AGRICULTURAL BIOLOGY,
2023, V. 58, Iss. 4, pp. 660-668
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.1.082.4:577.1

doi: 10.15389/agrobiology.2023.4.660eng
doi: 10.15389/agrobiology.2023.4.660rus

THE LEVEL OF SPERM PLASMA PROTEIN OXIDATIVE MODIFICATION ASSESSED IN STALLIONS (*Equus ferus caballus* L.) OF DIFFERENT AGES

M.M. ATROSHCHENKO[1] ✉, M.G. ENGALYCHEVA[1, 2], A.M. SHITIKOVA[1, 2]

[1]*All-Russian Research Institute for Horse Breeding,* Divovo, Rybnoe District, Ryazan Province, 391105 Russia, e-mail atromiks-77@mail.ru (✉ corresponding author);
[2]*Pavlov Ryazan State Medical University,* 9, ul. Vysokovol'tnaya, Ryazan, 390026 Russia, e-mail: mariyanaaber@yandex.ru, anyakudlaeva@mail.ru
ORCID:
Atroshchenko M.M. orcid.org/0000-0001-6023-0332 Shitikova A.M. orcid.org/0000-0002-4004-9058
Engalycheva M.G. orcid.org/0000-0002-9719-036X
The authors declare no conflict of interests
Acknowledgements:
The equipment of the Center for Collective Use the Collection of Genetic Resources of the All-Russian Research Institute of Horse Breeding was used
Supported financially by the Russian Science Foundation, grant No. 20-16-00101-П
Final revision received September 8, 2022
Accepted November 1, 2022

Abstract

Cryopreservation of stallion semen is a modern widespread method in horse breeding for preserving the genetic material of animals. Freezing and thawing reduces the reproductive characteristics of spermatozoa. Oxidative stress that causes damage to macromolecules is a factor contributing to damage to germ cells. With age, the oxidative stress and the amount of damaged proteins increase. In this work, for the first time, we quantified products of oxidative modification of proteins (OMP) in semen plasma in stallions of different ages. There is a significant increase in the content of protein carbonylation products in older animals compared to younger ones, mainly due to neutral aldehyde derivatives. This study is the first to assess the reserve-adaptive potential (RAP) of the seminal plasma of stallions. It was found that the ability to withstand oxidative stress in young stallions is significantly higher than in older stallions. The aim of this study was to assess the level of spontaneous OMP, induced OMP and the RAP values for stallion spermatozoa as influenced by the animal age. The study was carried out in 2020 on 40 purebred Arabian and Soviet draft stallions (*Equus ferus caballus* L.) (AO Tersk breeding stud No. 169, Stavropol Territory; Perevozsky and Pochinkovsky stud farms, Nizhny Novgorod Province). Three ejaculates of each stallion were collected with a 48-hour interval. The stallions of group I (n = 20) were from 14 to 21 years of age (mean age 15.8±1.9 years), of group II (n = 20) from 3 to 5 years of age (4.3±0.6 years). In each ejaculate, the volume and concentration of spermatozoa in 1 ml of semen was determined. Then the ejaculate was divided into two parts, one was diluted with lactose-chelate-citrate-yolk (LCCY) medium in a ratio of 1:3 and the progressive motility (PM) and survival of spermatozoa were determined at 4 °C. To assess the survival of spermatozoa during hypothermic storage of sperm, its PM was determined with a 24-hour interval up to a decrease in PM to 5 %. Sperm was frozen in liquid nitrogen vapor in 18 ml aluminum tubes according to the standard of the All-Russian Research Institute for Horse Breeding and stored in liquid nitrogen at –196 °C. The cryopreserved sperm was thawed in a water bath at 40 °C for 90 s, followed by the determination of the spermatozoa PM and survival at 4 °C. Another part of the ejaculate was centrifuged at 3500 rpm for 20 min. After microscopy of the supernatant, aliquots of seminal plasma free of spermatozoa were frozen in 2.0 ml Eppendorf tubes at –18 °C. To quantify the OMP, we used the spectrophotometric analysis of 2,4-dinitrophenylhydrazones formed by the interaction of protein carbonyl derivatives (aldehydes and ketones) with 2,4-dinitrophenylhydrazine. The total amount of carbonyl derivatives was recorded in a native sample of biological material (spontaneous OMP) and after in vitro induction of protein oxidation of biological material with a reaction mixture containing solutions of iron(II) sulfate and hydrogen peroxide (metal-catalyzed induced OMP). From metal-catalyzed and spontaneous OMP, RAP was evaluated to characterize the OS resistance. Spectrophotometric measurements were carried out at 14 wavelengths, at 260-280 nm for neutral aldehyde-dinitrophenylhydrazones, at 258-264 and 428-520 nm for basic aldehyde-dinitrophenylhydrazones, at 363-370 nm for neutral ketone-dinitrophenylhydrazones, and at 430-434 and 524-535 nm for basic ketone-dinitrophenylhydrazones. Statistically significant differences in sperm quality between animals of two age groups were found only in the survival rate of spermatozoa during hypothermic storage of diluted ($p < 0.05$) and

