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Effect of three extenders on the motility and morphological characteristics of spermatozoa in diluted Muscovy semen stored at 4°C up to 120 hours

Vasko Gerzilov[1*] and Madlena Andreeva[2,3]

[1]*Agricultural University, Department of Animal Science, 4000 Plovdiv, Bulgaria*
[2]*Bulgarian Academy of Sciences, Institute of Biology and Immunology of Reproduction "Acad. Kiril Bratanov", 1113 Sofia, Bulgaria*
[3]*Bulgarian Academy of Sciences, Institute of Neurobiology, 1113, Sofia, Bulgaria*
[*]*Corresponding author:* v_gerzilov@abv.bg

Abstract

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The present study evaluated the total and progressive motility, as well as sperm morphological characteristics of diluted semen with three different extenders (1:3 v/v) and stored at 4°C for 3, 6, 24, 30, 48, 54, 72, 78, 96, 120 h. Total (TSM) and progressive mobility (PSM) gradually decrease with prolonging storage time. Up to the 30 h of sperm storage the sperm maintain high TSM – over 80% in semen diluted with IMV Canadyl and AU extenders and over 68% when diluted with HIA-1 extenders. With increasing *in vitro* sperm storage time, PSM and rapid sperm cells decrease significantly after the 3rd h, while non-progressive motility increases until the 48th h, after which it also begins to decrease. For velocity parameters VCL, VAP, VSL are reported a more rapid trend of decrease between 3 and 30 h of cold storage, after which the decline is smoother ($P < 0.05$). As storage time is extended, the pathological forms of the sperm cells increase, with those affecting the head of the sperm cells predominating – crooked-neck and swelling forms.

Keywords: Muscovy duck; semen; extender; liquid storage; sperm motility; sperm morphology

Introduction

In the last few years, Bulgaria has firmly positioned itself as one of the three countries in the world – along with France and Hungary – with the largest production of *foie gras* obtained from mule ducks.

Commercial production of the intergeneric hybrid mule ducks relies entirely on artificial insemination (AI), as the observed ethological differences between both parental forms Muscovy drake (*Cairina moschata*) and the common duck (*Anas platyrhynchos*) that they show rare mating affinity. In AI, egg fertility rates significantly increase and vary between 65 and 80% with two inseminations weekly (Gerzilov, 2003; Marie-Etancelin et al., 2008). It has been demonstrated that sperm cell concentration in Muscovy ejaculates is high, i.e. the semen is viscous (Gerzilov, 2011; Łukaszewicz et al., 2020 a, b). In AI, diluted semen is increasingly used in order to increase the number of inseminated birds. According to Penfold et al. (2001), cold storage of drake semen provides an effective means of short-term preservation with no loss of fertility.

The prolonged storage time of diluted and cooled semen allows it to be transported to remote farms and used to inseminate large groups of females and to facilitate the utilization of sperm from superior male birds (Siudzińska & Łukaszewicz, 2008). However, on the other hand, it exercises some

adverse effects on sperm motility and viability and causes negative changes in the structure and function of sperm cells.

This study aimed to evaluate the changes in the sperm motility and morphological characteristics of sperm cells in diluted muscovy semen with three different extenders, stored at 4°C during 3 to 120 h.

Material and Methods

Birds, semen collection and housing

The ejaculates were sampled from 10 Muscovy drakes (autosexing line CF 80, originating from Grimaud Frères Sélection, France) during their first reproductive period. The males were clinically healthy, kept individually in spacious metal cages with dimensions 0.6×0.8×0.6 m, placed in a semi-open shed in natural light situated in the Poultry Division at the Agricultural University of Plovdiv. The ejaculates were collected individually twice a week from May to July by placing a female (teaser method) in the male's cage using an artificial vagina – by the method of Tan (1980) modified by Gerzilov (2000). The artificial vagina consisted of a rubber muff and a measuring test-tube.

