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REPRODUCTIVE QUALITY OF ROOSTERS WITH NORMAL AND MODIFIED GENOME

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Abstract

To date, progress has been made in the production of transgenic poultry: effective gene constructs have been obtained, and efficient systems for recombinant DNA delivery into target cells have been created. However, when breeding a genetically modified poultry, problems may arise with transgenic offspring, in particular its low viability or limited number. This paper is the first to report on histological structure of the testes and the composition of spermatogenic cells in the seminiferous tubules of transgenic roostersand on the quality and fertilizing ability of their semen. The study was carried out on transgenic roosters which were obtained by us in different ways based on chicken (*Gallus gallus* L.) Pervomaiskaya breed and their non-transgenic analogues (vivarium of Ernst Federal Science Center for Animal Husbandry, 2017-2018). group I was transgenic roosters after introduction of the lentiviral vector pWRSV into chicken embryos in vivo (n = 4); group II was transgenic roosters obtained by transplantation of transformed in vitro donor spermatogonia into the testes of sterile recipient roosters (n = 5). The control group included non-transgenic roosters selected as analogues (breed, age). Sperm was collected once every 2-3 days in penicillin vials heated to 30 °C by abdominal massage carried by the same technician. The following semen indicators were investigated: ejaculate volume, sperm motility and concentration in the ejaculate, the head and acrosome area, total spermatozoa length, flagella length. The criteria for evaluation of the fertilizing capacity of semen were the egg fertilization and the hatching of the younger generation. The histological structure of the seminiferous tubules and the composition of spermatogenic cells were also studied. Our experiments show that the quantitative and qualitative indicators of sperm in transgenic roosters decrease compared to the control. The volume of ejaculate, the concentration and motility of spermatozoa were 19, 15 and 1 % lower in group I and 38, 29 and 2 % lower in group II. However, there are no deviations in the safety of the acrosome in transgenic roosters when compared to the control individuals. Histological analysis of testes of the transgenic and non-transgenic roosters also reveals no significant pathological disturbances in the seminiferous tubules. Nevertheless, an insignificant decrease in the number of spermatogenic cells in transgenic individuals occurs when compared to the control ones (up to 19 %). The fertilizing capacity of the transgenic roosters' semen is also lower than that of the control roosters. In group I the differences with the control group for the percentage of chick hatching were 15 %, in the group 2 — 10 % ($p < 0.05$), which may indicate some negative effect of the integration of the transgene on the functional state of the germ cells in the studied genetically modified individuals.

Keywords: roosters, *Gallus gallus* L., transgenesis, gene constructs, transplantation, gene-transformed spermatogonia, acrosome, spermatozoon, semen quality, fertilizing ability, hatching

Purposeful modification of the poultry genome is a promising modern biotechnology, which is considered as an alternative to conventional breeding

[1]. This approach significantly accelerates the production of populations of individuals with the desired and fundamentally new properties, which is impossible by standard breeding methods. However, the techniques used to change the genome of transgenic farm animals are ineffective in creating genetically modified poultry because of the peculiarities of bird's reproduction and development [2]. At the same time, the biology of this phylum gives an opportunity to significantly expand a set of methods for the effective introduction of recombinant DNA into the target cells. Embryos of birds develop ex vivo, which facilitates access to them when performing genetic engineering manipulations. Blastoderm cells [3, 4], primordial germ cells [5, 6], and spermatogonia [7] are considered as promising targets for the introduction of recombinant DNA.