cryopreserved ($p < 0.01$) sperm. The total amount of spontaneous OMP products in the seminal plasma of older stallions was statistically significantly higher than in young stallions (531.7 and 384.3 ODU/g protein, respectively, $p < 0.05$). In addition, in group I, there was a shift in the absorption spectrum towards neutral aldehyde derivatives the content of which in animals of group II was significantly lower (367.6 and 255.8 ODU/g protein, $p < 0.05$). The evaluation of metal-catalyzed (induced) OMP also revealed a higher total amount of carbonyl derivatives in older stallions, but its increase under the influence of an oxidizing mixture was much higher vs. the initial spontaneous OMP in young stallions. The RAP value for the seminal plasma of young stallions significantly exceeds that of mature stallions ($p < 0.05$), which can positively affect the reproductive characteristics of native and cryopreserved sperm.

Keywords: *Equus ferus caballus*, stallions, sperm, seminal plasma, cryopreservation, oxidative stress, protein oxidative modification

Oxidative stress (OS) leads to sperm damage [1, 2] and a decrease in the quality of native and cryopreserved sperm [3-5]. The cause of OS development is excessive production of reactive oxygen species (ROS), depletion of the body's antioxidant capacity, or a combination of these factors. As a result of the predominance of pro-oxidant processes over the antioxidant capabilities of cells, ROS interact with basic macromolecules, causing irreversible damage to nucleic acids, lipid peroxidation (LPO), and oxidation of proteins, including the most important enzymes and structural proteins [6].

Quantification of oxidative modification of proteins (OMP) is one of the markers of the OS severity, since this indicator reflects the degree of damage to amino acid residues of proteins by free radicals of oxygen and nitrogen, as well as lipid peroxidation products [7]. OMP products appear in cells earlier than other derivatives of oxidative damage to macromolecules, they are stable and accessible for laboratory detection [8].

Sperm and sperm plasma proteins, like other proteins in the body of stallions, are prime targets for ROS and RNS due to their high sensitivity to free radicals [9, 10]. Numerous and structurally diverse free radicals are produced from enzymatic and non-enzymatic redox reactions, photochemical and ionizing effects. Oxidative modification of amino acid residues of proteins yields carbonyl derivatives, the aldehydes and ketones. Aldehydes are early markers of the oxidative destruction of proteins, and ketone derivatives are later ones [6, 8, 9]. It is important to remember that OMP occurs not only because of an increase in the concentration of free radicals, but also as a result of a shift in the balance between antioxidant and prooxidant systems in favor of the latter.

With age, the production of ROS increases in the body and a significant amount of OS products accumulates. This is associated with mitochondrial dysfunction, high production of ROS under depletion of the body's antioxidant capacity, disruption of the proteasome system, and degradation of damaged proteins [11].

