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MORPHOLOGICAL ANALYSIS OF BOAR SPERMATOZOA BY AGE AND BREED*

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SUMMARY: The total of 56 boars from 12 farm units (3 to 7 boars per farm) were used for cytological and morphological examination of semen. Large White (LW, n = 18); Swedish Landrace (SL, n = 11), Duroc (OA, n = 12); German Landrace (NL, n = 6), crossbreeds (OST, n = 9) boars were used for examination. Sperm was stained with eosin/nigrosine in one step. According to the findings of spermatozoa with protoplasmic droplets (PPD), boars were divided into groups with ≤ 10% of the PPD and the group with > 10% of the PPD. The impact of the PPD rate to number of live born piglets per litter and correlation of PPD rate and findings of live sperm with intact akcrosoma (LIA), or normal apical ridge (NAR) were investigated. Farrowin rate and abnormal sperm with tail deformities was significantly (p <0.05) lower in the boars younger than two years, compared to the boars older than two years (farrowing rate: 74.32% vs. 62.82%). Statistically significant correlations were found between the findings of protoplasmatic droplets (PPD) on the tail of spermatozoa in native semen and number of live born piglets per litter (r = 0.44, p = 0.001). The medium correlation within these parameters were found in the Large White (r = - 0.57, p <0.05, n = 18), and Duroc boars (r = 0.68, p <0.05, n = 12). Other boar breds did not have significant correlation. The finding of cytoplasmic droplets on boar sperm tail is very stable and relatively easy to establish. It should be used as a practical mathod for control the quality of sperm as a selection parameter.

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INTRODUCTION

Artificial insemination is an inseparable biotechnology in modern swine production. Quality control of native and diluted semen includes macroscopic and microscopic evaluation. Morphological analysis of sperm structure is rarely practiced. Only a few papers have been published on cytoplasmic droplets and their effect on the fertility of different species. It seems more profitable to discard abnormal ejaculate than to use the ejaculate with many transient morphological abnormalities (eg, cytoplasmic droplets). Thus, the boars with abnormal ejaculate should be excluded from the breeding until production of normal sperm begins, or until tests indicate that the abnormalities are normal species variation with no negative effect on fertility. In addition, it is difficult to set a final diagnosis of subfertility, as most animals tend not to be constantly subfertile, and there are many factors, other than male, which contribute to poorer pregnancy and small litter size (Althouse, 1998).

On the farms, collecting of boars sperm for artificial insemination, the following measures are recommended: data about health condition, implementation of clinical and laboratory practice standards, and estimation of potential breeding value of boars are necessary in centers reproduction (Jovičin et al., 2008). Creating good practice with optimal hygienic conditions and installation of air conditioners will provide better utilization of genetic resources of imported boars (Jovičin et al., 2003).

According to Rouge (2004), the results of sperm morphology are expressed as the percentage of normal sperm present in ejaculate. Anomalies may be classified as abnormalities of the head, the connecting part or tail. The primary anomalies are more difficult and it is thought that they were created when the sperm was in testicular seminal tubules. Secondary abnomalities occur during the sperm passage through the epididymal channel or within semen handling process in vitro. Miljkovic (1969) emphasizes the importance of lipoprotein sperm membrane consisting of colloidal epididymal secretion channels. It makes sperm resistful and fertile, and it is the carrier of electrical load. Immature spermatozoa forms have proximal or distal protoplazmatic droplet (PPD) which is located below the head or the tail. It is the residue of protoplasm and lipoproteins. Immature forms of spermatozoa are poorly resistant and infertile. Hancock (1957, cit. Miljković, 1969) believes that the sperm of fertile boars normally contain 5.4% sperm with proximal droplet and 3.4%. with distal droplet. Constant number of spermatozoa with a droplets was found in some boars ejaculate, with no effect on the fertility (Paredis, 1961). The semen of fertile boars rarely contains more than 10% spermatozoa with protoplasmic droplets (Rothe, 1963). The boars that produce unacceptable ejaculate should be tested once a week. If improvement in the quality of the ejaculate is not reached within 3 months, the animal should than be excluded from the breeding program.