Currently, the methods have been developed for effective directed transfer of recombinant genes into embryonic and somatic chicken cells, i.e. the introduction of recombinant DNA into embryonic cells with the help of lentiviral [8] and retroviral [9] vectors, transplantation of transformed donor primordial germ cells [10, 11] and spermatogonia [12, 13], transformation of primordial germ cells [14] and spermatogonia [15] in vivo. With the use of these approaches, transgenic chickens which produce with egg protein marker proteins [16, 17] and recombinant human proteins [18, 19] were hatched. However, in further breeding of such individuals, the problems with transgenic offspring are possible (reproductive disorders, limited number, and low viability) due to the low fertility of the original parental forms. A number of studies have shown a decrease in the fertilizing ability of the sperm and libido among males of transgenic animals as compared to non-transgenic individuals [20, 21]. Such surveys were virtually not carried out on transgenic poultry. In this regard, it is interesting to study the effect of transgenesis on the functional state of germ cells of genetically modified birds, in particular, roosters.

This paper is the first to report on the histological structure of the testes and the composition of spermatogenic cells in the seminiferous tubules of transgenic roosters obtained using different methodologies. The decrease in the fertilizing ability of the sperm among transgenic roosters as compared to the control is found. For the first time, a decrease in the number of spermatogenic cells in the seminiferous tubules of roosters after transplantation of donor spermatogonia is revealed. At the same time, no significant pathological disorders in the histological structure of the testes were found.

The work objective was to evaluate the reproductive performance of transgenic roosters in comparison with their non-transgenic analogs, namely in the study of qualitative and quantitative indicators of semen, its fertilizing ability, the histostructure of the seminiferous tubules of the testes, and the quantitative composition of spermatogenic cells in them.

Techniques. Transgenic roosters (*Gallus gallus* L.) were derived from Pervomaiskaya breed. Group I was transgenic roosters after the introduction of the lentiviral vector pWRSV into chicken embryos in vivo ($n = 4$); group II was transgenic roosters obtained by transplantation of transformed in vitro donor spermatogonia into the testes of sterile recipients ($n = 5$). The control group ($n = 5$) included non-transgenic roosters aged 8-9 months which were selected as analogs. Transgenic and non-transgenic poultry was kept in single cages (the physiological yard, Vivarium of Ernst Federal Science Center for Animal Husbandry, 2018) and fed with complete feed.

Sperm was collected from roosters once every 2-3 days by abdominal massage. For conditioning sperm collecting, the roosters were prepared within 1-2 weeks. The ejaculate volume was measured with a graduated pipette (up to 1.0 ml). Sperm concentration was calculated in a Goryaev's chamber (Nikon Ni-U

microscope, Nikon Corporation, Japan; magnification ×400). Cell motility was assessed on the warm table at 38-40 °C; the ejaculate was previously diluted 5-fold. The fresh sperm preparations were fixed in methanol for 10 min, and the morphometric analysis (at magnification ×400) was carried out with (Nikon DS-Qi2 digital camera, Nikon Corporation, Japan; 4908×3264 px resolution). The morphometric parameters (area of the head and acrosome, the total length of the sperm, flagella length) were calculated with built-in software NIS-Elements BR 4.30 (Nikon Corporation, Japan). At least 100 sperm cells from each rooster were examined. The state of acrosomes was studied in fixed sperm preparations with the kit for differential staining Diakhim-Diff-Kvik (NPF Abris+, Russia).

The criteria for evaluation of the fertilizing capacity of semen were the egg fertilization and the hatching of the younger generation.

Histological preparations of testes were prepared according to the generally accepted method [22]. Tissue samples were fixed in Bouin's solution (picric acid:acetic acid:formalin in a ratio of 15:1:5) for 48 hours. The preparations were stained with hematoxylin and eosin. For the analysis, only seminiferous tubules with a lumen and a rounded shape (in cross-section) were selected. Spermatogenic cells were identified by morphology [23]. At least 30 seminiferous tubules from each rooster were examined. Images were processed and analyzed with NIS-Elements software (Nikon Corporation, Japan). The diameter of the seminiferous tubules, the number of spermatogenic cells in them, and the cellular composition of the population were determined.

The obtained data were processed statistically with Microsoft Excel program. The arithmetic mean values (*M*) and standard errors of means (±SEM) are presented in the tables. The significance of differences was assessed according to Student's *t*-criterion. Differences were considered statistically significant at $p < 0.05$.