Here, for the first time, we measured the content of products of oxidative modification of proteins in the spermoplasm of stallions of different ages and revealed a statistically significant increase in the content of protein carbonylation products in aged animals compared to younger ones, mainly due to neutral aldehyde derivatives. The reserve-adaptation potential (RAP) of stallion spermoplasm has been studied for the first time. Our investigation revealed that the ability to withstand oxidative stress in young stallions is significantly higher than in older ones.

The purpose of this study was to evaluate spontaneous and induced oxidative modification of proteins, and to assess the reserve-adaptation potential of spermoplasm in stallions of different ages.

Materials and methods. In 2020, sperm of 40 stallions (*Equus ferus caballus* L.) of purebred Arabian and Soviet draft breeds (JSC Tersky Pedigree Stud

No. 169, Stavropol Territory; Perevozsky and Pochinkovsky Stud Farms, Nizhny Novgorod Province) was obtained during the breeding season (March-April) for a mare in heat using an artificial vagina. Three ejaculates were obtained from each stallion with an interval of 48 h. During the experiment, the feeding and housing conditions complied with the established standards for the stallions.

To assess the age dependence of OMP and reserve-adaptation potential (RAP) of spermoplasm, stallions were assigned to two groups, $n = 20$ each, in group I, animals aged from 14 to 21 years (15.8±1.9 years), in group II, from 3 to 5 years (4.3±0.6 years).

After sperm collection, each ejaculate was filtered through a sterile gauze pad to remove the secretion of the vesicular glands. The volume of ejaculate (in ml) after filtration was assessed using a graduated cylinder. Sperm concentration was measured (an SDM1 photometer, Minitube GmbH, Germany). Then the ejaculate was divided into two parts, one was diluted at 1:3 with lactose-chelate-citrate-yolk (LCY) medium, and the progressive motility (PM) and survival of sperm at 4 °C were assessed.

Progressive motility (PP) was assessed using the Argus CASA system (ArgusSoft Ltd., Russia) and a Motic BA 410 microscope (Motic, China) in a Makler chamber at 37 °C.

To assess the survival of sperm during hypothermic storage of sperm, their progressive motility was determined at 24-hour intervals until the PP decreased to 5%. Sperm was frozen in liquid nitrogen vapor in 18 ml aluminum tubes using standard technology of the All-Russian Research Institute of Horse Breeding and stored in liquid nitrogen at –196 °C [12]. After thawing in a water bath at 40 °C for 90 s, the progressive motility and survival of cryopreserved spermatozoa at 4 °C were determined.

The other part of the ejaculate, immediately after sperm collection, was centrifuged at 3500 rpm for 20 min (ELMI CM-6M, ELMI, Latvia). After microscopy of the supernatant, aliquots of sperm-free seminal plasma were frozen in 2.0 ml Eppendorf tubes at –18 °C until testing.

To quantify OMP, spectrophotometric analysis of 2,4-dinitrophenylhydrazones resulting from the reaction of protein carbonyl derivatives (aldehydes and ketones) with 2,4-dinitrophenylhydrazine was performed as per a patented procedure [13]. The total amount of carbonyl derivatives was assessed in a native biomaterial to measure the actual content of carbonyl derivatives formed in vivo due to spontaneous OMP and after in vitro induction of protein oxidation with a reaction mixture containing iron(II) sulfate and hydrogen peroxide (metal-catalyzed induced OMP). The oxidizing mixture promoted additional formation of carbonyl derivatives. If in the native sample there was a reserve of antioxidant systems and there were few amino acid residues that could quickly oxidize, then the induced OMP had minimal difference from the spontaneous one. Comparison of metal-catalyzed and spontaneous OMP levels gives estimates of the reserve-adaptive potential (RAP), that is, the ability to withstand OS.