During the reproductive period, the birds were fed with a commercial pelleted mixture for breeding drakes comprising: 11.5 MJ/kg metabolic energy, 15.7% crude protein, 4.5% crude fibres, 2.1% crude fats, 1.03% calcium, 0.75% total phosphorus, 0.8% L-lysine and 0.42% DL-methionine + cysteine. The daily ration was 200-230 g per bird. The intake of water was provided *ad libitum.*

Semen processing

Only ejaculates of good quality were mixed, i.e. color – pearly-white; purity – free of any contamination with cloacal products; volume – above 0.3 mL; sperm mobility – above 70% under light microscope Nikon Alphaphot-2YS2 (10×40). The ejaculates were pooled to avoid the effects of individual differences among males. No less than 10 pooled semen samples were used for the experiments.

The pooled semen sample was thoroughly mixed with the use of an automatic pipette, divided into three equal aliquots and diluted to semen: extender ratio of 1:3 (v/v) with IMV Canadyl, HIA-1 and AU extenders, respectively.

The IMV Canadyl extender was a commercial product from IMV-Technologies, France (IMV–Technologies, 2021).

Both the HIA-1 and AU extenders were developed by Gerzilov (2003).

The HIA-1 extender consists of 0.25 g D–glucose, 0.25 g D–fructose, 0.07 g saccharose, 0.50 g sodium citrate, 0.9 g sodium chloride, and 100 mL double distilled water. The osmolarity was 290 mOsmol/kg and pH – 7.00.

The AU extender consisted of the following components: 0.40 g of D–glucose, 0.80 g of D–fructose, 0.80 g of sugar, 0.90 g of sodium citrate, 0.84 g of sodium glutamate, 0.40 of glycocoll, 0.04 g of ethylenediaminetetraacetic acid disodium salt dihydrate, and with 100 mL double distilled water. The osmolarity was 320 mOsmol/kg (pH 7.00).

The diluted semen samples were places in an electric cool box car refrigerator (with a capacity of 25 L) at 4-5°C and transported from the Poultry division at the Agricultural University of Plovdiv to the Institute of Biology and Immunology of Reproduction "Acad. Kiril Bratanov" at the Bulgarian Academy of Sciences, Sofia for sperm analysis and evaluation.

Sperm analysis to determine sperm motility and velocity parameters

Sperm motility and morphological status were examined with a computerized sperm analyzer CASA (Sperm Class Analyzer [SCA] 5.0. Microptic, Barcelona, Spain) using the manufacturer's settings for the animal species. The studies were performed at 3, 6, 24, 30, 48, 54, 72, 78, 96 and 120 hours, with the ejaculates stored in refrigerated conditions at 4°C.

Sperm samples were loaded into a Leja 20 chamber (Leja Products B.V., Nieuw-Vennep, The Netherlands) and examined using a hot-stage microscope (Nikon, Tokyo, Japan). In addition to the total number of motile sperm, the SCA software also measured the speed of movement, the width of the sperm head trajectory, the frequency of change in the direction of the sperm head, and the kinematics for each sperm analyzed.

The following velocity parameters were recorded:

– Curvilinear velocity (VCL, μm/s – the average path velocity of the sperm head along its actual trajectory);

– Straight-line velocity (VSL, μm/s – the average path velocity of the sperm head along a straight line from its first to its last position);

– Average path velocity (VAP, μm/s – the average velocity of the sperm head along its average trajectory);

– Percentage of linearity (LIN, %) – the percentage of the VSL from the VCL;

– Percentage of straightness (STR, %) – the percentage of the VSL from the VAP.

– Percentage of the wobble (WOB, %) – reflects the measure of oscillation of the actual path about the average path.

– Amplitude of lateral head displacement (ALH, μm) – defined as twice the maximum displacement of a sperm head from its fitted moving axis in a track segment.

– Beat cross frequency (BCF, Hz) – defined as the frequency at which the sperm head moves across the middle plane of the "straightened" trajectory.