The aim of this paper was to demonstrate the importance of morphological examination of boar semen diluted in assessing the quality of sperm used for insemination of gilts and sows.

MATERIALS AND METHODS

This study included data for a total of 56 boars, from 12 farms in the South Backa and Srem district (Serbia). The selection was made by using a stratified representative sample of active boar population, randomly selected within the group of boars from own breeding, purchased on our farms and from the imported boars. The data from 3-7 boars per farm were processed. The first division was made according to the breed, and the animals were divided in four groups: Large White (LW, n = 18); Swedish Landrace (SL, n = 11), Duroc (OA, n = 12); German Landrace (GL, n = 6); the fifth group consisted of crossbreeds (OST, n = 9). The second division was made according to age: boras younger than 2 years (n = 24) and older than 2 years (n = 32).

The score of sexual desire (*libido sexualis*) was determined in a scale 1 to 5, based on measuring the time for mounting and collecting the sperm. The samples of native sperm and diluted semen were taken in order to evaluate the progressive motility of spermatozoa and carry out cytological and morphological analyses. The analyses were performed aseptically in sterile plastic tubes. Supravital sperm staining and making of smears for morphological analysis was done by eosin-nigrozine staining technique in one step.

For the analyses 10 μL of well-mixed native sperm or 20 μL of diluted semen was taken and mixed with one droplet (20 μL) of eosin-nigrozina solution (Björndahl et al., 2003), a modified prescription by Mortimer (1994) using adjustable automatic micro-pipette (0 0.5- 10 μL Eppendorf Research adjustable-volume pipette, Eppendorf, Germany). The suspension was incubated at room temperature (20°C) for 30 seconds on the pre-heated microscopic glass plate. A doplet of the mixture (10 μL) was transferred to microscope marked slides, with prepared smears, spread the drops with a glass rod and then air dried. The smears were examined using the microscope Olympus BX 40 with a phase-contrast microscopy, 1000 × magnification, under immersion, with a 100 × lens.

The third division was made according to the morphological feature. The findings of spermatozoa with protoplasmic droplets (PPD) was performed on a group of boars (n = 36) and the values were ≤ 10% PPD, and a group of boras (n = 24) with the values> 10% PPD.

Livestock Selection Service has collected and processed data of the sows and breeding gilts inseminated with the sperm of these boars. The impact of the PPD on live born piglets per litter was analyzed and correlation between the PPD findings and findings of live sperm with intact acrosom (SIA), i.e. normal apical ridge (NAR).

Statistical analysis were done using the programs Prism and Sigma (Pad Prism. v 5.0, Graph Pad Software Inc., San Diego, CA, USA, and SigmaStat 3.5, v. 3.5.0.54, Systat Software, Inc., San Jose, CA, USA). T-test, the variance analysis of variance and correlation analysis according to race and for all boars were carried out.

RESULTS

The examined boars (n = 56) were 161 to 1535 days of age (0.38 to 4.21 years, with average body condition score (BCS) = 3.09 ± 0.50 (2.0 to 4.0). The score of sexual desire (*libido sexualis*) was 3.39 ± 1.23. Time required to mount the sow lasted in average 140.79±93.11 seconds, or 2.35±1.55 minutes (0.42 to 8,00). Semen collecting lasted 382.61±154.21 seconds, or 6.38±2.57 minutes (2.92 to 19.52).

The volume of native ejaculate, after filtering through a double layer gauze, was 258.84 ± 103.41 mL (60-680). The number of spermatozoa in native semen was on average 251.66 ± 125.21 × 10^6/mL (84-510). After diluting, in ratio 1:2 to 1:13, 362 insemination doses were formed. Progressive motility of spermatozoa in native semen (active movements, AK) was 76.34% ± 18.79% (10-95%). Progressive motility of diluted semen was 71.54% ± 18.49% (1-90%).

The division according to age:

Including boars ≤ 2 (N = 32) and > 2 years of age (n = 24).

