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Influence of Spermatozoid Qualitative Indicators on Cow Fertility when Applying Artificial Insemination and Oocyte Fertility under in Vitro Conditions

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ABSTRACT

The aim of the research was to study the influence of the sperm cells' qualitative parameters on cow fertility at artificial insemination and fertilization of oocytes under in vitro conditions. The research methodology provides two independent experiments: the first one is to assess the influence of sperm qualitative indicators on the fertility of cows when applying artificial insemination; the second one is to assess the influence of qualitative indicators of sperm production during fertilization of oocytes in vitro. Studies were carried out on the qualitative parameters of sperm (sperm activity, number of live and dead sperm, acrosome integrity) from 6 different breeding bulls, as well as the effect of these indicators on the fertility of cows in the estruation stage and the fertility of oocytes in vitro. In the course of the first experiment, it was found that the bulls' sperm samples had an activity of 30.0±2.72% - 40.0±2.71%; indicators of live and dead sperm were 0.8-1%; the acrosome integrity corresponded to the norm (from 15.7±1.5 to 23.7±3.9%). Qualitative sperm indicators of the bull named Bokar-M (sperm activity (40.0±2.71%), a low number of sperm cells with a damaged acrosome (15.7±1.5%)) positively influenced the fertility of cows inseminated at the first menstruation (50%), their service period was 92.5±1.8 days. In the course of the second experiment, it was found that sperm samples from bulls had an activity of 33.0±2.3% - 42.0±2.10%, indicators of live and dead sperm when stained with eosin from 0.8-1%; the integrity of the sperm acrosome corresponded to the norm and was on average marked at the level from 25.2±1.8 to 36.3±2.2%. Better results at in vitro fertilization of oocytes (50% of fertilization) were obtained using the semen of the bull named Matthias-4593.

Keywords
Frozen semen; Sperm activity; Artificial insemination; Service period; Acrosome; Stain test kit; Fertility; Oocyte.

Introduction

For the livestock industry successful development, an important factor is considered to be good reproductive characteristics of heifers, cows and servicing bulls. Most farms raise and evaluate cows and heifers independently at all technological stages, and have an opportunity to form animal groups according to the desired phenotype and genotype, to conduct a comprehensive assessment of the animal, including its productive potential and reproductive capacity, and, based on the data obtained, make a decision on its further use (Gorelik, Brjanzev, Safronov, Gritsenko, & Shakirov, 2021; Gritsenko, 2016; Morrell & Rodriguez-Martinez, 2009).

An important indicator of the efficient cattle reproduction is the ability of sperm from servicing bulls to fertilize an oocyte. Sperm products are usually purchased at livestock breeding enterprises specializing in breeding servicing bulls. At breeding enterprises, breeders conduct a comprehensive assessment and work to improve the economically useful qualities of animals.

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Sperm products are obtained from bulls that have passed the assessment and received permission for use in accordance with the technologies provided by the enterprise. When obtaining semen, it is important to comply with all technological conditions, since the quality indicators are influenced by many factors, which include feeding and keeping bulls, hygiene of semen collection, composition of diluents, methods of freezing and thawing, and other factors. Sperm products obtained from breeding bulls are usually delivered to livestock enterprises frozen in liquid nitrogen in various forms (granules, straws) (Alm-Kristiansen et al., 2018; Iqbal et al., 2016; Luno et al., 2014; Morrell & Wallgren, 2014).

Freezing and storing sperm in liquid nitrogen is the most important method of animal reproductive biotechnology; however, the quality indicators and fertilizing ability of frozen and then thawed sperm suffer greatly, and thus, the sperm remain incapable to fertilize an oocyte both in artificial cattle insemination and in modern methods of oocyte fertilization *in vitro* (Mostek, Dietrich, Słowińska, & Ciereszko, 2017; Murphy, Eivers, O'Meara, Lonergan, & Fair, 2018; Naresh, 2016; Nongbua, Al-Essawe, Edman, Johannisson, & Morrell, 2018; Paudel et al., 2018; Tkachev, Tkacheva, Zubova, Pleshkov, & Smolovskaya, 2020b).

Despite the fact that research on improving the quality indicators of frozen-thawed sperm is being carried out by scientists all over the world and many positive points have already been achieved, this factor of reproduction requires a study and a scientifically substantiated proposal to livestock breeders to increase fruitful inseminations using various methods (Bagirov, Iolchiev, Tadzhieva, & Klenovitsky, 2015; Kumar, Kumar, Singh, Yadav, & Yadav, 2016; Pleshakov, 2004; Tkachev et al., 2020a, 2020b).

