-3] and chimeric [4-6] poultry is an alternative to traditional selection and genome modification. Due to the peculiarities of poultry reproduction and embryology, this technology opens up new opportunities for directed genome modification [7-9] and the reconstruction of valuable breeds and lines using cryopreserved material [10-12].

Primary embryonic cells (PES) are the precursors of the gamete cells. In embryogenesis, they differentiate into male and female germ cells. This greatly

expands the possibilities of realizing their potential while creating transgenic and chimeric individuals with prescribed properties. Primordial germ cells of chicken embryos are formed in the epiblast and migrate through the hypoblast into the gonads via blood [13]. Accordingly, when donor PEC are introduced into the dorsal aorta of recipient embryos during the migration of own PEC from the blood into the gonads, the donor cells may colonize gonads recipients [14, 15].

The transplantation efficiency of donor PEC can be increased through recipients embryos preconditioning focused on the eliminating own primordial germ cells. There are several methods to eliminate endogenous embryonic cells of different vertebrate species, such as γ -irradiation, x-radiation [16-18], chemosterilization [19]. In the latter, busulfan is used. It is an alkylating agent which causes DNA damage in target cells that leads to deactivation of all cellular mechanisms and cell destruction. Tests on laboratory and farm animals showed selective effect of busulfan on the male cells [20-22].

In this research, we studied the efficiency of administering busulfan to remove primordial germ cells in gonads of chicken embryos. This is the first comparative estimates of the primordial germ cell elimination when busulfan solution was prepared and injected to chicken embryos in different ways and at different concentrations.

Within the framework of developing technology to produce transgenic individuals with prescribed properties, this paper suggested optimization of methodology for elimination of primordial germ cells in chicken embryo gonads using busulfan.

Techniques. Pervomaiskaya breed chicken embryos were studied. To eliminate endogenous primordial germ cells, a solution of busulfan in dimethylsulfoxide (DMSO) and nutritional medium DMEM (Dulbecco's modified Eagle's Medium) (Invitrogen, USA) was used. The resulting solutions were sterilized by filtration through filter with a 22 μ m pore size. Before busulfan introduction, the eggshells were treated with 70 % alcohol as disinfectant. Further manipulations with embryos were carried out under sterile conditions (in a laminar box). The introduction of 10 μ g busulfan per embryo was carried out in two ways: through puncture in the shell at the blunt end of the egg before incubation or by administration of the solution to the embryonic disc through the eggshell hole at the blunt end of an egg after 24 hours of incubation. In the first case the injectate volume was 100 μ l, and in the second case it was 50 μ l. Eggs were incubated (Rcom Maru 190 Deluxe, Rcom, Korea) at 37.5 °C and 55 % humidity in compliance with the standard for chicken eggs. In determining busulfan dose optimal for the effective removal of primordial germ cells, their presence in gonads was studied when administrated busulfan amounted 40, 70, 100, 150, 200 and 250 μ g per embryo.

The busulfan efficiency in embryonic gonads was evaluated histologically. Untreated embryos served as control. The embryos were isolated on day 7 of incubation. Fixation was carried out in Bouin's Fixative Solution (picric acid:acetic acid:formalin, 15:1:5) for 48 hours. Histological specimen were prepared according to a common methods, involving tissue dehydration in increasing concentration alcohols, impregnating in a mixture of xylene paraffin and embedding into paraffin [23]. The preparations were stained with hematoxylin and eosin. Embryo cross-sections in the gonad area were used for the analysis, (the lumbar region, the place of primary kidneys localization). The histological analysis was carried out with a Ni-U microscope (Nikon, Japan), with interpretation and analysis of images using NIS-Elements software (Nikon, Japan). PES on histological sections was identified immunohistochemically with avidin-biotin systems (Vector Laboratories, USA) [21]. Primary antibodies were anti-SSEA-1

(stage-specific embryonic antigen-1). The antigen-antibody complex was identified using horseradish peroxidase detected by 3,3-diaminobenzidine tetrachlorate (DAB) (Vector Laboratories, USA).

Statistical data processing was performed in MS Excel using variation statistics methods. The tables show average values (\bar{X}) and mean errors ($\pm x$).

Results. Prior to administration of busulfan, we optimized composition of its diluent. Busulfan is commonly dissolved in dimethylsulfoxide (DMSO) as, when using aqueous solutions, busulfan can partially precipitate. Given DMSO cell toxicity, we studied the effect of the busulfan dissolved in DMSO and DMEM in different ratios on the development of embryos and the efficiency of primordial germ cell elimination (Table 1).

1. Development of Pervomayskaya breed chicken embryos and efficiency of primordial germ cell elimination under different composition of busulfan solution and administration techniques ($\bar{X} \pm x$)

Composition of injected solution	Embryos treated, n	Embryos developed to day 7 of incubation, n (%)	Tested gonads, n	Gonad diameter, μm ,	PEC number in gonads (histological section), n
Injection before egg incubation (100 μl per embryo)					
Control (untreated)	15	15 (100)	30	218 \pm 10	14 \pm 1
Bu + DMSO	20	15 (75)	30	185 \pm 11	8 \pm 1
Bu + 90 % DMSO + 10 % DMEM	20	16 (80)	32	191 \pm 9	8 \pm 1
Bu + 70 % DMSO + 30 % DMEM	20	17 (85)	34	190 \pm 10	10 \pm 1
Bu + 50 % DMSO + 50 % DMEM	20	17 (85)	34	201 \pm 12	10 \pm 2
Injection in 24 hours of incubation (50 μl per embryo)					
Control (untreated)	15	14 (93)	28	211 \pm 9	12 \pm 1
Bu + DMSO	20	12 (60)	24	172 \pm 12	7 \pm 1
Bu + 90 % DMSO + 10 % DMEM	20	14 (70)	28	178 \pm 8	7 \pm 1
Bu + 70 % DMSO + 30 % DMEM	20	15 (75)	30	188 \pm 10	9 \pm 1
Bu + 50 % DMSO + 50 % DMEM	20	16 (80)	32	190 \pm 14	10 \pm 1

Note. Bu — busulfan (10 μg per embryo), PEC — primordial embryonic cells.

