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Effect of prooxidants and chelator Desferal on the oxidative status and sperm motility of Muscovy semen

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ABSTRACT

This study aimed to establish the sensitivity of Muscovy duck semen to oxidative stress (OS) and the effect of Desferal, applied as an antioxidant. The effect of three prooxidant systems in presence and absence of Desferal were tested on the motility and kinetic parameters (determined using CASA system), as well as the level of lipid peroxidation (LPO) and glutathione (tGSH) of Muscovy semen. The semen was diluted (1:3 v/v) with four extenders (saline solution, IMV Canadyl, HIA-1, and AU) and stored at 4 °C for 6 h. The cooled semen was divided into aliquots (50×10^6 sperm cells/mL), which were incubated at 37 °C for 30 min with one of the following prooxidative agents: ferrous sulfate ($FeSO_4$, 0.1 mM), hydrogen peroxide (H_2O_2, 1 mM), and Fenton system ($FeSO_4(Fe^{2+})$, 0.1 mM + H_2O_2, 1 mM), in the presence or absence of Desferal (0.1 mM). The addition of $FeSO_4$ + H_2O_2 or $FeSO_4$ regardless of the used extender, as well as the addition of H_2O_2 to the diluted semen with saline solution significantly increased the levels of LPO ($P < 0.05$). With the lowest prooxidant effect was H_2O_2. The application of Desferal reduced significantly ($P < 0.05$) the LPO levels induced by $FeSO_4 + H_2O_2$ or $FeSO_4$ and in a weaker degree by H_2O_2. Among all prooxidants, $FeSO_4 + H_2O_2$ decreased in the greatest extent the tGSH concentration in semen diluted with each used extenders in comparison to the relevant control. The addition of Desferal in semen diluted with HIA-1 extender and incubated with $FeSO_4$, and H_2O_2, showed the best restoration of tGSH level, close to that of respectively controls. The studied prooxidants significantly reduced total, progressive, and kinetic sperm motility ($P < 0.05$). Although the inclusion of Desferal reduced the sperm OS, it did not improve the impaired by OS sperm motility.

1. Introduction

Muscovy duck (*Cairina moschata*) is used as the paternal form, and common duck (*Anas platyrhynchos D.*), mainly the Pekin breed, as the maternal form in the inter-generic crossbreeding for receiving of the F_1 sterile hybrid mulard, better known as mule duck. The mulards are reared in poultry farming to the production of meat and the delicious gastronomic product – foie gras. The EU produces approximately 90 % of the world's foie gras (https://www.eurofoiegras.com/en/home).

Between both parental forms (Muscovy drakes and Pekin ducks) exist ethologic differences therefore spontaneous mating is rare and leads to no more than 45 % fertile eggs [1–3]. At present, artificial insemination is used with great success and fertility rates vary between 65 and 80 % with two inseminations per week [3,4]. Sperm cell concentration in Muscovy semen is high *i.e.*, it is viscous [5,6]. In practice, diluted and short-term stored semen is increasingly used in order to increase the number of inseminated birds.

Studies have shown that sperm preparation, processing and handling lead to increased free radical generation and induction of oxidative stress (OS) [7]. The severity of the OS depends on many factors as continuance of *in vitro* storage, temperature, extenders used, dilution level, centrifugation, oxygen partial pressure, light, freezing-thawing cycles [8–11]. In addition, OS can also be provoked *in vivo* in organisms by various factors such as diseases, parasites, poisons, pollutants, climatic factors (cold, heat), and growing conditions [12].

Avian semen is particularly susceptible to OS because of high level of

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polyunsaturated fatty acids (PUFAs) in spermatozoa. The high proportions of PUFAs in the lipid fractions of sperm cells reflect the need to maintain high membrane fluidity and the flexibility required for sperm motility and fusion with the oocyte [13–15]. According to Surai et al. [13,15] duck spermatozoa contained the highest proportion of PUFAs (mainly 20:4n-6; 22:4n-6 and 22:6n-3) and the highest peroxidizability index compared to other five avian species - chicken, turkey, guinea fowl, goose spermatozoa. PUFAs represent a major part (54.4 %) of the total fatty acids in duck spermatozoa. Presumably, the very high proportion of long chain PUFAs in the avian spermatozoa including duck spermatozoa predisposes them to lipid peroxidation. PUFAs in the plasma membrane create favourable conditions for the formation of peroxidative products, leading to changes in membrane features and ultimately resulting in reduced semen quality, incl. impaired motility, reduced acrosomal reaction, morphological changes [16–18]. Furthermore, in birds, mammals and human, the OS can lead to protein damage as enzyme inhibition and histone modifications, and DNA damage with base modifications, changes in methylation patterns, deletions, chromosome rearrangements, fragmentation and ultimately cell death [7, 19–22]. Thus, the induction of OS, both in *in vitro* and *in vivo* (under adverse and stressful environmental impact or xenobiotics) conditions, is considered as one of the major causes which may ultimately impair male fertility [12,23,24]. Antioxidant protection has a crucial meaning in the maintenance of sperm membrane integrity and their fertilizing ability. For this reason, the application of antioxidants is a good strategy to preserve reproductive capabilities. The effects of various antioxidants, either dietary supplemented or as ingredients of extenders, on avian semen characteristics are well studied [12,15,25–28].

