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Influence of Selected Extenders for Liquid Storage at 4ºC of Breeding Chinchilla (*Chinchilla lanigera*) Semen on Sperm DNA Integrity*

Piotr NIEDBAŁA, Olga SZELESZCZUK, Marta KUCHTA-GŁADYSZ, Maria JONECZEK, Małgorzata DOBRZYŃSKA, and Dorota MAJ

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The influence of two commercial and two laboratory oriented extenders on survival rate and DNA integrity of chinchilla (*Chinchilla lanigera*) sperm was determined during liquid storage. Semen was collected using an electroejaculator from 6 adult male chinchillas. Ejaculates (n=16) were diluted with extenders to obtain a concentration of 40×10^3 sperm/5 µl. After dilution the semen samples were stored at 4ºC. The percent motility, progressive motility, and morphology were assessed conventionally, whereas DNA integrity was evaluated by Single Cell Gel Electrophoresis (comet) assay at 0 (just after dilution), 24, 48 and 72 h. Conventional assessment of sperm quality showed that commercial extenders are characterized by the lowest sperm survival parameters out of the investigated extenders. In commercial extenders spermatozoa lost their capacity for progressive motility compared to laboratory extenders. After 24 h storage, from 21.67% to 30% of motile sperms were observed in commercial extender whereas total sperm motility was 63.33% (41.67% with progressive motility) in samples in which stallion semen extender was used. After 72 h storage, 10% of sperm were motile in stallion semen extender while no sperm movement was observed in tubes containing the commercial extender. Furthermore, a lower percentage of damaged spermatozoa in laboratory oriented extenders was demonstrated. It was also stated that along with the extended time of semen storage at 4ºC, commercial extenders lost their protective action. An analysis of DNA content in the heads of sperm cells and tail moment (TM) showed that the most useful extender for liquid preservation of chinchilla semen was the extender for stallions.

Key words: Chinchilla, sperm, extender, comet test.

Piotr NIEDBAŁA, University of Agriculture in Kraków, Institute of Animal Sciences, Department of Swine and Small Animal Breeding, Mickiewicza 24/28, 30-059 Kraków, Poland.
E-mail: p.niedbala@ur.krakow.pl
Olga SZELESZCZUK, Marta KUCHTA-GŁADYSZ, Maria JONECZEK, University of Agriculture in Kraków, Institute of Veterinary Science, Department of Animal Anatomy, Mickiewicza 24/28, 30-059 Kraków, Poland.
E-mail: rzszeles@cyf-kr.edu.pl
m.kuchta-gladysz@ur.krakow.pl
Małgorzata DOBRZYŃSKA, National Institute of Public Health – National Institute of Hygiene, Department of Radiation, Hygiene and Radiobiology, Chocimska 24, 00-791 Warszawa, Poland.
E-mail: mdobrzynska@pzh.gov.pl
Dorota MAJ, University of Agriculture in Kraków, Department of Genetic and Animal Breeding, Mickiewicza 24/28, 30-059 Kraków, Poland.
E-mail: d.maj@ur.krakow.pl

Chinchilla (*Chinchilla lanigera*) belongs to the Chinchillidae family and is native to South America. The organized breeding of chinchilla was launched in 1923 in California. Chinchillas were imported to Poland in 1956 by Władysław and Elwira Rżewski. Since then, the breeding of chinchillas has been an important branch of agricultural production. Moreover, these animals are also commonly kept as pets (BARABASZ 2008).

It is particularly important to continue studies on the improvement of semen preservation in a wide range of animal species. Such experiments are

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conducted on a continuous basis. Efficient storage of chinchilla semen in a liquid state with the use of different diluents has practical significance. It will be particularly important to use extenders already applied in semen preservation of other species. Such semen can be quickly used either in commercial farms or in hobby breeding and may increase the number of wild chinchillas, and may thus contribute to the protection of critically endangered species (BUSSO *et al.* 2012).

Research on collecting, assessing and possible preservation of semen of breeding chinchilla (*Chinchilla lanigera*) has been conducted since 1967 (HEALEY & WEIR 1967) and continued by HEALEY and WEIR (1970), BARNABE *et al.* (1994), PONCE *et al.* (1998a), PONCE *et al.* (1998b), CARRASCOSA *et al.* (2001) NIEDBAŁA *et al*. (2010), PONZIO *et al.* (2011) NIEDBAŁA *et al*. (2012) and DOMINCHIN *et al.* (2014).