Neutral aldehyde-dinitrophenylhydrazones (ADNPHn) was assessed at 260-280 nm, basic aldehyde-dinitrophenylhydrazones character (ADNPHb) at 258-264 and 428-520 nm, neutral ketone-dinitrophenylhydrazones (KDNPHn) at 363-370 nm, basic ketone-dinitrophenylhydrazones (KDNFGb) at 430-434 and 524-535 nm (SF-2000 spectrophotometer, OKB Spectr, Russia). The area under the curve of the absorption spectrum of dinitrophenylhydrazine (DNPH) derivatives of protein carbonyl derivatives (S) was measures. The obtained extinction values were expressed as optical density units E_{OD} per 1 g of spermoplasm protein content determined by the Lowry method.

Statistical analysis was performed using Statistica 13.3 (StatSoft, Inc.,

USA) and Microsoft Office Excel 2016. Normality of distribution was determined by the Shapiro-Wilk test. The statistical significance of differences between independent samples was assessed by the Mann-Whitney U test. The result was considered statistically significant at $p < 0.05$. Data are presented as median (*Me*) and quartiles (Q1; Q3).

Results. A comparative analysis of spermograms (Table 1) revealed that the rate of sperm survival during hypothermic storage was significantly lower in older stallions than in young animals. Both diluted and cooled sperm (66.0 h for adult stallions, 107.0 h for young stallions, $p < 0.05$) and sperm thawed after cryopreservation (17.3 h and 49.0 h, respectively, $p < 0.01$) showed similar patterns. A similar dependence of sperm survival on age was found not only in stallions [14, 15], but also in men [16, 17] and in bulls [18]. The likely reason for this is the accumulation of irreversible damage to nucleic acids with age, which negatively affects reproductive properties [14, 19].

1. Comparative characterization of sperm quality in purebred Arabian and Soviet draft stallions (*Equus ferus caballus* L.) of different ages (Me [Q1; Q3]; AO Tersky Pedigree Stud No. 169, Stavropol Territory; Perevozsky and Pochinkovsky Stud Farms, Nizhny Novgorod Province, 2020)

Parameter	Group I	Group II
Number of stallions, n	20	20
Age, years	15.8 [14.0; 21.0]	4.3 [3.0; 5.0]
Native and diluted sperm ($n = 60$)		
Ejaculate volume, ml	47.4 [15.0; 80.0]	61.9 [39.0; 105.0]
Sperm concentration, million/ml	153.9 [83.0; 245.0]	159.1 [105.0; 214.0]
Progressive mobility, %	48.0 [35.0; 60.0]	50.3 [40.0; 65.0]
Sperm survival rate, h	66.0 [36.0; 120.0]	107.0* [84.0; 120.0]
Frozen-thawed sperm ($n = 60$)		
Progressive motility, %	17.1 [10.0; 30.0]	21.8 [13.0; 33.0]
Sperm survival rate, h	17.3 [6.0; 72.0]	49.0** [12.0; 84.0]

* and ** Differences between sperm survival during hypothermic storage of diluted semen in stallions of groups I and II are statistically significantat $p < 0.05$ and $p < 0.01$, respectively.

The sperm survival during hypothermic storage (4 °C) is one of the main quality indicators. Spermatozoa survival is their ability to maintain progressive motility after hypothermic storage of diluted chilled or cryopreserved sperm. The survival of sperm is statistically significantly correlated with their progressive motility and the frequency of mares' pregnancies [20].

The total spontaneous OMP of the spermoplasm of stallions of group I was statistically significantly higher than in group II (531.7 and 384.3 E_{OD}/g protein, respectively, $p < 0.05$) (see Table 1). This indicates more pronounced oxidative stress in group I and more significant damage to amino acid residues of spermoplasmic proteins in older stallions compared to young animals. Besides, in group I, a shift in the absorption spectrum towards neutral aldehyde derivatives occurred, and their content in group II was lower (367.6 vs. 255.8 E_{OD}/g protein, $p < 0.05$).

A small amount of ROS in sperm is necessary for the transmission of intracellular signals, hyperactivation and acrosomal reaction, which provides fertilizing ability. However, excess production of ROS inevitably leads to the development of OS and damage to macromolecules [21].