Currently, much attention is focused on the study of genetic material and drugs for compliance with their quality standards, including the study of semen. It has been reliably proven that in Russia and abroad, most of the producers supply farms with low-quality biotechnological sperm from servicing bulls. Sperm either die during thawing or freezing, or lose their fertilizing ability from other reasons (Alm-Kristiansen et al., 2018; Iqbal et al., 2016; Luno et al., 2014; Morrell & Wallgren, 2014; Tkachev et al., 2020b).

Development of recommendations for determining the sperm acrosome integrity using the stain test kit *Diff-Quick*, produced by the All-Union State Scientific Control Institute of Veterinary Drugs together with the All-Russian Scientific Research Institute of Animal Husbandry named after L.K. Ernst, allows performing a more accurate prediction of the sperm fertilizing ability. Scientists note that "the staining technique specified in GOST 32277-2013 does not meet modern requirements for determining the integrity of the sperm acrosome, and the eosin/nigrosine stain is advised to be used only to establish quantitative indicators of dead and live sperm" (Nikulin et al., 2018, p. 33).

For the successful development of animal husbandry, both with the use of the artificial insemination method and the introduction of modern biotechnological methods of reproduction, it is important to take into account the qualitative characteristics of the semen that affect the fertilizing ability along with the assessment of bulls by their offspring quality (Borunova et al., 2017; Chugunov, Popova, & Protopopova, 2020; Eskin, 2008; Tkachev & Tkacheva, 2017).

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In this regard, when used in methods of artificial insemination and in vitro fertilization, it becomes relevant to study the qualitative characteristics of bulls' sperm production in order to develop a scientifically substantiated method of using frozen-thawed sperm.

Purpose of the research

The research was aimed at studying the influence of the sperm qualitative parameters the cow fertility when applying artificial insemination and the oocyte fertilization under in vitro conditions.

Tasks

1. To conduct a study of frozen-thawed sperm samples for motility (activity), quantitative indicators of live and dead sperm, as well as to determine the sperm acrosome integrity in the test samples.
2. To determine the influence of the sperm cells' quality indicators on the cows' fertility and service period when applying artificial insemination.
3. To determine the influence of the sperm cells' quality indicators on the fertilization of oocytes in vitro.

Materials and Methods

Studies of the frozen-thawed semen samples were carried out on the semen of 6 breeding bulls, tested for the quality of the offspring. The supplier of semen products for the study was Kemerovoplem, JSC (Kemerovo region, Yasnogorskiy settlement). Sperm was delivered frozen in a Dewar vessel at a temperature of -196 ºC. Within the study, there were examined 10 semen samples from each bull.

Since the purpose of the study was to determine the sperm qualitative indicators for fertilizing ability in different production conditions, 2 independent studies of frozen-thawed sperm samples from different bulls were carried out: 1 - artificial insemination of cows, 2 - fertilization of oocytes in vitro. Since the experimental animals selected for artificial insemination and the oocyte fertilization were advised to a high class and maximally excluded the manifestation of other factors on the success of fertilization, the determination of the qualitative characteristics of the test sperm samples played a key role in determining the percentage of successful fertilization.

Artificial insemination of cows was carried out in *Mikhailovskoye LL*C, Prokopyevsky district, Kemerovo region, where the semen of the bulls Canon-M SA0011329467, Houdini-M CA0011595031, and Bokar-M CA0011397828 was delivered.

Experimental studies on fertilization of oocytes in vitro were carried out in the Research Laboratory "Biochemical, Molecular Genetic Research and Selection of Farm Animals" of the FSBEI HE Kuzbass State Agricultural Academy, where frozen sperm of bulls-producers of the Holstein Black-and-White breed (Allegro-106303101; Matthias-4593; and Nabeg-1896) was delivered.

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Semen was tested for activity according to GOST 32277-2013 (Federal Agency for Technical Regulation and Metrology, 2014). The quantitative ratio of spermatozoa with rectilinear translational motion to their total number was determined visually in a crushed sperm drop by a microscope.

Sperm is allowed for use, if the content of spermatozoa with rectilinear translational movement is not lower than 40% (GOST 26030-2015) (Federal Agency for Technical Regulation and Metrology, 2015).