BUSSO *et al.* (2005a) worked out a method to obtain semen by electroejaculation under anaesthesia. The volume of semen taken from the chinchilla was comparatively small as it ranged from 20 to over 70 µl (BUSSO *et al*. 2005b), averaging between 20 µl (SZATKOWSKA *et al.* 1996) and 70 µl (NIEDBAŁA *et al.* 2009b). Slightly higher volumes (0.1-0.5 ml) were obtained by BARNABE *et al.* (1994), PONCE *et al.* (1998b) and SPOTORNO *et al.* (2004) from wild chinchillas in captivity. The concentration of sperm cells averaged $2145.9 \pm 365.3 \times 10^6 ml^{-1}$ (BUSSO *et al.* 2005b) and 4154×10^6 ml^{-1} (NIEDBAŁA *et al.* 2009b). The total number of sperm cells in the collected ejaculates ranged between 25 and over 100×10^6 sperm cells per ejaculate (BUSSO *et al.* 2005b). A considerably higher number of spermatozoa per sample (340×10^6 sperm cells) was obtained by NIEDBAŁA *et al.* (2009b).

Therefore, chinchilla semen obtained from males in assisted reproduction should be diluted before it is used. Extenders, which prolong the life of sperm cells, contain various buffering, nutritional and protective substances. They also contain cryoprotectants, which enable sperm preservation at low temperatures. These studies were carried out, among others, by BARNABE *et al*. (1994), PONCE *et al.* (1998a), PONCE *et al.* (1998b), and PONZIO *et al*. (2008). BARNABE *et al.* (1994) used a diluent with or without glycerol containing sodium citrate, egg yolk and fructose. According to PONCE *et al.* (1998a) and PONCE *et al.* (1998b), the most useful extender was that containing TES, Tris, sodium citrate, egg yolk, fructose and glycerol. Further research was carried by PONZIO *et al.* (2008) based on extender containing TES, Tris, sodium citrate, egg yolk, fructose but with glycerol or ethylene glycol as a cryoprotectant. Only CARRASCOSA *et al*. (2001) investigated the effect of the same diluents on the parameters of spermatozoa during their preservation in liquid state at 4° C. However, they used glycerol or ethylene glycol as cryoprotectant. Glycerol could also have a negative effect on sperm (JURKA 1996). The degree of the toxic effect of glycerol on sperm depends on its concentration and contact time with the sperms. Glycerol denatures the sperm membrane glycoprotein complex. This is manifested through an intensive release of cell enzyme proteins associated with plasmalemma or those located in acrosome and middle piece to the external environment ("dilution effect") (STRZEŻEK 1998).

As mentioned above, the addition of cryoprotectants to extenders is required during preservation of semen at low temperatures. Extenders without cryoprotectants were used for liquid preservation of semen from several animal species in cold storage at 4°C. These studies were conducted with the semen of stallions (PAGL *et al.* 2006; ZHANDI & GHADIMI 2014), rabbits (SARIÖZKAN *et al.* 2013; JOHINKE *et al.* 2014), dogs (PONGLOWHAPAN *et al.* 2004; VERSTEGEN *et al.* 2005; KASIMANICKAM *et al.* 2012), goats (SALVADOR *et al.* 2006), and even elephants (GRAHAM *et al.* 2004).

Research has shown that chromatin structure and DNA integrity have a substantial influence on the function of germ cells (SAKKAS *et al.* 2003; FRASER & STRZEŻEK 2004). BOGUCKA *et al*. (2006) stated that DNA integrity is a crucial index in determining suitability of sperm cells for *in vitro* fertilization, which can also have an influence on the number of pregnancies obtained and development of embryos by assisted reproductive techniques. Such methods, along with the standard method, also enable the ultrastructure of the sperm cell to be controlled.

One of these methods is the comet assay (SCGE, Single Cell Gel Electrophoresis assay) (BOGUCKA *et al*. 2006). It is a very sensitive and specific method which monitors the presence of breaks in the structure of the DNA chain of the sperm and somatic cells. The SCGE technique has been applied in the investigation of human sperm cells (BAUMGARTNER *et al*. 2009) as well as animal ones (LOVE *et al*. 2002; FRASER & STRZEŻEK 2004; DOBRZYŃSKA 2005; PRINOSILOVA *et al.* 2012).