1. The ability to inseminate was significantly lower in the boars younger than two years, compared to boars > 2 years (74.32% - 62.82% = 11.50%, t-test, p <0.05;*).
2.The percentage of secondary abnormal sperm, with tail deformities, in the samples of native sperm was significantly higher in the first group, i.e. in the boars younger than two (1.17% -0.44% = 0.73%, t-test, p <0.05;*). Other differences were statistically unimportant.

The division according to the findings of protoplasmatic droplets (PPD):

Including the spermatozoa with tail droplets in diluted semen: boars with ≤ 10% of PPD (n = 36) and boars with >10% of PPD (n = 20).

1. The younger animals had the finidings ≤ 10% PPD (678.42 days - 932.20 days = 253.78 days, t-test, p <0.01;*). The average age was 1.86 year and more than 2.56; the difference was 8.46 months.
2. Number of live born piglets per litter was lower in boars with the finding ≤10%PPD (9,88–10,41=–0,53, t-test, p<0,05; *).
3. The findings of native sperm: progressive sperm motility (active movements):
   (AK-nat): 80.56–68.75=11.81%, t-test, p<0.05;
   SIA%: 54.6136.35=18.26%, t-test, p<0.001;
   PPD%: 4.38–25.60=21.21%, t-test, p<0.001; ***
   I ABN%: 5.9–-11.80=5.86%, t-test, P<0.05; *
   II ABN%: 0.38-1.40=1.01%, t-test, p<0.01; **
   PAT%: 6.33–12.85=6.52%, t-test, p<0.5; *
4. The findings of diluted sperm:
   SIA%: 48.19–27.90=20.29%, t-test, p<0.001; ***
   PPD%: 3.1924.50=–21.31%, t-test, p<0.001; ***
   I ABN%: 5.44–14.20=8.76%, t-test, P<0.01; **
   II ABN%: 0.39–1.35=0,96%, t-test, p<0.01; **
   PAT%: 5.83–15.55=9.72%, t-test, p<0.01; **
   Other differences were statistically insignificant.

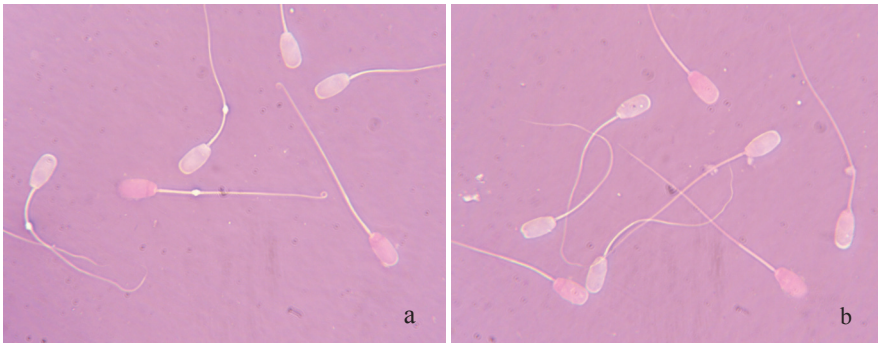
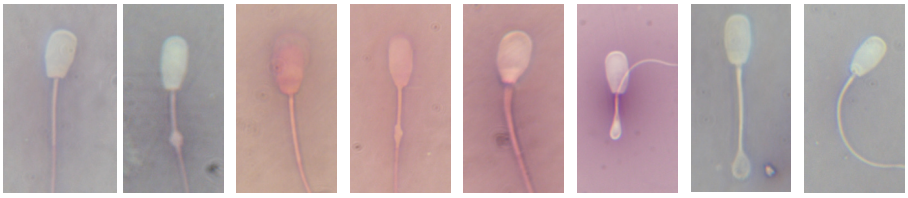


Fig. 1. Supravital colored sperm smear of boar. a) The above are the four living (not stained) below are the two-colored (eosinophilic) spermatozoa, three spermatozoas with bright protoplasmatic droplets on the tail; b) Five of live spermatozoas, a contrasting with the head, two in the middle of the picture right, have protoplasmatic drop on the tail, three are dead, with shady and unclear contours of the head. Staining with eosin / nigrosine, 400 × magnification