OMB of spermoplasm causes changes in their secondary and tertiary structure which negatively affects the functional state of proteins which means the morphological and reproductive characteristics of sperm deteriorate (22). Damaged proteins can undergo processes of aggregation and fragmentation due to protein-protein interactions.

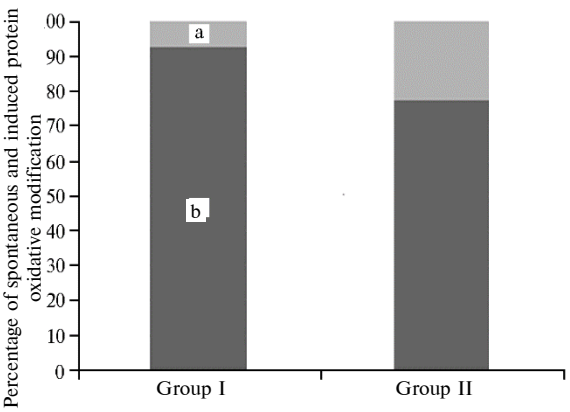
Accumulation of OMP products, protein aggregates and derivatives resistant to proteolysis disrupts cell metabolism, leading to apoptosis or necrosis [22,

23]. It should be noted that antioxidant and proteolytic systems prevent the development of oxidative stress and OMP, therefore, some damage to protein molecules is reversible. Enzymatic antioxidant systems include superoxide dismutase, glutathione reductase, catalase, and peroxidase. Non-enzymatic antioxidants are vitamins E and C, glutathione, carotenoids, ubiquinone and other metabolites [21, 22]. However, with age, pro-oxidant systems increasingly predominate over protective ones, and the degree of OMP is growing. Besides, antioxidant enzymes themselves, like other proteins, are subject to oxidation which disrupts their functioning [24].

2. Absorption spectrum (EOD/g protein) **of products derived from spontaneous and metal-catalyzed protein oxidative modification** (OMP) **in spermoplasm of purebred Arabian and Soviet draft stallions (*Equus ferus caballus* L.) of different ages** (Me [Q1; Q3]; AO Tersky Pedigree Stud No. 169, Stavropol Territory; Perevozsky and Pochinkovsky Stud Farms, Nizhny Novgorod Province, 2020)

Carbonyl derivatives	Group I (aged from 14 to 21 years; n = 20)		Group II (aged from 3 to 5 years; n = 20)	
	spontaneous OMP	induced OMP	spontaneous OMP	induced OMP
SADNFGn	367.6 [162.8; 500.5]	374.7 [236.5; 785.9]	255.8 [122.8; 328.1]	315.3 [193.8; 349.5]
SKDNFGn	83.9 [47.4; 131.5]	105.3 [74.9; 386.4]	69.9 [30.4; 84.3]	76.4 [66.0; 122.5]
SADNFGb	64.6 [40.7; 141.1]	84.0 [66.1; 323.4]	49.6 [29.5; 71.1]	71.4* [62.4; 98.6]
SKDNFGb	11.3 [6.9; 25.2]	13.8 [10.8; 43.0]	9.6 [5.1; 12.3]	11.8 [11.4; 19.4]
Stotal	531.7 [264.6; 787.6]	562.8 [364.2; 1519.7]	384.3 [172.6; 490.4]	484.7* [334.9; 598.4]

N o t e. SADNFGn, SKDNFGn, SADNFGb, SKDNFGb are the area under the curves of the absorption graphs of neutral aldehyde dinitrophenylhydrazones, neutral ketone dinitrophenylhydrazones, basic aldehyde dinitrophenylhydrazones, basic ketone dinitrophenylhydrazones, respectively. Stotal — the total area of the figure formed when plotting the absorption curve of DNPH derivatives of proteins carbonyl derivatives at different wavelengths.
* Differences between metal-catalyzed and spontaneous protein oxidative modification in stallion spermoplasm are statistically significant at $p < 0.05$.