The aim of this research was to determine the usability of selected extenders without cryoprotectants for liquid preservation of the semen of breeding chinchillas (*Chinchilla lanigera*), on the basis of the integrity of sperm DNA using the SCGE assay.

Material and Methods

Animals

Six sexually mature breeding chinchilla males, weighing 573 ± 49 g, were used in the study. Animals were individually housed in stainless steel standard cages (0.40 m wide, 0.50 m long, 0.34 m high) exposed to controlled temperature (18-20°C), natural fluctuations in photoperiod from the middle of March to the middle of September and modified (12L:12D) during the remaining period of the year.

Pelleted chinchilla food (RABA, Myślenice, Poland) and water were provided *ad libitum*, and a cube of compressed alfalfa was given twice weekly. A spoonful of talc powder was added to the sand bath (Sepiolite - SMG Spain) in each cage on a regular basis so that animals could perform a dust bath to keep their fur dry and uncompressed. All experiments were conducted in accordance with the EC Directive 86/609/EEC for animal experiments with approval of the First Local Ethical Committee on Animal Testing at the Jagiellonian University in Kraków – Act No. 15/2007 decision of 22nd February 2007 and decision of 10th March 2010.

Semen collection and evaluation

Semen from anaesthetized males was obtained by electroejaculation in the morning from 22nd February to 13th July 2011 at five-week intervals. Semen was collected five times from each male. The anaesthetic contained a mixture of Ketamine (Bioketon, BIOWET Puławy, Poland, up to 0.05 ml/kg body weight) and Xylazine (Sedazin, BIOWET Puławy, Poland, up to 0.1 ml/kg body weight) at a ratio of 1 to 2, according to the advised dosage (FEHR 2009). In order to calculate an appropriate dose of the pharmacological agent, animals were weighed on WPT-2 laboratory scales (RADWAG, Radom, Poland). Semen was collected using an electroejaculator. The semen was placed into Eppendorf tubes at room temperature (18-20°C).

Directly after ejaculation, semen was submitted to standard analysis encompassing semen volume, sperm motility and progressive motility, and sperm concentration (BIELAŃSKI 1977). Sperm morphology (viability) was evaluated by semen smears stained with 5% eosin (Sigma Aldrich) and 10% nigrosin (Sigma Aldrich) in water soluble under a light microscope at 1500 × immersion magnification (Bioval, Poland). Sperm concentration was measured in a Bürker chamber, and sperm motility was evaluated in a Blom chamber placed over a thermostatized plate (SEMIC Warszawa, Poland) to keep the sperm at 37°C during the evaluation. Assessments were performed under a light microscope at 200-400 × magnification (Bioval, Poland). For further analysis (as soon as it was possible – up to fifteen minutes after collection) the semen was diluted to a concentration of 40×10^3 sperms/5 µl, in two commercial (E-1 and E-2) and two lab oriented (E-3 and E-4) extenders: E-1 (SAFE CELL PLUS for boars, IMV-TECHNOLOGIES, France), E-2 (GALAP for rabbits, IMV-TECHNOLOGIES, France), E-3 for canine semen consisting of Tris-24.98 mM, Citric acid-8.85 mM, Fructose-6.94 mM and distilled water up to 100 ml (MORTON *et al.* 1989), E-4 for canine and equine semen consisting of Tris-19.81 mM, Citric acid-7.29 mM, Glucose-4.44 mM and distilled water up to 100 ml (PROVINCE *et al*. 1984). Benzylpenicillin (0.30 mM) and dihydrostreptomycin sulfate (0.17 mM) were added to the lab oriented diluents. Just after adding extenders (0 h) and in the diluted semen kept at a temperature of 4ºC through 24 h, 48 h and 72 h, motility, morphology, and DNA integrity of individual sperm cells were assessed.