Slika 1. Razmaz supravitalno obojene sperme nerasta. a) gore su četiri živa (neobojena); dole su dva obojena (eozinofilna) spermatozoida; tri spermatozoida su sa svetlim protoplazmatičnim kapljicama na repu; b) pet živih spermatozoida, sa kontrastnom glavom; dva u sredini slike desno, imaju protoplazmatičnu kapljicu na repu; tri su mrtva, sa mutnim i nejasnim konturama glave. Bojenje eozin/nigrozinom, uvećanje 400×



a b c d e f g h

Picture 2. Supravital colored sperm smear of boar. a) live normal sperm forms with intact edge of acrosom (LIA) ; b) live sperm with protoplasmatic drop (PPD); c) dead sperm with swollen - damaged acrosom (DA); d) live sperm with damaged acrosom (LDA) and protoplasmatic drop; e) live sperm with primary abnormality of the head and a connecting part (I ABN); f) live sperm with secondary abnormality of the tail (II ABN); g) live sperm with tail curved into a loop (II ABN); h) live sperm with simple curved tail (II ABN). Staining with eosin / nigrosine, 1000 × magnification

Slika 2. Razmaz supravitalno obojenih spermatozoida nerasta. a) živ spermatozoid normalne forme sa intaktnim rubom akrozoma (ŽIA); b) živ spermatozoid sa protoplazmatičnim kapljicom (PPK); c) mrtav spermatozoid sa nabubrelim – oštećenim akrozomom (OA); d) živ spermatozoid sa oštećenim akrozomom (ŽOA) i protoplazmatičnom kapljicom; e) živ spermatozoid sa primarnom abnormalnošću glave i spojnog dela (I ABN); f) živ spermatozoid sa sekundarnom abnormalnošću repa (II ABN); g) živ spermatozoid sa repom savijenim u petlju (II ABN); h) živ spermatozoid sa jednostavno savijenim repom (II ABN). Bojenje eozin/nigrozinom, uvećanje 1000×.

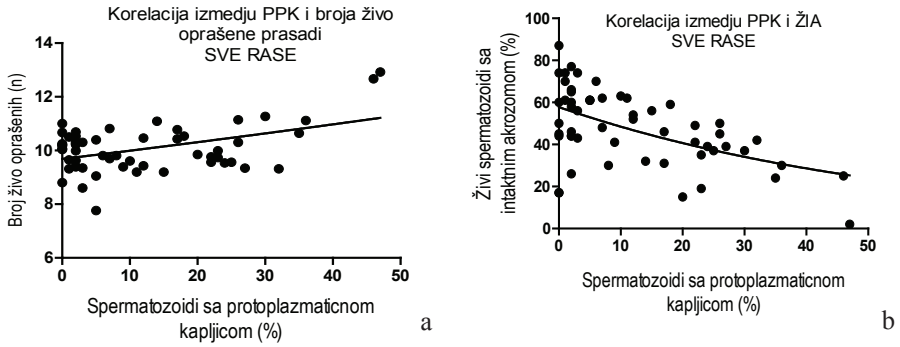


Fig. 1. a) influence of findings protoplasmatic droplets on boar sperm tail to the number of live born piglets per litter (p<0,05). b) mutual influence of the total findings of protoplasmatic drops on tail and intactness akrozoma in living boar sperm (n=56, p<0,0001)

Grafikon 1. a) uticaj nalaza protoplazmatičnih kapljica na repu spermatozoida nerastova (n=56) na broj živo oprašene prasadi u leglu (p<0,05). b) međusobni uticaj ukupnog nalaza protoplazmatičnih kapljica na repu i intaktnosti akrozoma kod živih spermatozoida nerastova (n=56, p<0,0001)

The division according to boars breed:

The variance analysis showed statistically no significant differences in the examined fertility parameters in the morphology of spermatozoa, neither in native nor in diluted sperm.