Sperm analysis to determine the morphological status of sperm

A solution prepared at the Agricultural University of Plovdiv was used for the staining of the morphological smears. To the 2 mL of staining solution (1.6 g eosin + 6 g nigrosin dissolved in 100 mL 2.9% sodium citrate solution) 20 µl of diluted semen were added with a micropipette in a dropwise manner and the mix was incubated in an Eppendorf vial for 2 min before being spread on a microscope slide.

The stained microscopy slide was examined using the software program of a computer sperm analyzer (SCA Microptic, Barcelona, Spain) at 100× magnification with a light microscope with an immersion lens using cedar oil according to the requirements of Microptic Systems (Spain) and recorded on the software of SCA, Morphology. The semen was differentiated sequentially in several visual fields – 200 spermatozoa were counted. The study was performed at 3h, 6h, 24h, 30h, 48h, 54h, 72h, 78h, 96 h and 120 h.

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) followed by t-test to determine the level of significance among mean values. The results are presented as mean ± SEM. The significant differences among mean values were determined by Duncan's multiple range test at significance level $P < 0.05$.

Results and Discussion

The results showed that as storage time increases in cooled and diluted semen, sperm total motility (TSM) and sperm progressive motility (PSM) decrease (Table 1). TSM remains high (over 80%) in the diluted semen with IMV Canadyl and AU extenders for up to 24 h of storage. TSM in diluted semen with HIA-1 extender is high for up to 6 h (over 87%). The results show that TSM in the different sperm samples varies in a very wide range, due to which, despite its decrease up to 30 h vs. 3 h, no proven differences were found ($P > 0.05$). The decrease in PSM is significant at 24 h vs. 3 h ($P < 0.05$). Diluted semen with these extenders maintained high PSM – over 62% up to 3 h of storage. In addition, TSM is higher for up to 120 h in cold-stored and diluted semen with the two extenders AU and IMV Canadyl vs. HIA-1 – with a significant difference ($P < 0.05$).

At 120 h extended period of storage time, the highest TSM was found in diluted semen with AU extender (42.55±3.51%) and the lowest with HIA-1 extender (21.75±3.34%) – $P < 0.05$. Łukaszewicz et al. (2020b) find that the semen extenders (Schramm's extender and Watanabe's extender) and storage time (0 h, 3 h and 6 h) caused

Table 1. Sperm motility of Muscovy drake spermatozoa in diluted semen stored at 4°C for up to 120 h (means ±SEM)