To determine the sperm acrosome integrity, a Diff-Quick stain kit was used. The kit consisted of a fixative, solution 2-pink, solution 3-blue, and buffer D (1 bottle each), it was produced by the research and production company "ABRIS +", Russia, St. Petersburg. The results were evaluated according to GOST R ISO 5725-1-2002 (State Committee of the Russian Federation for Standardization and Metrology, 2009).

Oocytes for the experiment were obtained from donor cows by aspiration from the ovaries of live animals under ultrasound control (ovum-pick-up (OPU)) at the livestock breeding enterprise “Selyana”, Kemerovo region.

In the laboratory, oocytes were washed 3 times in the medium TC-199, it contained 5%-fetal bovine serum (FBS), 10 μg/ml of heparin, 0.2 mM of sodium pyruvate, and 50 μg/ml of gentamicin. For evaluation, there were selected oocytes of a rounded shape, with a homogeneous cytoplasm, a pellucid zone uniform in width, surrounded by a multilayer compact cumulus. For fertilization, preliminarily evaluated oocytes of good and excellent quality were selected (Golubets et al., 2010).

Activity related to the production, preparation and fertilization of oocytes in vitro was carried out according to the method "In vitro fertilization of cattle oocytes" (Novikova, Koba, Skorikov, & Alravashdeh, 2019).

Statistical processing of the data was carried out on a personal computer using the Microsoft Office Excel program with confirmation of the reliability by the Student's t-test in the following values: *p <0.05; **p <0.01; ***p <0.001.

Results

The results of the first experiment showed that the sperm samples of the bull Canon-M CA0011329467 had an activity of 31.0±2.46% with rectilinear-translational movement of sperm (3.1 points), semen of the bull Bokar-M CA0011397828 had an activity of 40.0±2.71% (4.0 points) with translational movement, and semen of the bull Houdini-M CA0011595031 had an activity of 30.0±2.72% (3 points) with translational movement (Table 1).

Table 1. Results of determining sperm activity

No	Name and number of the servicing bulls	Average activity indicator, %	Average indicator, score
1	Canon-M CA0011329467	31.0±2.46	3,1
2	Bokar-M CA0011397828	40.0±2.71	4,0
3	Houdini-M CA0011595031	30.0 ±2,72	3,0

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When determining the indicators of living and dead sperm, the following results of dead sperm were obtained: Bocar-M CA0011397828 - 1%, Canon-M SA0011329467 - 0.8%, Houdini-M CA0011595031 - 0.9%. Indicators of motility and live-dead sperm were evaluated in accordance with GOST 32277 - 2013.

To identify the frequency of morphological damage to the acrosome structure during sperm thawing and the degree of its influence on cows' fertilizing ability, a stain test kit "*Diff-Quick*" was used. One frozen semen granule was taken and the content of spermatozoa with damaged acrosome was determined. Each semen sample (10 samples from a bull) was examined: immediately after thawing, 1 and 2 hours after thawing (Table 1). Spermatozoa of cattle with a damaged acrosome, when using the "*Diff-Quick*" kit, were colored in a light pink color, normally - in brown (Borunova et al., 2017).

The indicators presented in table 2 shows a significant difference in the studied indicators between the groups. It was found that a lower percentage of spermatozoa with damaged acrosomes was in the semen samples of the bull Bokar-M; on average, this indicator was recorded at the level of 15.7±1.5%, which may indicate a high sperm quality. The highest average indicators of acrosomes damage (greater percentage of damage) were observed in the sperm samples of the bull Canon-M, where this indicator was 23.7±3.9%, which was by 8% higher than in the Bocara-M samples ($p<0.001$) and by 4.7% ($p<0.01$) than in the Houdini-M samples, where this indicator was noted at the level of 19±2.6%. There was also a significant difference between the semen samples from the bulls Bocara-M and Houdini-M, where it was 3.3% ($p<0.05$). Thus, it can be concluded that the samples of the bull Bokar-M is distinguished by the best characteristics of sperm in determining the acrosome integrity.

Based on the research results, for all 3 parameters (mobility, number of live-dead sperm, acrosome integrity), the sperm of the bulls Cannon-M, Bocar-M and Houdini-M corresponded to the norm.