Reserve adaptive potential of spermoplasm in purebred Arabian and Soviet draft stallions (*Equus ferus caballus* L.) of different ages: a — induced oxidative modification of proteins, b — spontaneous protein oxidative modification; Group I — adult stallions (from 14 to 21 years of age), Group II — young stallions (from 3 to 5 years of age) (*Me* [Q1; Q3]; AO Tersky Pedigree Stud No. 169, Stavropol Territory; Perevozsky and Pochinkovsky Stud Farms, Nizhny Novgorod Province, 2020).

The predominance of neutral aldehyde derivatives in older stallions (Table 2) indicated oxidative damage to the amino acid residues of cysteine, glutamine, asparagine, tryptophan, tyrosine, methionine, leucine, and proline. Due to oxidation of the thiol group of cysteine, disulfide cross-links are formed [21, 25]. In this case, the structural and functional state of proteins containing cysteine residues changes. A number of CRISPS (cysteine-rich secretory proteins) are present in the sperm of stallions [26]. CRISPs, found in the testes and epididymis of mammals, are involved in fertilization. During spermatogenesis, the CRISP2 protein is incorporated into the acrosome, where it is believed to participate in the adhesion of germ cells to Sertoli cells. CRISP2 also serves as part of the sperm tail and is involved in the regulation of flagellar beating. The CRISP3 is found in seminal fluid isolated from the prostate, and its function is unknown [26-28]. Oxidative modifications of cysteine residues can negatively affect the functioning of these proteins.

An assessment of induced OMP also revealed a higher total amount of

carbonyl derivatives in group I, but an increase in the amount of carbonyl derivatives when exposed to an oxidizing mixture was much higher compared to the initial level of spontaneous OMP in young stallions (see Table 2, Fig.). Our findings indicate that in the spermoplasm of young stallions there are amino acid groups that could potentially be subject to oxidative stress. However, this did not happen in vivo, probably due to the active antioxidant systems that prevent damage to protein molecules [29]. This is also confirmed by the fact that during induced oxidation, the proportion of neutral and basic aldehyde derivatives (the early markers of oxidative destruction) increased significantly. The results obtained indicate high RAP in young stallions.

In group I, the added oxidative mixture increased the content of carbonyl derivatives (see Fig.), but not so significantly when compared to the initial values. This indicates a significant accumulation in vivo of damaged amino acid radicals of sperm plasma proteins and depletion of RAP in adult stallions.

Thus, the total oxidative modification of spermoplasm proteins in older purebred Arabian and Soviet draft stallions is higher than in younger stallions ($p < 0.05$). The reserve-adaptive potential of the spermoplasm of young stallions significantly exceeds that of older animals ($p < 0.05$), which can positively influence the reproductive quality parameters of native and cryopreserved sperm. A higher content of sperm plasma protein carbonylation products in older stallions semen indicates oxidative stress which may cause a decreased sperm viability.