Time of storage, hours	Total sperm motility, %			Progressive sperm motility, %			Non-progressive sperm motility, %		
	IMV Canadyl	HIA-1	AU	IMV Canadyl	HIA-1	AU	IMV Canadyl	HIA-1	AU
3	97.82±1.88^{a}	99.02±0.83^{a}	99.64±0.23^{a}	65.73±12.49^{a}	62.62±12.64^{a}	66.20±10.55^{a}	32.09±11.21^{bc}	36.40±12.13	33.63±10.50^{b}
6	90.86±2.81^{a}	87.41±4.56^{a}	91.08±2,76^{ab}	36.30±7.18^{ab}	35.73±7.46^{ab}	40.25±6.37^{ab}	54.56±4.78^{abc}	51.68±5.84	51.37±5.31^{ab}
24	82.35±9.04^{ab}	68.6±8.26^{ab}	87.29±5.26^{abc}	16.18±5.78^{b}	16.76±9.48^{b}	17.97±4.70^{bc}	64.85±3.43^{abcB}	51.74±5.40^{A}	69.92±3.42^{aB}
30	62.29±5.90^{ab}	65.78±8.25^{ab}	84.00±3.90^{abc}	13.69±3.35^{b}	6.91±3.36^{b}	12.09±3.27^{bc}	68.65±5.08^{abcAB}	58.86±6.10^{A}	71.90±2.67^{aB}
48	56.44±9.62^{bc}	64.65±16.87^{ab}	83.09±8.00^{abc}	9.66±3.42^{b}	7.71±3.22^{b}	14.68±3.40^{bc}	52.63±6.22^{abc}	56.94±13.74	68.42±5.75^{a}
54	52.36±4.11$^{bc,d\,A}$	47.05±10.59$^{bcA.}$	73.89±6.56^{abcdB}	7.14±1.13^{b}	3.25±1.43^{b}	11.13±4.38^{bc}	49,30±4.09^{abcAB}	43,71±9.21^{A}	62.76±4.32^{abB}
72	47.43±5.04^{cdA}	40.31±11.98^{bcA}	72.41±4.88^{bcdB}	5.47±0.87^{b}	3.76±1.74^{b}	7.84±2.32^{bc}	47.45±4.11^{abcA}	36.54±10.23^{A}	64.58±3.62^{abB}
78	46.93±10.47^{cdA}	37.09±6.32^{bcA}	63.11±8.35^{cdeB}	5.20±1.56^{bB}	3.34±0.37^{bA}	6.21±1.63^{cB}	42.23±9.00^{abcA}	35.22±5.97^{A}	56.89±6.89^{abB}
96	30.36±2.88^{deA}	36.12±2.51^{bcA}	55.27±5.95^{deB}	4.86±0.65^{bB}	1.87±0.61^{bA}	5.10±1.59^{cB}	40.82±2.43^{abcA}	33.57±1.87^{A}	50.17±4.51^{abB}
120	30.36±4.66$^{deA.}$	21.75±3.34^{eA}	42.55±3.51^{eB}	1.46±0.14^{b}	1.80±0.42^{b}	2.25±0.66^{d}	28.90±4.54^{cA}	21.61±4.59^{A}	40.30±3.98^{abB}

Note: Average values in the respective column within one extender with different lowercase letters, differ significantly: a, b…e – $P<0.05$. The significant difference has a smaller value when the letters occupy a closer position in the alphabet. The presence of identical letters between two indicators means that there is no significant difference. Average values in the respective line, within one storage time (hour), with different uppercase letters, differ significantly: A and B – $P < 0.05$

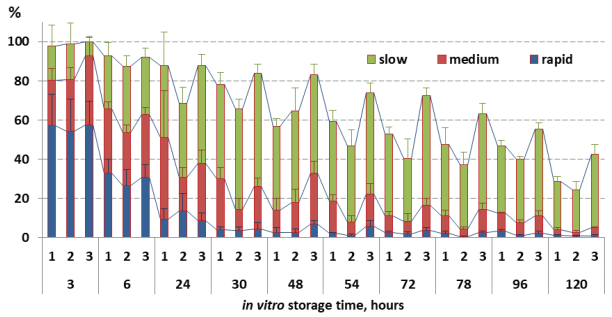
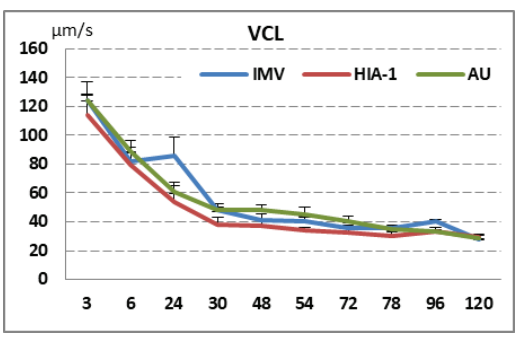


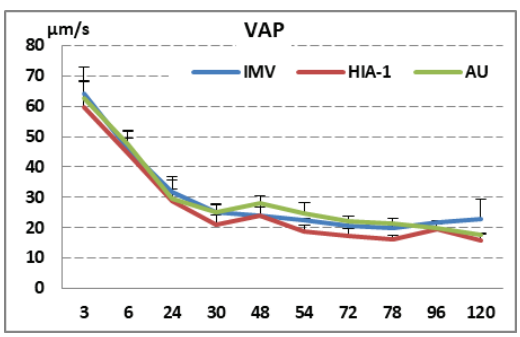
Fig. 1. Velocity motion of spermatozoa in semen diluted with (1) – IMV Canadyl, (2) – HIA-1, (3) – AU extender