SCGE procedure

The cells were briefly suspended on an object glass in a gel of 0.5% LMPA (Sigma Aldrich) between two layers of 0.5% NMPA (Sigma Aldrich). In order to remove proteins, a 24 h lysis of cells in an alkaline buffer (2.5 M NaCl, 100 mM Na_2EDTA, 0.4 M Tris-HCl, 1% Sodium N-Lauroyl Sarcosinate, 10% Triton X-100, 1% DMSO, pH = 10) was conducted. After a 20 min incubation in a strongly alkaline TBE buffer (10 N NaOH, 200 mM EDTA, pH≥13), electrophoresis was conducted (25 V, 300 mA, 20 min) (SINGH *et al.* 1988). Finally, the preparations were neutralized with 0.4 M Tris-HCl (Sigma Aldrich) and dyed with ethidium bromide (200 µg ml^{-1}).

Analysis of SCGE assay results

Sperm DNA integrity was assessed based on the percentage of nuclear DNA in the head of the comet and the value of the Tail Moment (TM) index. Analysis of preparations was made on a microscope with Zeiss epifluorescence, 400× magnification. To assess the degree of damage to the sperm DNA in semen just after dilution and chilling, one gel preparation was made for particular samples. In each preparation 50 cells were analysed. Measurements of comets were made in the CASP 1.2.0 program. Comets observed in the chilled semen were assessed with regards to degree of damage and then they were classified according to the Gedik scale (GEDIK *et al.* 1992).

Statistical analysis

Results from all parameters were analysed using a one-way ANOVA with repeated measures, and post-hoc analyses were carried out using Tukey's test. Statistical procedures were performed using the SAS program (SAS/STAT 2001). All P values of less than 0.05 were considered statistically significant. All values were expressed as mean ± standard error of mean (SEM).

Results

Sperm motility and morphology

Thirty trials of chinchilla semen collection were conducted. Sixteen of 22 ejaculates obtained were studied. Afterwards three samples of semen were selected from 6 males. From the remaining ones, two samples each were taken. Ejaculates in which an average of 86.67% sperm cells were moving, and 73.33% of those showing progressive movement, were used for preservation (Table 1). The average concentration of sperm cells in the ejaculate was 4383.33×10^6 ml^{-1}, and the proportion of damaged sperm cells was 15.67%. After dilution a gradual decrease in the proportion of mobile sperm in all extenders was noticed (Table 2). But the differences in total and progressive sperm motility were statistically significant ($P \leq 0.05$) only between fresh semen and semen diluted with E-1 extender. After 24 h, reduction of total sperm motility (53.33 and 63.33%, respectively) and proportion of sperm cells with progressive movement (35 and 41.67%, respectively) was lowest in E-3 and E-4 extenders. Also after 48 h the highest percentage of sperm cells with total and progressive movement was noticed in the same extenders. All decreases of total and progressive sperm motility in all extenders were statistically significant ($P \leq 0.05$). Our results showed that motility of sperm was not observed in the two commercial extenders (E-1 and E-2) after 72 h. During this time the highest number of mobile cells after dilution was observed in the semen diluted in E-4 (10.83%), but only in 4.17% of sperm cells was progressive movement observed.

The level of damaged sperm cells in fresh semen averaged 15.67%. Immediately after dilution, a distinct increase in proportion of damaged sperm from 21.67% in E-4 to 29.33% in E-1 were observed. The differences for fresh semen and semen diluted with E-4 extender were statistically insig-

Table 1

Characteristics of chinchilla semen used for liquid storage at 4ºC immediately after ejaculation (n=16)

Parameter	Mean	SEM
Seminal volume (µl)	114.5	7.74
Sperm concentration ($\times 10^6$ml^{-1})	4383.33	696.18
Total sperm motility (%)	86.67	2.11
Progressive sperm motility (%)	73.33	4.22
Intact spermatozoa (%)	84.33	1.15

Table 2

The effect of extenders on sperm motility during liquid storage at 4ºC of chinchilla semen (n=16). Extenders: E-1 – SAFE CELL PLUS for boars, IMV-TECHNOLOGIES, France, E-2 GALAP for rabbits, IMV-TECHNOLOGIES, France, E-3 for canine semen (MORTON *et al.* 1989), E-4 for canine and equine semen (PROVINCE *et al*. 1984). Means in the same row with different superscripts (ABCD) differ significantly ($P \leq 0.05$). Means in the same column with different superscripts (abc) differ significantly ($P \leq 0.05$)