Metal chelators are also accepted as antioxidants, since they prevent the generation of the hydroxyl radicals (O˙H) that are assumed as the most damaging agents within cells [29]. Transition metals (mainly iron or copper) catalyse the formation of ˙OH *via* Fenton reaction from superoxide anion radicals ($O^{\bullet}_2$-) and H_2O_2 that naturally occur as a consequence of the cell metabolism. Having in mind that almost any mixture used in the laboratory practice contains metal ions contamination that even in very low concentrations are able to generate O˙H, it could be hypothesized that the use of chelators during sperm manipulation will ameliorate the spermatozoa oxidative damage.

Thus, the aim of the present study was to determine the effect of Desferal, on the levels of lipid peroxidation, total glutathione (tGSH), and motility, and kinetic parameters of semen from Muscovy ducks diluted and stored for 6 h at 4 °C and incubated with different prooxidants – $FeSO_4$ (Fe^{2+}), H_2O_2 or Fenton system ($FeSO_4$ (Fe^{2+}) + H_2O_2). In the present study, Desferal (Deferoxamine) was selected as a specific chelator of iron ions. The latter are involved in the generation of hydroxyl radicals, which are considered to be the most damaging agent of biomolecules by the Fenton reaction mechanism. In addition, Desferal has antioxidant properties that are independent of its ability to bind iron. It has been demonstrated that Desferal is able to donate an electron or hydrogen atom from its hydroxamate center, inhibiting oxidation [30–32].

2. Material and methods

2.1. Birds and housing

The experiment was conducted with 10 previously selected Muscovy drakes with well manifested sexual desire (line CF 80 autosexing, a product of Grimaud Frères Sélection, France) during first reproductive period from May to July. The males were clinically healthy, kept individually in spatial, metal cages with size 0.6 × 0.8 × 0.6 m placed in a semi-open shed under natural light situated in the Poultry Division at the Agricultural University of Plovdiv. The birds were fed with a pelleted mixture for breeding drakes comprising: 11.5 MJ/kg metabolize energy, 15.7 % crude protein, 4.5 % crude fibres, 2.1 % crude fats, 1.03 % calcium, 0.75 % phosphorus total, 0.8 % L–lysine and 0.42 % DL–methionine + cysteine. Daily ration was 200−230 g per bird. The intake of water was provided *ad libitum*.

2.2. Semen collection

The semen was collected regularly two times per week individually by placing a female (teaser method) in the male's cage using an artificial vagina – by the method of Tan [33] modified by Gerzilov [34]. On the particular day of collection, the volume of pooled ejaculates varied from 6.4–12.2 mL (rather about 8−10 mL on average). The intense sexual excitation of the drake is manifested by the characteristic tail movement in a horizontal plane. The male brings the swollen cloaca as close as possible to that of the female, seeking a mating. In fact, the male's tail is directed down semi vertically and always to the left of the female's tail.

Instant before touching both cloacae by pressing at the base and side of the male cloaca, the copulatory organ erects in the artificial vagina (previously focused on the cloaca) and ejaculates for about 3–4 seconds. The artificial vagina consisted of a rubber muff and a graduated test-tube.

Only good quality ejaculates were mixed (color – pearly-white; purity – free of any contamination with cloacal products; volume – above 0.3 mL; sperm motility – above 70 % under light microscope Nikon Alphaphot-2YS2 (10 × 40) with phase contrast condenser CETI. The ejaculates were pooled to avoid the effects of individual differences among males.The pooled semen sample was gingerly mixed with the use of automatic pipette and divided into four equal parts and diluted at a ratio 1:3 (v/v, semen: extender) with saline solution, IMV Canadyl, HIA-1 and AU extenders.

The IMV Canadyl extender is commercial product from IMV-Technologies, France (https://www.imv-technologies.com/product/canadyl).