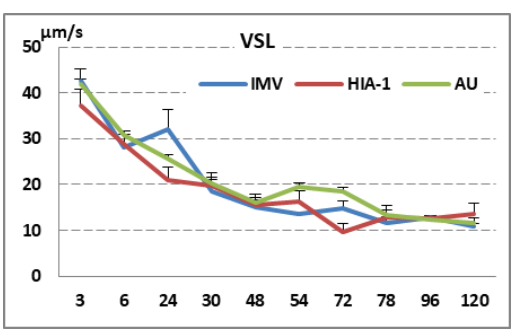
significant changes in sperm morphology in Muscovy semen stored at 4°C. Similar studies of rooster semen cold-stored for 3h, 6h, 24 h and 48h affect sperm morphology, livability, acrosomal integrity, plasma membrane integrity and motility (Siudzińska & Łukaszewicz, 2008; Rakha et al., 2016). In general, well-selected extenders are essential during in vitro storage of sperm cells.

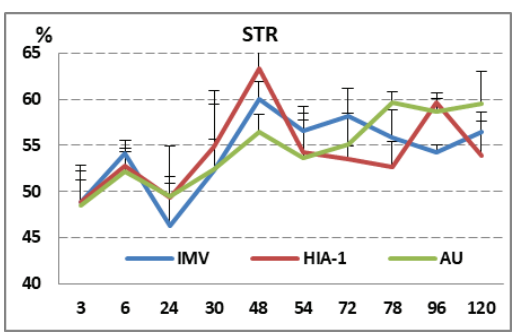
Total sperm motility (TSM) is the sum of rapid, medium and slow motility of spermatozoa. The velocity motion of the sperm in the three extenders is shown in Figure 1. Rapid spermatozoa predominate at 3 h of storage – AU (57.66 ± 10.76%), IMV Canadyl (57.18 ± 14.79) and HIA-1 extender (54.30 ± 14.86%). With increased storage time, rapid spermatozoa decreased and slow spermatozoa increased. At 24 hours versus 3 hours, rapid spermatozoa decreased significantly ($P < 0.05$). Velocity motion of spermatozoa is an important indicator and it affects the fertilization of eggs.

The CASA is an important method for examining the kinematic parameters of semen. Kinematic evaluation allows each individual sperm cell to be determined according to the trajectories obtained from the analysis (Luna et al., 2015). In accordance with storage time, the changes in the velocity parameters VCL, VAP and VSL are similar regardless of the used extender (Figure 2). A more rapid downward trend is reported between 3 and 30 h of cold storage, after which the decline is smoother ($P < 0.05$). At the respective time, insignificant higher levels of VCL, VAP and VSL were reported in the semen diluted with IMV Canadyl and AU, and lower in the semen diluted with HIA-1 extender ($P>0.05$). We share the findings of Mortiner (2000) that VAP is similar to VSL when sperm perform a linear motion with very little lateral deviation. The three velocity parameters LIN, STR and WOB as well as the other two ALH and BCF compared with each other, show a similar trend of change from 3 to 120 h. The velocity parameters ALH and BCF decrease with increasing *in vitro* storage time. In general, the results for the velocity parameters of sperm cells depending on the duration of their *in vitro* storage are consistent with those obtained from a previous study by Gerzilov et al. (2011). The results confirm the positive correlation established by Pilane and Mapeka (2021) between motility and VCL, VAP and VSL in frozen-thawed rooster semen.