Extender	Items	Total sperm motility (%)				Progressive sperm motility (%)			
		0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Fresh semen	Mean	86.67^{a}				73.33^{a}			
	SEM	2.11				4.22			
E-1	Mean	56.67^{Ab}	21.67^{Ba}	6.67^{Ca}	0^{Ca}	38.33^{Ab}	9.17^{Ba}	3.33^{Ba}	0^{Ba}
	SEM	8.03	5.43	3.33	0	9.46	2.39	1.67	0
E-2	Mean	66.67^{Aab}	30^{Bac}	10.83^{Cac}	0.83^{Da}	50^{Aab}	13.33^{Ba}	4.17^{Ca}	0^{Ca}
	SEM	6.15	6.32	4.17	0.83	6.32	3.33	1.54	0
E-3	Mean	83.33^{Aa}	53.33^{Bbc}	28.33^{Cbc}	6.67^{Da}	66.67^{Aa}	35^{Bb}	13.33^{Cb}	3.33^{Da}
	SEM	3.33	8.03	7.49	3.33	4.22	5.63	3.07	1.67
E-4	Mean	85^{Aa}	63.33^{Bb}	33.33^{Cb}	10.83^{Db}	70^{Aa}	41.67^{Bb}	12.67^{Cb}	4.17^{Da}
	SEM	3.42	5.58	3.33	3.00	5.16	6.01	1.67	2.01

Table 3

The effect of extenders on sperm morphology during liquid storage at 4ºC of chinchilla semen (n=16). Extenders: E-1 – SAFE CELL PLUS for boars, IMV-TECHNOLOGIES, France, E-2 – GALAP for rabbits, IMV-TECHNOLOGIES, France, E-3 for canine semen (MORTON *et al.* 1989), E-4 for canine and equine semen (PROVINCE *et al*. 1984). Means in the same row with different superscripts (ABCD) differ significantly (P≤0.05). Means in the same column with different superscripts (abc) differ significantly (P≤0.05)

Extender	Items	Damaged spermatozoa (%)			
		0 h	24 h	48 h	72 h
Fresh semen	Mean	15.67^{a}			
	SEM	1.15			
E-1	Mean	29.33^{Ab}	33^{Aa}	43^{Ba}	49.5^{Bab}
	SEM	3	3.06	3.61	4.5
E-2	Mean	27.5^{Ab}	34.67^{Ba}	43.83^{Ca}	54.5^{Da}
	SEM	2.88	3.06	2.65	7.5
E-3	Mean	27.33^{Ab}	31^{Aa}	34.83^{Ba}	42.5^{Cb}
	SEM	2.72	3.01	2.18	3.5
E-4	Mean	21.67^{Aab}	28.67^{Ba}	32.83^{Ca}	46.5^{Dab}
	SEM	2.58	2.56	3.08	0.5

nificant (P>0.05). The highest number of sperm cells with secondary changes after 24, 48 and 72 h in ejaculates diluted with E-2 (34.67, 43.83 and 54.5%, respectively) were observed (Table 3). Similar results were obtained for E-1. The smallest increase in sperm with secondary changes after 48 h was in E-4 (to 32.83%). However, after 72 h the smallest proportion of damaged sperm cells was observed in E-3 (42.5%).

Sperm DNA integrity

Microscopic analysis of SCGE preparations showed that the proportion of comet-detected spermatozoa with damaged DNA in fresh semen was negligible (Fig. 1). The proportion of spermatozoa with damaged DNA was observed to increase directly after dilution (0 h), and continued to increase (P>0.05) along with storage time, as was the case with all extenders. The highest increase in TM index occurred for semen diluted with extenders E-1 (0.23-1.29) and E-2 (0.22-0.88), and the differences were statistically significant (P≤0.05). In another case (E-4) the increase in TM value was statistically insignificant (P>0.05). TM results are presented in Table 4.