Statistically significant correlations were detected between the findings of protoplasmatic droplets on spermatozoa tail in native sperm and the number of newborn piglets. In the Large White breed the correlation was moderate ($r=-0.57$; $p<0.05$; $n=18$), in Duroc breed the correlation was moderate ($r=0.68$; $p<0.05$; $n=12$), and in other breed no significant correlation was detected. In all 56 boars moderate correlation was detected ($r=0.44$; $p=0.001$; $n=56$).

Highly significant positive correlations were found between native protoplasmatic droplets in diluted sperm: the Large White breed =0.73; $p<0.001$; $n=18$; in Swedish Landrace $r=0.95$; $p<0.0001$; $n=11$; in Duroc $r=0.82$; $p<0.01$; $n=12$; in German Landrace $r=0.96$; $p<0.01$; $n=6$; in crossbreeds $r=0.95$; $p<0.001$; $n=9$. In all 56 boars the correlation was $r=0.85$; $p<0.001$; $n=56$.

DISCUSSION

The volume of native sperm without gel fraction was 258.84 ± 103.41 mL, while the spermatozoa count was 251.66 ± 125.21 × 10^6/mL. The volume of ejaculate and the spermatozoa count determined in our experiment were suitable in the production of boars in conditions of intensive exploitation (Hafez and Hafez, 2000). Examining the effect of the breed on the sperm characteristics, some authors have found that the volume of ejaculate in Large Yorkshire was on average 238.0 ± 110.2 mL, which is in agreement with our findings (Gerfen et al., 1994).

Progressive spermatozoa motility in native semen was 76.34% ± 18.79%. In diluted sperm progressive motility was 71.54 ± 18.49%. Gerfen et al. (1994) had similar

results. They detected progressive mobility of 74.3 ± 1.8% in Large White breed.

The ability to inseminate was statistically significant in the boars up to at the age of two, i.e. comparing to the boars >2 years ($p<0.05$). Younger boars were with the findings ≤10% PPD ($p<0.01$). The number of live born piglets per litter was smaller in the boars witht the findings ≤10% PPD ($p<0.05$). The percent of secondary abnormal spermatozoide, with tail defomities, in the samples of native sperm were significanlty higher in the first group, i.e. in the boars younger than two ($p<0.05$).

Sonderman et al. (2008) compared the effects of breed and genetic lines of boars on sperm production and fertility. They noted that the ejaculates of genetically pure boars produce ejaculate with less spermatosoide (8 to 15 doses per ejaculate, ie. in a week) compared to the crossbreeds of same age. The aging increases semen volume of pure breed boars, but does not increase the number of sperm.

Comparing the progressive motility of shortly preserved semen (Sonderman et al., 2008) 5% better progressive sperm motility was found in Landrace breed after storage of 1 day compared to the crossbreed and other breeds.

Statistically significant correlations were found between the findings of protoplasmatic droplets on the tail of spermatozoa in native sperm and the number of live born piglets per litter: in Large White breed the mean correlation was ($r=-0.57$; $p<0.05$; $n=18$), in Duroc the average corellation was ($r=0.68$; $p<0.05$; $n=12$), and in other breeds no significant correlation was noted. In all 56 boars mean correlation was ($r=0.44$; $p=0.001$; $n=56$).

Highly significant positive correlations were found between the findings in native protoplazmatskih droplets in diluted semen: the Large White breed $r=0.73$; $p<0.001$; $n=18$; in Swedish Landrace $r=0.95$; $p<0.0001$; $n=11$; in Duroc $r=0.82$; $p<0.01$; $n=12$; in German Landrace $r=0.96$; $p<0.01$; $n=6$; in crossbreeds $r=0.95$; $p<0.001$; $n=9$. In all 56 boars the correlation was $r=0.85$; $p<0.001$; $n=56$.

Shortly after the first sperm is collected, terminal boar lines (hybrids and purebreds) usually produce usable ejaculate. They are usually produce weak sperm - 8 and 15 doses (motile sperm per dose - 2.5×10^9) are produced, but they meet the minimum criteria regarding the concentration and quantity. The maternal line boars seem to lag for about 2-8 weeks, before they begin to meet the minimum criteria of concentration and sperm count. The factors regarding the differences between the breeds should be taken into accounts in the centers in their decision-making processes, in order to ensure adequate quality of boars, and to meet the satisfaction of customers (Sonderman et al., 2008).