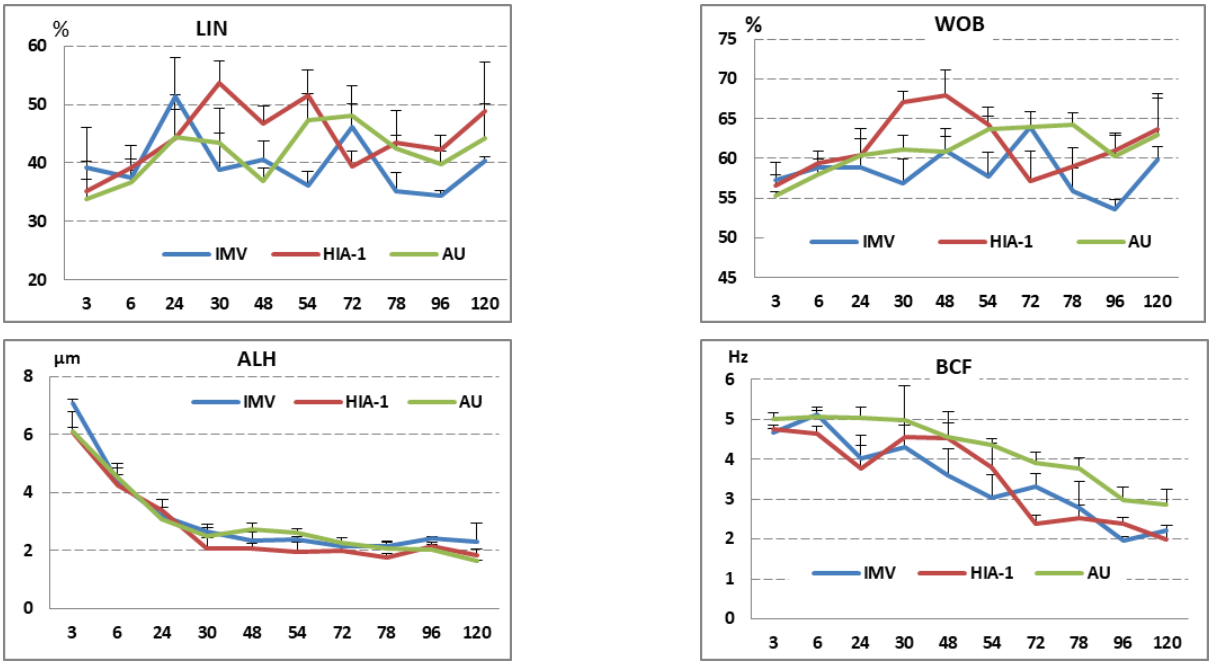


Fig. 2. Velocity parameters of Muscovy drake spermatozoa in diluted semen stored up to 120 h at 4°C

Table 2. Morphological characteristics of Muscovy drake spermatozoa in diluted semen stored up to 120 h at 4°C (means ±SEM), in % from live sperm cells