Four groups of sperms were created on the basis of the GEDIK *et al.* (1992) scale. The first group (a) includes undamaged sperm cells or those with damage of less than 5%. The second (b) contains sperm cells with degree of nuclear DNA fragmentation ranging from 5% to 25%. The third (c) and fourth groups (d) contain, respectively, sperm cells with disorder of DNA integrity (a – from 25 to

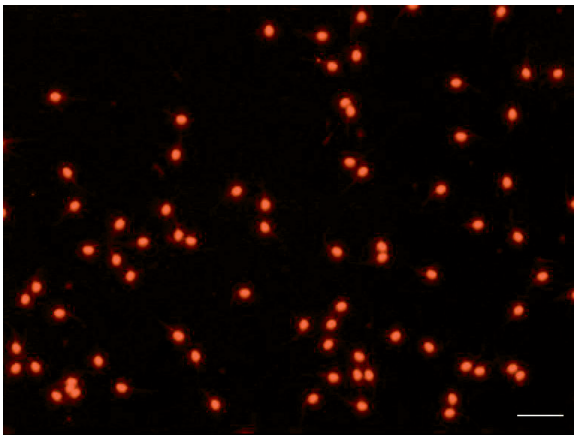

Fig. 1. Microscopic image of sperm cells in fresh chinchilla semen (400 × magnification).

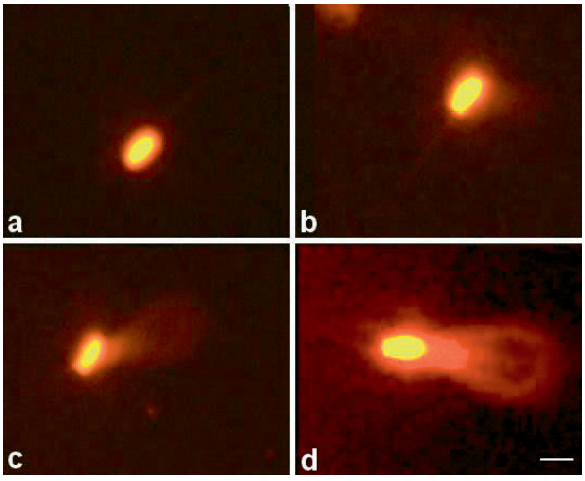

Fig. 2. Fluorescence microscopy displaying spermatozoa: a – without damaged DNA; b, c, d – with damaged DNA, as shown by a comet test (1250 × magnification).

Table 4

Tail moment (TM) of sperm with damaged DNA during liquid storage at 4ºC of chinchilla semen (n=16). Extenders: E-1 – SAFE CELL PLUS for boars, IMV-TECHNOLOGIES, France, E-2 – GALAP for rabbits, IMV-TECHNOLOGIES, France, E-3 for canine semen (MORTON *et al.* 1989), E-4 for canine and equine semen (PROVINCE *et al*. 1984). Means in the same row with different superscripts (ABC) differ significantly ($P \leq 0.05$). Means in the same column with different superscripts (abc) differ significantly ($P \leq 0.05$)

Extender	Items	Sperm with damaged DNA (%)			
		0 h	24 h	48 h	72 h
E-1	Mean	0.23^{Aa}	0.36^{Aa}	0.67^{Ba}	1.29^{Ca}
	SEM	0.06	0.08	0.09	0.25
E-2	Mean	0.22^{Aa}	0.33^{Aa}	0.50^{Bab}	0.88^{Cb}
	SEM	0.05	0.05	0.03	0.14
E-3	Mean.	0.28^{Aa}	0.62^{Bb}	0.68^{Ba}	0.69^{Bbc}
	SEM	0.09	0.06	0.13	0.12
E-4	Mean	0.31^{Aa}	0.43^{Aab}	0.45^{Ab}	0.50^{Ac}
	SEM	0.04	0.10	0.04	0.05