Both extenders HIA-1 and AU were developed by Gerzilov [4].

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 following components: 0.40 g D–glucose, 0.80 g D–fructose, 0.80 g sugar, 0.90 g sodium citrate, 0.84 g sodium glutamate, 0.40 glycocol, 0.04 g ethylenediaminetetraacetic acid disodium salt dihydrate, and plus 100 mL double distilled water. The osmolarity was 320 mOsmol/kg and pH - 7.00.

The diluted semen was transported in an electric cool box car refrigerator 25 L at 4 °C from Poultry division at Agricultural University of Plovdiv to Institute of Biology and Immunology of Reproduction "Academician Kiril Bratanov" at Bulgarian Academy of Sciences, Sofia.

2.3. Sperm analysis

The sperm analysis was performed in a specialized laboratory of the Institute of Biology and Immunology of Reproduction, Sofia, Bulgaria. The assessment of semen quality parameters and various kinematic parameters of motile spermatozoa was carried out with a CASA system (Sperm Class Analyzer [SCA] 5.0. Microptic, Barcelona, Spain). Sperm concentration was adjusted to SCA analysis by dilution, loaded into a Leja 20 chamber (Leja Products B.V., Nieuw-Vennep, The Netherlands) and examined using a microscope with warm stage (Nikon, Tokyo, Japan). The diluted semen in volume of 10 μL was placed on a glass slide, covered, and examined in five different fields. The standard parameter settings for poultry semen were used: total motility (%) – % of progressive and non-progressive spermatozoa; progressive movement of spermatozoa (%); sperm velocity of movement (%) – static spermatozoa (under 10 μm/s), slow velocity (10−50 μm/s), medium velocity (50−100 μm/s), rapid velocity of sperm cells (over 100 μm/s). In addition, the software of SCA also measured the following velocity parameters were recorded:

- The curvilinear velocity (VCL, μm/s - the average path velocity of the sperm head along its actual trajectory);
- The 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);
- The average path velocity (VAP, μm/s - the average velocity of the sperm head along its average trajectory);
- The percentage of linearity (LIN, % - the ratio between VSL and VCL);
- The percentage of straightness (STR, % - the ratio between VSL and VAP).
- The percentage of the wobble (WOB, % - which reflects the measure of oscillation of the actual path about the average path.

2.4. Determination of oxidative stress of sperm diluted with different extenders in the presence of oxidants: ferrous sulphate, hydrogen peroxide, and Fenton system

Sperm aliquots (50×10^6 sperm cells/mL final concentration) were incubated with the prooxidative agents (in corresponding final concentrations) – $FeSO_4$ (0.1 mM), H_2O_2 (1 mM) and $FeSO_4$ (0.1 mM) + H_2O_2 (1 mM) (Fenton system) in presence or absence of Desferal (0.1 mM, final concentration) at 37 °C for 30 min. Thus, the following groups according to the given type of extenders, prooxidants (with or without) and Desferal (with or without) were obtained: Control groups, containing diluted semen in saline, IMV Canadyl, HIA-1 or AU; $FeSO_4$ groups, containing semen diluted in saline or in the respective extender, incubated with $FeSO_4$; H_2O_2 groups, containing semen diluted in saline or in the respective extender, incubated with H_2O_2; Fenton groups, containing semen diluted in saline or in the respective extender, incubated with $FeSO_4$ + H_2O_2 (Fenton system). Each of above mentioned groups was tested in the absence and presence of the iron chelator Desferal (Deferoxamine; formula – $C_{25}H_{48}N_6O_8$; commercial product from Novartis Bulgaria, multinational pharmaceutical corporation based in Basel, Switzerland). Thus, the total number of groups was 32 in determining LPO and tGSH, and 36 in determining sperm motility and velocity of spermatozoa before and after incubation.

2.4.1. Lipid peroxidation assay

Lipid peroxidation and amount of total glutathione that are widely used as markers of oxidative stress were assessed. Lipid peroxidation was determined by the amount of the thiobarbituric acid reactive substances, according to the method of Hunter et al. [35]. After the incubation of semen with prooxidants ± Desferal, to the sperm aliquots were added 0.6 mL of a mixture, containing 2.8 % trichloroacetic acid: 5 N HCl: 2% thiobarbituric acid in 50 mM NaOH (2:1:2 v/v). For color development, the samples were heated at 100 °C for 15 min. After cooling and centrifuging at 1000×g for 10 min the absorbance of supernatants was read at 532 nm using a Jenway 6305 Single Beam UV/Visible Spectrophotometer (Bibby Scientific Ltd, UK). The values were expressed in nanomoles of malondialdehyde (MDA)/mL sperm, using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.4.2. Measurement of amount of total glutathione

Total glutathione (tGSH) concentration was determined according to Rahman et al. [36]. The assay is based on the reaction of GSH with 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB), producing colored substance 5′-thio-2-nitrobenzoic acid (TNB) with absorbance pick at 412 nm. The speed of TNB formation is proportional to the concentration of tGSH in the sample. The tGSH concentration was calculated by using a reference standard and expressed as ng/mg protein.