Time of storage, hour	Extender	Normal spermatozoa	Pathlogical (abnormal) spermatozoa			
			Changes in head		Midpiece abnormality	Tail abnormality
			Crooked-neck forms	Swelling forms		
	IMV Canadyl	95.33±0.63^{a}	1.75±0.34	1.50±0.35^{a}	0.50±0.20^{ab}	0.92±0.24
3	HIA-1	92.92±0.55^{b}	2.42±0.27	2.42±0.20^{b}	1.00±0.26^{a}	1.25±0.26
	AU	94.50±0.44^{ab}	2.08±0.30	2.33±0.30^{b}	0.33±0.15^{b}	0.75±0.26
	IMV Canadyl	90.17±0.67	3.50±0.27	4.08±0.42	0.58±0.20	1.67±0.32
6	HIA-1	89.17±0.98	4.33±0.52	3.83±0.51	1.00±0.26	1.67±0.27
	AU	89.83±1.09	4.08±0.40	3.50±0.52	1.08±0.35	1.50±0.35
	IMV	82.42±0.90	5.42±0.44^{a}	5.58±0.79	1.75±0.43	4.83±0.56
24	HIA-1	79.92±0.97	6.92±0.65^{ab}	6.83±0.59	1.67±0.30	4.67±0.41
	AU	81.33±0.73	7.17±0.63^{b}	6.08±0.83	1.50±0.30	3.92±0.66
	IMV Canadyl	70.50±0.73^{a}	8.58±0.73	8.58±0.57	4.08±0.45	7.83±0.56
48	HIA-1	66.75±1.36^{b}	9.92±0.74	9.50±0.80	4.92±0.52	8.92±0.71
	AU	69.17±1.33^{ab}	8.50±0.57	9.33±0.67	4.08±0.54	8.92±0.65
	IMV Canadyl	52.92±1.23^{a}	11.17±0.79^{a}	12.00±1.18^{a}	10.58±0.82^{a}	13.33±1.07^{a}
72	HIA-1	41.25±0.92^{b}	14.50±0.95^{b}	14.58±0.73^{b}	12.83±0.63^{b}	16.83±0.65^{b}
	AU	46.17±0.50^{c}	13.25±0.67^{ab}	14.25±0.52^{ab}	9.58±0.44^{a}	16.75±0.56^{b}
	IMV Canadyl	34.17±1.19^{a}	17.17±0.69	18.75±0.78	13.17±0.63	16.75±0.80
96	HIA-1	26.92±1.03^{b}	18.58±0.51	22.25±0.77	13.50±0.65	18.75±1.25
	AU	30.67±0.82^{c}	18.25±0.48	18.17±0.71	14.17±0.67	18.75±0.95
	IMV Canadyl	13.25±0.84	20.50±0.64	24.83±1.07	17.42±0.54	24.00±1.31^{a}
120	HIA-1	8.83±0.40	19.67±0.73	24.67±0.96	18.00±0.72	28.83±0.75^{b}
	AU	10.92±0.42	20.58±0.81	23.42±1.04	18.00±0.45	27.08±0.49^{b}

Note: Average values in the respective column, within one period of storage time, with different lowercase letters, differ significantly: a, b – $P < 0.05$

Morphological assessment of spermatozoa is essential for assessing semen quality. Numerous studies have found a positive correlation between egg fertility and the percentage of sperm cells with normal morphology (Bakst, 1980; Blesbois and De Reviers, 1992; Blesbois & Brillard, 2007).

With increasing storage time, we found a decrease in the percentage of normal live spermatozoa. Within the respective storage hours, the highest percentage of normal spermatozoa was found in semen diluted with IMV Canadyl, and the lowest in semen diluted with HIA-1 – differences were found at 3, 48, 72 and 96 hours ($P < 0.05$) (Table 2). By the 48 h of storage of the diluted semen, normal sperm cells predominate ie. they retain their morphofunctional integrity. The analysis of the pathological forms showed that the most common deformities are in the tail and head of sperm cells. Our results are consistent with those of Łukaszewicz et al. (2020 b).

The different disabilities we observed are presented in Figures 3-8. The decrease in the percentage of normal spermatozoa with increased storage time is due to ongoing met-

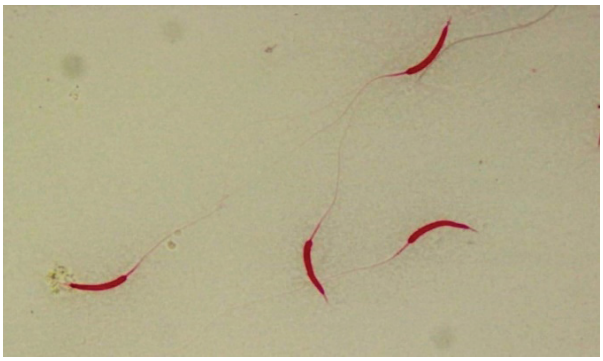


Fig. 3. Normal live spermatozoa

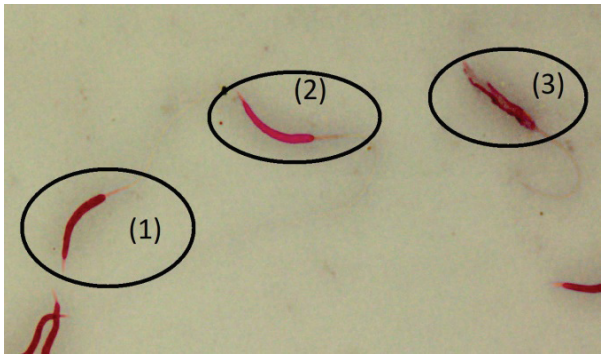


Fig. 4. Normal live spermatozoon (1); dead spermatozoon (2); dead spermatozoon with damage plasma membrane (3)