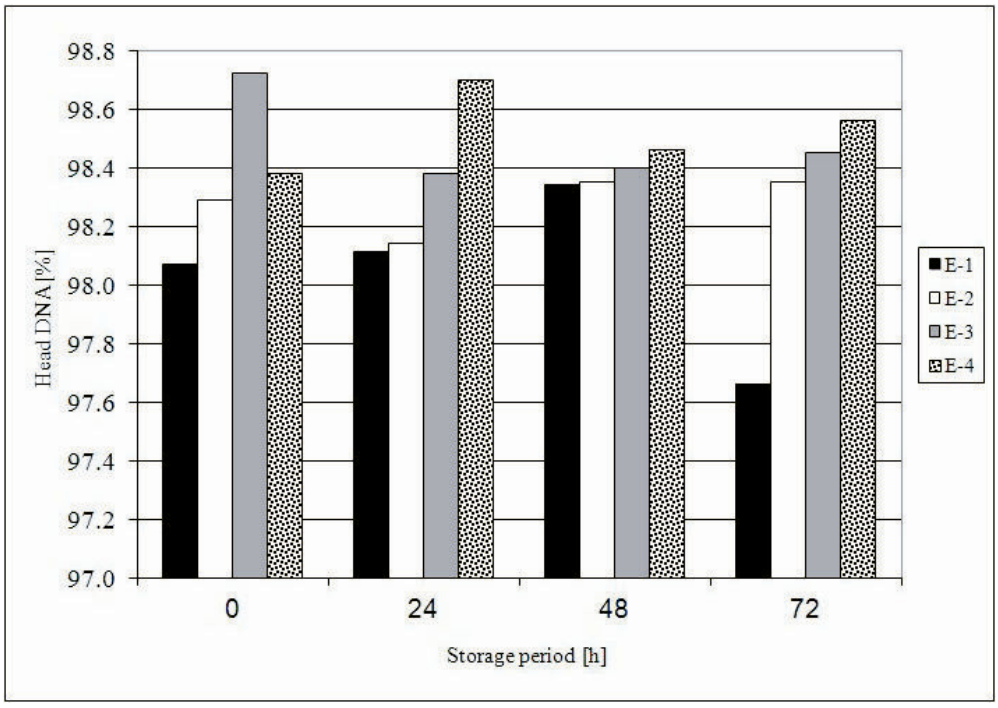

Fig. 3. Effect of liquid preservation on the percentage of head DNA in comets of chinchilla spermatozoa during liquid stored at 4ºC. Extenders: E-1 – SAFE CELL PLUS for boars, IMV-TECHNOLOGIES, France, E-2 – GALAP for rabbits, IMV-TECHNOLOGIES, France, E-3 for canine semen (MORTON *et al.* 1989), E-4 for canine and equine semen (PROVINCE *et al*. 1984) .

40% and b – from 40 to 95%) in the tail of the comet. Fig. 2 contains a graphic representation of the four isolated groups of DNA damage.

When estimating the percentage of nuclear DNA in the heads of sperm cells, the best preserved DNA integrity was found for the semen extended in E-4, for which the percentage of DNA in heads after 72 h storage was still high and exceeded 98.56%. Moreover, analysis of the SCGE assay demonstrated that the worst protector of chinchilla semen was the E-1 extender, for which DNA content in the head after 72 h was the lowest at 97.7%. Detailed data are presented in Fig. 3.

Discussion

Chinchilla (*Chinchilla lanigera*) is characterized by numerous species-specific traits. Semen parameters also belong to the specific traits of a given species. The small volume of ejaculate (10-70 µl) usually obtained from males of this species was characterized by a white colour, creamy consistency, and a hardly noticeable smell (HOLT 2000). In our laboratory we have been conducting extensive research to improve methods for collection, assessment and preservation of semen while introducing the most recent biotechnological techniques (NIEDBAŁA *et al.* 2009a; NIEDBAŁA *et al.* 2012).

One of the basic parameters which proves the usefulness of the ejaculate in reproduction is an appropriate proportion of motile sperm, especially those with progressive movement. The generally accepted standards for semen intended for further usage is that it should have at least 70% motile sperm, 60% of which should show progressive movement (BIELAŃSKI 1977; WIERZBOWSKI 1999; STRZEŻEK 2007). Ejaculates in which an average of 86.67% of sperm cells were motile and 73.33% showed progressive movement were used in this study. The average concentration of sperm cells in the ejaculate amounted to 4383.33×10^6 ml^{-1}. At least 84% of spermatozoa had intact morphological structure. Concerning the sperm concentration, the proportion of ejaculates with undamaged sperm obtained in our study differed slightly from those reported by BUSSO *et al*. (2005a), BUSSO *et al*. (2005b), PONZIO *et al*. (2011) and DOMINCHIN *et al*. (2014). However, their volumes were higher and the percentages of total and motile and progressive motile sperms were lower.