Results. Visual evaluation of semen samples from transgenic roosters and males of the control group did not reveal any significant deviations: the color and smell of the ejaculates met the requirements. However, the differences between the experimental groups in terms of semen quality occur (Table 1).

1. Semen quality of transgenic and non-transgenic Pervomaiskaya roosters (*Gallus gallus* L.) (*M*±SEM, the physiological yard of Vivarium of Ernst Federal Science Center for Animal Husbandry, 2018)

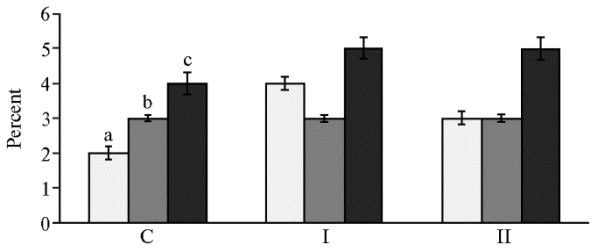
Indicator	Group		
	control	I	II
Ejaculate volume, ml	0.31±0.11	0.25±0.09	0.19±0.08
Sperm concentration, bln/ml	2.98±0.76	2.51±0.65	2.11±0.74
Sperm motility, %	86±6	85±7	84±8
The proportion of sperm with abnormal morphology, %	9±3	12±4	11±3

N o t e. See the description of groups in the section Techniques.

Transgenic roosters have a tendency to decrease the volume of ejaculate and sperm concentration compared to the control, i.e. by 19 and 15 %, respectively, in group I, and by 38 and 29 % in group II. In addition, the sperm of transgenic roosters was less motile, though these differences were less significant and did not exceed 2 %.

Along with the decrease in the ejaculate volume and sperm concentration in the experimental groups, the proportion of sperm with abnormal morphology increased. Differences with the control on this indicator were 33 % in group I and 22 % in group II. The percentage of sperm with abnormal morphology of head, middle part, and flagellum of spermatozoa showes that the most frequent were violations in the flagellum area (Fig.). This indicator was 4 % for the control group and 5 % for the experimental group. In comparison

with this, the share of sperm with abnormal morphology of the head and the middle part in all experimental groups was 1-2 % lower.



The share of sperm with abnormal morphology of the head (a)**, middle part** (b)**, and flagellum** (c) **in the ejaculates of transgenic and non-transgenic Pervomaiskaya roosters (*Gallus gallus* L.):** C — control, I and II — experimental groups (the physiological yard of Vivarium of Ernst Federal Science Center for Animal Husbandry, 2018). See the description of groups in the section Techniques.

Sperm morphometric differences between the experimental groups are also found (Table 2). Among transgenic roosters of group I, the head area increased by 27 % compared to control ($p < 0.01$), of group II by 6 %. In addition, the length of the flagellum and, as a consequence, the total length of a sperm cell decreased slightly (up to 2 %). The acrosomes of transgenic individuals of group I decreased in size by 33 % compared to control, in group II by 25 % ($p < 0.05$). It should be noted, however, that these morphometric changes did not have a significant impact on the integrity of acrosomes: among all roosters, it was almost the same, i.e. 98.9%: for transgenic and 99.0 % for non-transgenic.

2. Semen morphometry of the ejaculates of transgenic and non-transgenic Pervomaiskaya roosters (*Gallus gallus* L.) (*M*±SEM, the physiological yard of Vivarium of Ernst Federal Science Center for Animal Husbandry, 2018)

Group	Area, μm²		Length, μm	
	head	acrosome	total	flagellum
Control	11.9±0.6	1.2±0.1	81±3	68±3
I	15.2±0.7**	0.8±0.1*	79±1	67±2
II	12.7±0.5	0.9±0.1*	80±1	66±3

N o t e. See the description of groups in the section Techniques.