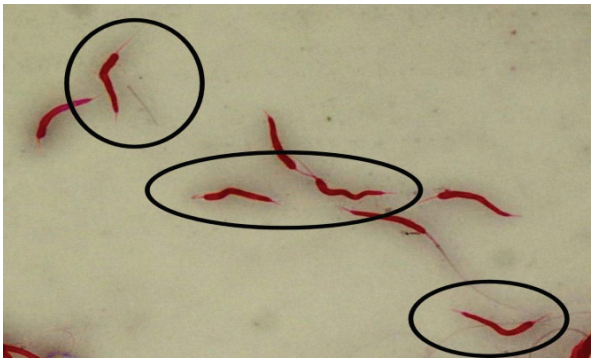


Fig. 5. Crooked-neck form

Fig. 6. Swelling form

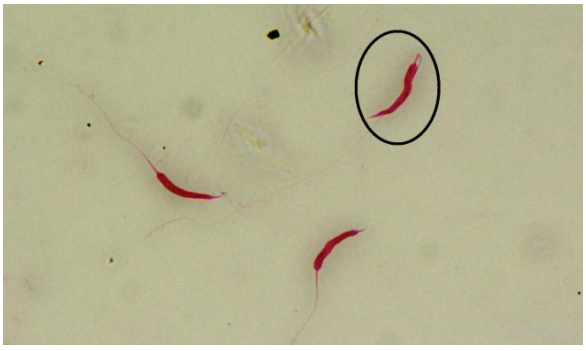


Fig. 7. Midpiece damage

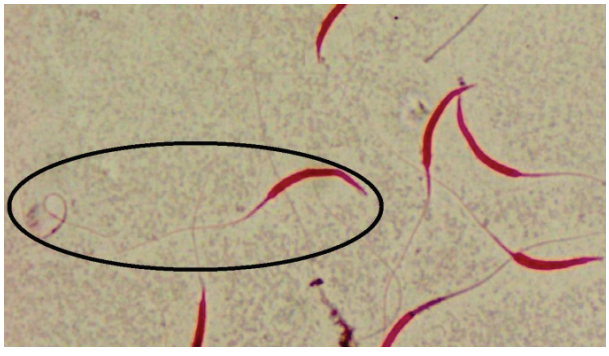


Fig. 8. Tail damage

abolic processes including sperm cell oxygen uptake (Atanasov et al., 2007), lipid content and peroxidation (Partyka et al., 2012), changes in pH and osmolarity (Clarke et al., 1984; Donoghue & Wishart, 2000; Siudzińska & Łukaszewicz, 2008; Sarkar, 2020).

The most common damages are localized in the head of the sperm cells – crooked-neck or swelling forms. The most likely reason for this is the higher lability and permeability of the cytoplasmic membrane (plasmalemma) in the area of the head and neck. We tend to accept the opinion of some authors (Saeki, 1960; Bakst, 1980; Klarke et al., 1986; Donoghue et al., 1996) that the appearance of the "crooked-neck" or "swelling" form is due to the change in the osmolarity of the diluted semen during storage (Figures 3, 4, 5, 6, 7, 8).

Conclusion

Total motility (TSM) is preserved relatively highly until the 30th h, while progressive motility (PSM), as well as sperm velocity parameters up to 3rd h of cooled storage (4°C) of diluted semen in all three extenders. Normal sperm predominate over pathological forms for up to 48 h of diluted semen. All the three investigated extenders are suitable for short-term in vitro storage of Muscovy semen.

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