Table 2. Results of sperm examination when determining the acrosome integrity

No.	Name and number of the servicing bulls	Average indicator (norm according to GOST is 50%)
1	Cannon-M CA0011329467	$23,7±3,9^{2***3**}$
2	Bocar-M CA0011397828	15,7±1,5
3	Houdini-M CA0011595031	$19±2,6^{2*}$

Note: hereinafter, the difference is significant at - *p<0.05; **p<0.01; ***p<0.001

For conducting artificial insemination, 30 cows were selected (10 heads for insemination with semen samples from each of 3 bulls) (Table 3). According to the physiological status, all selected animals were suitable for artificial insemination and were in the estruation period.

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Table 3. Results of selecting cows for artificial insemination

Parameter	**Insemination with the sperm sample from**		
	Cannon-M CA0011329467	**Bocar-M CA0011397828**	**Houdini-M CA0011595031**
Number of cows, heads	10	10	10
Breed	Black-and-White	Black-and-White	Black-and-White
Age of productive use, years	3-5	3-5	3-5
Live weight, kg	549±31.7	545±30.4	552±32.1
Months after calving	1-2	1-2	1-2
Diseases, incl. gynecological ones	no	no	no
Period	Active estruation	Active estruation	Active estruation

The results obtained in the experiment on the cow fertilization indicate that the highest rates were obtained in the estruation period, and the shortest service period was established for cows inseminated with the semen of the bull Bokar-M. It can be associated with a better indicator of sperm activity (40.0±2.71%) and a lower indicator of sperm cells, with a damaged acrosome (15.7±1.5%).

E.N. Novikova et al. (2019) noted that the main reason for animal infertility is violating the technology of keeping, feeding and insemination of cows. The optimal service period is from 60 to 110 days, and more than 140 days are observed in problem animals. Pregnancy from primary inseminations should be 50-60%. (p. 278).

Table 4. Fertility and service period of cows when rectocervical artificial insemination (n = 6)

Name and number of the servicing bulls	**Fertility**						**Service-period (days)**
	I estruation		**II estruation**		**III estruation**		
	heads	**%**	**heads**	**%**	**heads**	**%**	
Cannon-M CA0011329467	2	33.3	2	33.3	2	33.3	108.6±11.02
Bokar-M CA0011397828	3	50	3	50	-	-	92.5±1.8
Houdini-M CA0011595031	2	33.3	2	33.3	2	33.3	103.3±12.89

In the course of the first experiment, there were obtained the data indicating the sperm qualitative parameters of the bull Bokar-M: sperm activity (40.0±2.71%), the number of damaged acrosome sperm a (15.7±1.5%) and showing their positive effect on the fertility of cows inseminated by in the first estruation (50%) and on the service period duration (92.5±1.8 days).

The analysis of the second experiment results showed that the sperm samples of the bull Allegro-106303101 had an activity of 33.0±2.30% with a rectilinear-translational movement of sperm (3.3 points), the sperm of the bull Matthias-4593 had an activity of 42.0±2.10% with a translational movement (4.2 points), the sperm of the bull Nabeg-1896 had an activity of 35.0±2.5% with translational movement (3.5 points) (table 5). The sperm activity indicators of the bull Matias-4593 significantly exceeded the ones of the bulls Allegro-106303101 by 9% ($p < 0.01$) and Nabeg-1896 by 7% ($p < 0.05$).

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Table 5. Results of determining sperm activity

No	Name and number of the servicing bulls	Average activity indicator, %	Average indicator, score
1	Allegro-106303101	33.0±2.3	3,3
2	Matthias-4593	42.0±2.10[1**3*]	4,2
3	Nabeg-1896	35.0±2.5	3,5

Note: hereinafter, the difference is significant at - *p<0.05; **p<0.01; ***p<0.001

When determining the indicators of the living and dead sperm, the following results of dead sperm were obtained: Allegro-106303101 - 1%, Matthias-4593 - 0.8%, and Nabeg-1896 - 0.9%. Thus, the indicators characterizing sperm motility, the content of live and dead sperm in the samples corresponded to GOST No. 32277 – 2013 (Federal Agency for Technical Regulation and Metrology, 2014).

The indicators presented in table 6 indicate a significant difference in the studied indicator between the bull semen samples. It was found that in the samples of the bull Matthias-4593, a smaller percentage of sperm with damaged acrosomes was revealed. On average, this indicator is noted at the level of 25.2±1.8%, which may indicate a high quality of sperm.