Embryonic mortality for DMSO solution of busulfan was 18-23 % higher compared to the control. Introduction of DMEM to resultant solution of busulfan reduced the negative effect of DMSO on embryo development up to 20 %. However, as the DMEM content in the injectable solution increased to 30 % or more, the effectiveness of busulfan decreased. Optimal efficiency of embryonic development and primordial germ cell elimination resulted from the administration of 10 μg busulfan in 90 % DMSO and 10 % DMEM. In this case, the administration of busulfan after 24-hour incubation was more effective than when busulfane was used prior to incubation. The number of primordial germ cells in the gonads was 12.5 % less than in the embryos treated with busulfan at an earlier incubation time. In further studies, we used a busulfan solution contained 90 % DMSO and 10 % DMEM which were injected after 24-hour incubation of embryos.

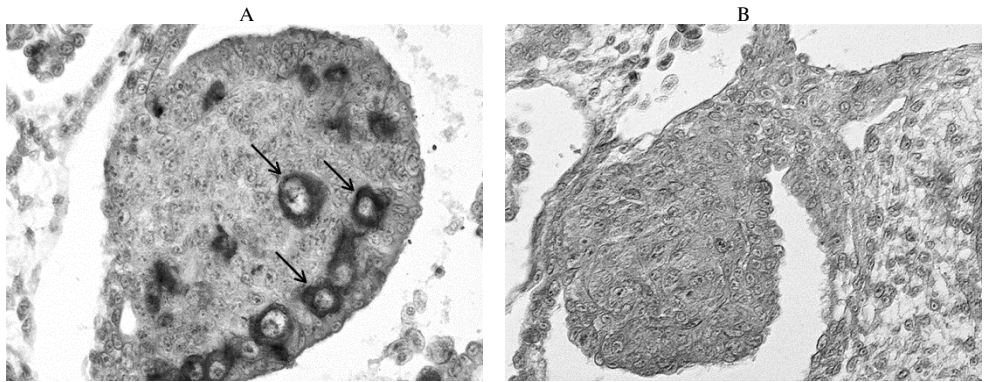
2. Efficiency of primordial germ cells (PEC) elimination in gonads of Pervomayskaya breed chicken embryos depending on a dose of busulfan ($\bar{X} \pm x$)

Parameter	Dose of busulfan, μg per embryo						
	control	40	70	100	150	200	250
Embryos treated, n	15	20	20	20	20	20	20
Embryos developed to day 7 of incubation, n (%)	14 (93)	10 (50)	8 (40)	5 (25)	2 (10)	1 (5)	0 (0)
Tested gonads, n	28	20	16	10	4	2	0
Gonad diameter, μm	211 \pm 9	176 \pm 6	154 \pm 4	111 \pm 7	99 \pm 3	96 \pm 1	
PEC number in gonads (histological section), n							
min	4	1	0	0	0	0	
max	22	13	11	2	1	1	
avg	12 \pm 1	6 \pm 1	4 \pm 1	1 \pm 1	1 \pm 1	1 \pm 1	

Histological study of 7-day-old chick embryos revealed a change in the

size of gonads and the presence of primordial germ cells which depended on the busulfan concentration (Table 2). So, at a dose of 40 µg busulfan, the average gonad diameter was 17 % less than that in the control. With increasing busulfan amount, the diameter was decreasing. The difference with the control reached 27, 47, 53 and 55 % for 70, 100, 150 and 200 µg per embryo, respectively. At 250 µg of busulfan, there was no further development of all 20 embryos treated.

Reducing gonad diameter was due to a significant decrease in the number of primordial germ cells (Fig.). At 40 and 70 µg of busulfan, this index was 2- and 3-fold lower, respectively, compared to the control (see Table 2). At a dose of more than 100 µg per embryo, there were single primordial germ cells in the gonads. It was also reported that busulfan decreased the size of the testicles and testicle tubules in laboratory animals and cocks [24, 25].



Immunohistochemical study of gonads in 7-day-old embryos of Pervomayskaya breed chicken: A — control (untreated), stained primordial cells are indicated by the arrows; B — administration of busulfan at 100 µg per embryo, no primordial germ cells found. Magnification $\times 400$, light microscopy (Nikon, Japan).

Thus, the data obtained indicate the efficacy of busulfan to remove primordial germ cells from gonads of chick embryos. The optimal dose of the agent is 100 µg per embryo. This amount of busulfan led to a 53 % reduce in the diameter of gonads and a 92 % decrease in the number of primordial germ cells. Higher busulfan amount, up to 250 µg, caused abnormalities in embryo development resulted in high embryonic mortality.

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