REFERENCES

1. Bisht S.. Faiq M.. Tolahunase M.. Dada R. Oxidative stress and male infertility. *Nature Reviews Urology*. 2017. 14(8): 470-485 (doi: 10.1038/nrurol.2017.69).
2. Wyck S.. Herrera C.. Requena C.E.. Bittner L.. Hajkova P.. Bollwein H.. Santoro R. Oxidative stress in sperm affects the epigenetic reprogramming in early embryonic development. *Epigenetics & Chromatin*. 2018. 11(1): 60 (doi: 10.1186/s13072-018-0224-y).
3. Lone F.A.. Naikoo M.. Khatun A.. Shah R.A.. Pampori Z.A.. Khan H.M.. Ahanger A.A. Idebenone improves quality of ram sperm by mitigating oxidative stress during cryopreservation. *Cryobiology*. 2019. 90: 15-20 (doi: 10.1016/j.cryobiol.2019.09.004).
4. Rodrigues R.B.. Uczay M.. Brito V.B.. Godoy A.C.. Moura D.J.. Vogel C.. Vasconcelos A.C.N.. Streit D.P. Jr. Oxidative stress and DNA damage of Zebrafish sperm at different stages of the cryopreservation process. *Zebrafish*. 2021. 18(2): 97-109 (doi: 10.1089/zeb.2020.1942).
5. Moubasher A.E.. Taha E.A.. Younis A.. Fakhry M.E.. Morsy H. Testicular tissue oxidative stress in azoospermic patients: Effect of cryopreservation. *Andrologia*. 2020. 52(11): e13817 (doi: 10.1111/and.13817).
6. Barati E.. Nikzad H.. Karimian M. Oxidative stress and male infertility: current knowledge of pathophysiology and role of antioxidant therapy in disease management. *Cellular and Molecular Life Sciences*. 2020. 77(1): 93-113 (doi: 10.1007/s00018-019-03253-8).
7. Teplov S.A.. Abalenikhina Yu.V.. Fomina M.A.. Matveeva I.V. *Nauka molodykh — Eruditio Juvenium*. 2016. 1: 50-54 (in Russ.).
8. Hawkins C.L.. Davies M.J. Detection. identification. and quantification of oxidative protein modifications. *The Journal of Biological Chemistry*. 2019. 294(51): 19683-19708 (doi: 10.1074/jbc.REV119.006217).
9. Čolak E. New markers of oxidative damage to macromolecules. *Journal of Medicak Biochemiastry*. 2008. 27(1): 1-16 (doi: 10.2478/v10011-007-0049-x).
10. Zvyagina V.I.. Shumaev K.B.. Bel'skikh E.S.. Uryas'ev O.M.. Akhmedova S.R.. Marsyanova Yu.A.. Shitikova A.M.. Suchkova O.N. *Rossiyskiy mediko-biologicheskiy vestnik imeni akademika I.P. Pavlova*. 2022. 30(4): 457-470 (doi: 10.17816/PAVLOVJ109410) (in Russ.).
11. Zhang H.. Davies K.J.A.. Forman H.J. Oxidative stress response and Nrf2 signaling in aging. *Free Radical Biology Medicine*. 2015. 88(Pt B): 314-336 (doi: 10.1016/j.freeradbiomed.2015.05.036).
12. Naumenkov A.I.. Roman'kova N.K. V sbornike: *Teoriya i praktika sovershenstvovaniya porod loshadey. Nauchnye trudy VNIIK* [In: Theory and practice of improving horse breeds. Scientific works of VNIIK]. Divovo. 1971. XXV: 128-132 (in Russ.).
13. Fomina M.A.. Abalenikhina Yu.V.. Fomina N.V.. Terent'ev A.A. *Sposob kompleksnoy otsenki soderzhaniya produktov okislitel'noy modifikatsii belkov v tkanyakh i biologicheskikh zhidkostyakh. Patent 2524667 RF. MPK G01N 33/52. Zayavl. 21.01.13. Opubl. 27.07.14* [Method for comprehensive assessment of the content of protein oxidative modification products in tissues and