A disparity in the ability to inseminate was noted and it ranged from 29.05% to 91.94%. The differences were also apparent in the average number of farrowed and weaned pigs.

The sperm of "stable boars", containing up to 10% of abnormal sperm (AS) should be used, especially for the elite purebred herds. The boars with high AS level should be timely excluded, as recommended by Cevoski (Cerovski et al., 2005). Prolonged maintainance of initial AS content probably is an evidence of hereditary phenomenon. Co-existence of different sperm subpopulations within the mammalian ejaculate is nowadays widely accepted by the scientists (Peña et al., 2005).

Sperm with morphological abnormalities are an indication of a disorder in spermatogenesis and maturation,but they may also occur during the handling. When a larger number of spermatozoa in the ejaculate show abnormal morphology, sperm vitality,

which is considered normal, comes into question, because both normal and abnormal spermatozoids, present in the given ejaculate, undergo spermatogenesis, maturation, and are handled at the same time. The evaluation of sperm morphology, therefore, should be primarily used as a parameter in the control of ejaculate quality. Having this in mind, the author recommends using only that ejaculate that has <15% proximal and distal cytoplasmic droplets. The sperm from boars that produce unacceptable ejaculate should be taken once a week. If there is no improvement in the quality of the ejaculate within 3 months, the animal should be excluded from the breeding program (Althouse, 1998).

Waberski et al. (1994) recommend that the content of spermatozoa with protoplasmic drop should not exceed 15%, especially when for insemination diluted and preserved semen is used, which is a common practice nowadays. In the Czech Republic the applicability of boar semen insemination is limited to findingof AS to 25%.

Bonet et al. (1991) found out that the frequency of ejaculation affects sperm quality and fertility of boars. Sperm motility decreased 76.3% in the first group of boars, and 35.2% in the boars from the second II ($p<0.01$).

1) The percentage of spermatozoa with proximal droplet increased from 3.5% in the first group of boars to 67.4% for boars in the second group ($p <0.01$).
2) The percentage of sperm with distal droplets decreased from 25.3% in boars in the first group to 1.2% in boars second group ($p <0.01$).
3) The percentage of mature spermatozoa decreased from 68.9% in the first group, to 25.1% for the boars from the second group ($p <0.01$).
4) The percentage of abnormal sperm increased from 1.6%, in the first group of boars, to 6.7% in the second group of boars ($p <0.01$). The number of spermatosoa with twisted or folded tail is much biggee in the ejaculate of the boars in the second group than in the first group. Fertility is reduced from 73.5% in the boars from the first group to 7.7% in the boars from the second group ($p <0.01$).

Eliasson (2010) points out that for the researchers in human medicine, involved in assisted reproductive technology (ART), it is important that the methods are easy to learn and inexpensive to perform. It is stated that spermatozoa morphology may be more sensitive instrument for measuring reproductive health and stress of testis (Menkveld et al., 2011). Measurement and evaluation of spermatozoa morphology remains very important tool in diagnosing of male potential fertility and in making decision for treating the boars with infertility problems, especially if it is known that reduction of dose volume and sperm number per dose, lead to a decrease in sows fertility, both after intracervical, and after intrauterine insemination (Stančić et al., 2010). Genetically determined morphological abnormalities in sperm are caused by stress (physiological, mental, and caused by the conditions in the environment), which are reversible, and it is possibility to stop that stress.