A greater percentage of damaged acrosomes was noted in the samples of the bull Allegro-106303101, it averaged 36.3±2.2%, which is by 11.1% higher than in the samples of Matthias-4593 (p<0.01) and Nabeg-1896 by 3.6%, where this indicator was noted at the level of 32.7±2.7%. The difference between the semen samples of the bulls Nabeg-1896 and Matias-4593 significantly differed and amounted to 7.5% (p<0.05). When determining the integrity of the acrosome, the semen of the bull Matthias-4593 has the best characteristics.

Table 6. Results of sperm examination in determining the acrosome integrity

No	Name and number of the servicing bulls	Average indicator (norm according to GOST is 50%)
1	Allegro-106303101	36.3±2.2[2**]
2	Matthias-4593	25.2±1.8
3	Nabeg-1896	32.7±2.7[2*]

Thus, the research results according to 3 parameters (mobility, rate of live-dead sperm, acrosome integrity) showed that the sperm of the bulls Allegro-106303101, Matias-4593, Nabeg-1896 corresponded to the norm, but there were certain differences in the studied indicators, which may have been associated with the conditions for obtaining and using frozen semen.

The results of in vitro fertilization (Table 7) showed a different fertilizing ability of the studied bulls' sperm. The results obtained in the experiment indicate that the highest rates of oocyte fertilization in vitro, using the sperm of the bull Matias-4593, where 5 successful fertilizations out of 10 (50%) were obtained. The higher rates of the obtained percentage of fertilization in vitro with the sperm of the bull Matias-4593 can be associated with the better indicator of sperm activity (42.0±2.10%) and the lowest content of sperm with damaged acrosome (25.2±1.8%).

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Table 7. Results of in vitro fertilization of oocytes

Name and number of the servicing bulls	In vitro fertilization rates				
	number	normal fertilization	no fertilization (parthenogenesis or 1 pronucleus)	polyspermia (more than 3 pronuclei)	Total
Allegro-106303101	n	4	5	1	10
	%	40	50	10	100
Matthias-4593	n	5	3	2	10
	%	50	30	20	100
Nabeg-1896	n	4	4	2	10
	%	40	40	20	100

Conclusion

As the results of Russian and foreign researchers show, the quality of sperm production depends on a number of factors. Failure to comply with the conditions for obtaining, freezing, storage and thawing of sperm, the quality characteristics are markedly reduced, which has a negative effect on the fertilizing ability of sperm (Alm-Kristiansen et al., 2018; Kumar et al., 2014; Morrell & Rodriguez-Martinez, 2009; Sidorova, 2014; Tkachev & Tkacheva, 2017; Tkachev et al., 2020a).

Authors of the article carried out two independent experiments to assess the influence of sperm quality parameters on its ability to fertilize cows with artificial insemination, and under conditions of in vitro fertilization of oocytes.

The first experiment proved that:

- semen samples from the servicing bulls had activity from 30.0±2.72% with rectilinear-translational movement of sperm (3 points) to 40.0±2.71 % (4 points), indicators of live and dead sperm with eosin staining were from 0.8 to 1%, which corresponded to GOST 32277 - 2013. The acrosome integrity of all the 3 bulls corresponded to the norm (from 15.7±1.5 to 23.7±3.9%);
- qualitative indicators of the Bokar-M bull sperm: activity (40.0±2.71%), number of damaged acrosome sperm (15.7±1.5%), positively influenced the fertility of cows inseminated by it in the first estruation (50%), service period was 92.5±1.8 days.

The second experiment showed that:

- semen samples of bulls had activity from 33.0±2.3% (3.3 points) to 42.0±2.10% (4.2 points), indicators of live and dead sperm when stained with eosin were from 0.8 up to 1%, which corresponds to GOST 32277–2013. The sperm acrosome integrity of the samples corresponded to the norm as well. On average it was noted at the level of from 25.2±1.8 to 36.3±2.2%.
- better results when applying in vitro fertilization of oocytes (50% of fertilization) were obtained using the semen of the bull Matthias-4593. This factor was influenced by the quality indicators of sperm, such as activity (42.0±2.10%) and the number of sperm with damaged acrosome (25.2±1.8%).

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Thus, the results obtained indicate the dependence of the quality indicators of sperm fertilizing ability, both by artificial insemination of cows and byb fertilization of oocytes under in vitro conditions. The data obtained indicate the need for a qualitative assessment of sperm doses before use in farms and laboratories for embryo transplantation, which will increase the percentage of successful fertilization and increase the profitability of the livestock industry.

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