biological fluids. Patent 2524667 RU. MPK G01N 33/52. Appl. 01/21/13. Publ. 07/27/14] (in Russ.).
14. Castro R.. Morales P.. Parraguez V.H. Post-thawing sperm quality in Chilean purebred stallions: effect of age and seasonality. *Journal of Equine Veterinary Science*. 2020. 92: 03170 (doi: 10.1016/j.jevs.2020.103170).
15. Greiser T.. Sieme H.. Martinsson G.. Distl O. Breed and stallion effects on frozen-thawed semen in warmblood. light and quarter horses. *Theriogenology*. 2020. 142: 8-14 (doi: 10.1016/j.theriogenology.2019.09.033).
16. Moskovtsev S.. Willis J.. White J.. Mullen B. Sperm survival: relationship to age-related sperm DNA integrity in infertile men. *Archives of Andrology*. 2009. 53(1): 9-32 (doi: 10.1080/01485010600908330).
17. Kang W.. Harada Y.. Yamatoya K.. Kawano N.. Kanai S.. Miyamoto Y.. Nakamura A.. Miyado M.. Hayashi Y.. Kuroki Y.. Saito H.. Iwao Y.. Umezawa A.. Miyado K. Extra-mitochondrial citrate synthase initiates calcium oscillation and suppresses age-dependent sperm dysfunction. *Laboratory Investigation*. 2020. 100(4): 583-595 (doi: 10.1038/s41374-019-0353-3).
18. Anbaza Yu.V. *Vestnik KrasGLU*. 2018. 2: 286-292 (in Russ.).
19. Luo Q.X.. Liao Y.B.. Huang C.P.. Guo J.H.. Liang X.D. Correlation of sperm DNA fragmentation index with age and semen parameters and its influence on the outcomes of IVF-ET. *Zhonghua Nan Ke Xue (National Journal of Andrology)*. 2020. 26(8): 708-712.
20. Atroshchenko M.M.. Arkhangelskaya E.. Isaev D.A.. Stavitsky S.B.. Zaitsev A.M.. Kalaschnikov V.V.. Leonov S.. Osipov A.N. Reproductive characteristics of thawed stallion sperm. *Animals*. 2019. 9(12): 1099 (doi: 10.3390/ani9121099).
21. Piomboni P.. Stendardi A.. Gambera L.. Tatone C.. Coppola L.. De Leo V.. Focarelli R. Protein modification as oxidative stress marker in normal and pathological human seminal plasma. *Redox Report*. 2012. 17(5): 227-232 (doi: 10.1179/1351000212Y.0000000014).
22. Agarwal A.. Durairajanayagam D.. Halabi J.. Peng J. Vazquez-Levin M. Proteomics. oxidative stress and male infertility. *Reproduction Biomed Online*. 2014. 29(1): 32-58 (doi: 10.1016/j.rbmo.2014.02.013).
23. Caruso Bavisotto C.. Alberti G.. Vitale A.M.. Paladino L.. Campanella C.. Rappa F.. Gorska M.. Conway de Macario E.. Cappello F.. Macario A.J.L.. Marino Gammazza A. Hsp60 post-translational modifications: functional and pathological consequences. *Frontiers in Molecular Bioscience*. 2020. 7: 95 (doi: 10.3389/fmolb.2020.00095).
24. Angrimani D.. Nagai K.K.. Rui B.R.. Bicudo L.C.. Losano J.. Brito M.M.. Francischini M.. Nichi M. Spermatic and oxidative profile of domestic cat (*Felis catus*) epididymal sperm subjected to different cooling times (24. 48 and 72 hours). *Reproduction in Domestic Animals*. 2018. 53(1): 163-170 (doi: 10.1111/rda.13086).
25. Cai Z.. Yan L.J. Protein oxidative modifications: beneficial roles in disease and health. *Journal of Biochemical and Pharmacological Research*. 2013. 1(1): 15-26.
26. Gaikwad A.S.. Hu J.. Chapple D.G.. O'Bryan M.K. The functions of CAP superfamily proteins in mammalian fertility and disease. *Human Reproduction Update*. 2020. 26(5): 689-723 (doi: 10.1093/humupd/dmaa016).
27. Hu J.. Merriner D.J.. O'Connor A.E.. Houston B.J.. Furic L.. Hedger M.P.. O'Bryan M.K. Epididymal cysteine-rich secretory proteins are required for epididymal sperm maturation and optimal sperm function. *Molecular Human Reproduction*. 2018. 24(3): 111-122 (doi: 10.1093/molehr/gay001).
28. Cohen D.J.. Busso D.. Da Ros V.. Ellerman D.A.. Maldera J.A.. Goldweic N.. Cuasnicu P.S. Participation of cysteine-rich secretory proteins (CRISP) in mammalian sperm-egg interaction. *Int. J. Dev. Biol.*. 2008. 52(5-6): 737-742 (doi: 10.1387/ijdb.072538dc).
29. Zalesskaya T.I.. Aver I.I.. Mandrik K.A. *Zhurnal GrGMU*. 2007. 3: 66-68 (in Russ.).