Our findings showed large decreases in sperm motility (particularly in E-1 and E-2) at the very beginning of the experiment. Such observations were made for chilled semen of other animal species, such as nutria (SZELESZCZUK 1996). It was shown that the proportion of motile sperm can be reduced to 43%, especially in Kiev diluent containing EDTA and glucose. Similarly, high declines of 33% were observed by GOGOL and BOCHENEK (2003) in rabbit semen preserved with extender designed for boar semen. After 48 hours, they observed it to contain only 2% of motile sperm. Even in the Galap diluent designed for rabbit semen after 48 hours up to 80% less motile sperm can be observed (GOGOL & WIERZCHOŚ-HILCZER 2009), and the proportion of sperms with progressive motility may be lower than 30% (GOGOL 2012).

PONCE *et al.* (1998a) used five extenders and the best of them contained Tris and fructose. After thawing, total sperm motility was 55%. In the extender containing glucose, the percentage of those sperms was even lower and reached a level of 44%. However, they concluded that such a composition of the first extender constitutes the best protection, as it prolongs the life and activity of chinchilla sperm cells. Replacing glycerol with ethylene glycol in the extender with fructose allows obtaining higher sperm post thaw motility (PONCE *et al.* 1998b). However, apart from the applied assessment of semen, DNA integrity and chromatin structure were not checked.

As mentioned in the introduction only CARRASCOSA *et al.* (2001) investigated liquid preservation of chinchilla semen at a temperature of 4ºC. They tested the usability of extender based on TES, Tris, sodium citrate egg yolk and fructose with the addition of glycerol or ethylene glycol as a cryoprotectant. After 24 hours, they showed that the percentage of motile sperm was lower by about 30%. A similar decrease was observed in our study: 26% for extender E-4 and slightly higher (38%) for E-3. The same authors stated that although sperm cells chilled for 72 h are characterized by lower motility, their basic biological functions remain at the same level. However, they also did not assess nuclear DNA integrity. Correct DNA structure of fertilizing sperm itself is a basic condition for correct development of embryos (EVENSON 1999).

Technological procedures connected with preservation of semen (dilution, lowering or raising of temperature) can disturb the fertilizing ability of sperm cells causing heat stroke, which damages the usually correct biochemical structure of the plasmalemma. Moreover, heightened sensitivity of the chromatin condensation process in maturing sperm cells to factors associated with collection of semen was noticed (composition of extenders). Sperm cells with DNA damaged in such a way can show appropriate mitochondrial activity and mobility and even correct morphology (BOCHENEK *et al.* 2001). Moreover, the phenomenon of natural ageing of sperm cells influences the index of fertilization in both *in vivo* and *in vitro* conditions (BOGUCKA *et al.* 2006). The above-mentioned morphofunctional changes arising at the ultrastructural level of sperm cells in the preserved semen can be controlled by varying the conditions of semen preservation (temperature and composition of extender). The detailed characteristics of a species' semen are indispensable to create new possibilities for successful support of reproduction by means of biotechnological procedures, such as preservation of semen (FRASER & STRZEŻEK 2005).

Moreover, numerous irregularities and differences in the size of the sperm cell heads were observed among the analysed cells. TRIVEDI *et al.* (2010) assessed the correlation between morphology of the head of the cell and parameters of com-

ets in toxicological tests of rat spermatozoa. Using the cytostatic doxorubicin they observed substantial growth in the damage of DNA in germ cells with defects of head morphology. A substantial correlation between the morphology of cell heads and the results of the comet test was noted. Therefore damage to the genetic material of sperm cells can result in morphological defects of the head of the sperm cell. Therefore, an assessment of regularities in the morphology of the head of the sperm cell alone can be an insufficient criterion for assessment of semen extenders and cryoprotectants. Application of standard methods and SCGE in the assessment of fertilizing ability of stored reproductive cells seems to be a more accurate laboratory tool.

In summary the results of our study agree with the findings of NIEDBAŁA *et al.* (2012), who used a similar group of extenders and assessed them only on the basis of motility and morphological changes. They proved that E-4 extender (PROVINCE *et al.* 1984), in which a daily decrease in their proportion amounted only to 25% in comparison to other extenders, is the best protection for chilled semen. Taking into consideration DNA content in heads of sperm cells and TM value it was stated that the most useful extender for liquid preservation of chinchilla semen was the E-4 extender for canine and equine semen (PROVINCE *et al.* 1984), which contained TRIS, citric acid, glucose and distilled water. The diluents E-1 for boars and E-2 for rabbits were unsuitable for liquid preservation of chinchilla semen.

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