*, ** Differences with control are statistically significant at $p \le 0.05$ and $p \le 0.01$, respectively.

The fertilizing ability of the sperm of transgenic roosters was lower compared to the control roosters (Table 3). In the experimental group I, the percentage of fertilized eggs and hatched chickens were respectively 4 and 15 % lower than in the control poultry. In the II experimental group, the differences were 6 and 10 % ($p < 0.05$).

3. Embryo development and chicken hatching efficiency of transgenic and non-transgenic Pervomaiskaya roosters (*Gallus gallus* L.) (*M*±SEM, the physiological yard of Vivarium of Ernst Federal Science Center for Animal Husbandry, 2018)

Group	Eggs put for incubation, pcs.	Among them, pcs. (%)		Eembryos, %	Hatched chickens, %
		not fertilized	fertilized		
Control	50	3 (6)	47 (94)	90	85
I	50	5 (10)	45 (90)	84	70
II	55	7 (12)	48 (88)*	85	75

N o t e. See the description of groups in the section Techniques.

* Differences with control are statistically significant at $p \le 0.05$.

Similar data were obtained for transgenic farm animals, in particular, goats and rabbits. Among goats with the human lactoferrin gene *hLF*, there is a decrease in the quality of the semen and its fertilizing capacity (differences up to 13 % compared to the control group) [20, 24]. Among the male rabbits with the gene of the growth hormone of the bull *bGH*, the decrease in libido was observed [21]. At the same time, human lactoferrin transgenic mice had normal reproductive parameters [25]. Among sheep carrying the recombinant gene of

cattle chymosin in the genome the percentage of lambed ewes also corresponded to the regulatory indicators [26].

The decrease in the reproductive qualities of transgenic roosters compared to control was due to the multiple deterioration of semen quality, in particular, the decrease in the ejaculate volume, motility, and sperm concentration (see Table 1). Histological studies have confirmed the decrease in the concentration of sperm in the ejaculates obtained from transgenic roosters. Analysis of the histological structure of the seminiferous tubules showed a reduction in the number of spermatogenic cells in the seminiferous tubules of transgenic roosters compared to the control group (Table 4). These changes were most significant (by 19 %) among transgenic roosters after transplantation of donor spermatogonia (group II). Among transgenic roosters produced by lentiviral vector transduction (group I), the difference with the control group in the number of spermatogenic cells did not exceed 2.5 %.

4. Different types of spermatogenic cells in the seminiferous tubule (pcs.) **of transgenic and non-transgenic Pervomaiskaya roosters (*Gallus gallus* L.)** (*M*±SEM, the physiological yard of Vivarium of Ernst Federal Science Center for Animal Husbandry, 2018)

Group	Total	Sertoli cells	Spermatogonia	Primary and secondary spermatocytes	Spermatids	Spermia
Control	1198±115	18±3	69±5	613±20	218±14	280±17
I	1167±213	15±2	65±8	625±35	205±18	257±25
II	960±88	17±3	58±11	526±31	158±25	201±14*

N o t e. See the description of groups in the section Techniques.
* Differences with control are statistically significant at $p \leq 0.05$.

Comparison of semen quality in roosters upon application of different transgenic methodologies showed that group I was superior to group II (in particular, by 24 and 16 % for the ejaculate volume and sperm concentration, respectively). The semen fertilizing ability was also higher in using the lentiviral vector than after transplantation of transformed donor spermatogonia.

Thus, our investigations have shown that the integration of a transgene affects the functional state of germ cells of genetically modified roosters, with a decrease in the ejaculate volume, sperm concentration and motility, in the number of spermatogenic cells in the seminiferous tubules, and also in fertilizing ability of the semen. Nevertheless, any abnormalities in the integrity of acrosomes of transgenic poultry are not found. The analysis of testes of transgenic roosters and their non-transgenic analogs also did not reveal significant pathological disturbances in the histostructure.

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