Johnson, et al. (1981) carried out a field survey on 36 farms in the Netherlands to compare the fertilizing capacity of fresh and frozen and thawed boar sperm. Four hundred and fifty-one sows were artificially inseminated with sperm that was frozen and thawed according to the Beltsvils method or diluted in Kiev extender and inseminated on the day of collection. Twelve boars were Dutch Landrace and Dutch Large White. Farrowing rate, total number of piglets per litter and number of live born pigs per litter was higher ($P<0.0001$) for the sows inseminated with fresh sperm, than in the sows inseminated with frozen and thawed sperm (79.1%, 10.6 and 9.9 vs 47.0%, 7.4 and 7.1, respectively)

Rill (1989) states that a combination of these factors, and improvements in application techniques of doses, can be achieved by significant increase in the number of doses produced per ejaculate. Currently 2 to 3 × 10^9 spz / dose is applied for insemination of sows, but the numer of spermatozoa population must be drastically reduced in the near future.

CONCLUSION

Based on the results in the present study, it can be concluded:

1) The abbility to inseminate, and the findings of secondary abnormal spermatozoa, was considerably lower in the the boars in the first two years, comparing to boars > 2 years (74.32% - 62.82% = 11.50%, t-test, $p < 0.05$; *)
2) Statistically significant correlations were found between the findings protoplazmatic droplets (PPKD) in the tail of spermatozoa in native semen and the number of piglets born alive per litter, ($r = 0.44$, $p = 0.001$, $n = 56$). In Large White breed there was a medium correlation r=–0,57; p<0,05; n=18), in Duroc it was medium (r=0,68; p<0,05; n=12), but in other breeds there were sno significant corelations.
3) Highly significant positive correlations were found between the findings of protoplazmatic droplets in native and diluted semen ($r = 0.85$, p<0,001; n=56). In Large White r=0,73; p<0,001; n=18; in Swedish Landrace r=0,95; p<0,0001; n=11; in Duroc r=0,82; p<0,01; n=12; in German Landrace r=0,96; p<0,01; n=6; in crossbreed r=0,95; p<0,001; n=9. This was the correlation in all 56 boars.
4) The finding of cytoplasmic droplets on sperm tail was very stable and relatively easy to detect. Control of sperm quality has to be implemented as a tool in selection.

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MORFOLOŠKA ANALIZA SPERMATOZOIDA NERASTOVA PO UZRASTU I RASAMA

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Izvod

Izvršen je citološko-morfološki pregled sperme 56 nerastova, sa 12 imanja, po 3-7 iz svakog gazdinstva. U čistoj rasi su: Veliki jorkšir (VJ, n=18); Švedski landras (ŠL, n=11); Durok (OA, n=12); Nemački landras (NL, n=6); petu grupu su činili melezi (OST, n=9). Sperma je obojena eozin-nigrozinom u jednom koraku. Prema nalazu spermatozoida sa protoplazmatskim kapljicama (PPK), nerastovi su podeljeni na grupu sa ≤ 10% PPK i grupu sa > 10% PPK. Analiziran je uticaj nalaza PPK na broj živo oprašenih prasadi u leglu i međusobna povezanost nalaza PPK i nalaza živih spermatozoida sa intaktnim akrozomom (ŽIA), odnosno normalnim akrozomalnim rubom (NAR). Oprasivost je bila statistički značajno manja kao i nalaz sekundarno abnormalnih spermatozoida, sa deformitetima repa, kod nerastova u uzrastu do dve godine (≤ 2), u odnosu na nerastove sa(>2) godine (74,32% - 62,82%=11,50%, t-test, $p<0,05$). Statistički značajne korelacije su utvrđene između nalaza protoplazmatskih kapljica (PPK) na repu spermatozoida u nativnoj spermi i broja živorođene prasadi u leglu, ($r=0,44$; $p=0,001$; $n=56$). Kod rase Veliki jorkšir je srednja korelacija ($r=-0,57$; $p<0,05$; $n=18$), kod rase Durok srednja korelacija ($r=0,68$; $p<0,05$; $n=12$), a kod ostalih rasa nije bilo signifikantnih korelacija. Nalaz citoplazmatskih kapljica na repu spermatozoida nerasta je vrlo postojan i relativno lako se ustanovljava. Treba da se uvede u praksu kao parameter u kontroli kvaliteta sperme i kao selekcijski parametar.

Ključne reči: morfologija, spermatozoid, rasa, uzrast, nerast.

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