2.5. Statistical analysis

Data of sperm motility of diluted semen stored at 4 °C for 6 h were analyzed by one-way ANOVA. The interactions between main effects (extender × prooxidant × Desferal) influencing the OS markers (LPO and tGSH) and sperm motility after induction oxidative stress were analyzed by three-way analysis of variance (ANOVA). The significance of differences between the examined values was determined by applying post-hoc analysis and Tukey's test. Differences between the treatment values were considered statistically at $P \leq 0.05$. The analysis of variance (ANOVA) was performed in the IBM Statistics SPSS 24 statistical environment [37,38].

3. Results

The total and progressive motility of sperm cells stored *in vitro* at temperature 4 °C for 6 h was high (Table 1). With regard to the kinetic parameters of sperm motility, the same trend was observed (Table 2). Prior to the addition of the prooxidants, the *in vitro* stored spermatozoa had high rate parameters. No proven differences depending on the extender ($P > 0.05$) were found. This shows that the used four extenders are suitable for *in vitro* storage of sperm cells for 6 h under cool conditions.

The incubation of semen with $FeSO_4$ and combination $FeSO_4$+ H_2O_2 (Fenton system) led to significant increase of LPO, independently of the extender used (Fig. 1, left panel). The addition of H_2O_2 to the reaction medium increased statistically significantly only the LPO in the sperm, diluted with saline. When comparing the protective effect of extenders against prooxidative impact, in comparison to IMV and AU, HIA-1 inhibited significantly the induction of LPO by H_2O_2 and Fenton system (Fig. 1, left panel). HIA-1 showed a much better protective effect compared to other extenders under Fenton system conditions. In presence of $FeSO_4$ the three extenders used compared to saline had similar protective effect, reducing sperm LPO almost twice.

The addition of Desferal to the semen samples led to a significant ($P < 0.05$) reduction in sperm LPO induced by $FeSO_4$ and Fenton system and had no effect in control and H_2O_2 incubated groups compared to the chelator-free samples (Fig. 1, right panel). The beneficial effect of Desferal on LPO was most pronounced in samples diluted with saline, suggesting a protective effect of extenders that was potentiated by the Desferal when semen was exposed to $FeSO_4$ or $FeSO_4$+ H_2O_2 (Fenton system). A significant decrease in the tGSH levels was observed after addition of prooxidants (Fig. 2, left panel). The tGSH depletion was most significant in semen diluted with saline in presence of Fenton system. In the control groups, the level of tGSH was the lowest in sperm samples diluted with AU. Semen diluted with HIA-1 extender after addition of the prooxidants (FeSO4, H_2O_2, and Fenton system) showed higher tGSH values compared to those of samples diluted with the other extenders. There were no statistically significant differences in the tGSH levels between the semen diluted with IMV Canadil and AU extenders.

In presence of Desferal, the levels of tGSH in all groups incubated with prooxidants were elevated in comparison to the corresponding groups without chelator, as only in the samples diluted with AU this increase was insignificant (Fig. 2, right panel). The addition of Desferal in the groups of semen diluted in HIA-1 extender and incubated with H_2O_2, showed the best protection of semen tGSH concentration, close to that of the control group.

After incubation at 37 °C for 30 min with the prooxidants - $FeSO_4$, H_2O_2, and Fenton system, the total and progressive sperm motility in diluted semen decreased significantly ($P < 0.05$) while static sperm cells increased significantly ($P < 0.05$) – Table 3. There were statistically insignificant higher progressive motility of sperm diluted with saline, IMV and AU in the samples incubated with $FeSO_4$ compared to those incubated with H_2O_2 and Fenton system.

In contrast to our expectations, the results showed that the addition of the metal chelator Desferal to the reaction medium did not significantly increase sperm motility. The only statistically significant increase in progressive motile sperm and decrease in static sperm in the presence of Desfeal was found in samples diluted in saline and incubated with $FeSO_4$.

The sperm velocity parameters were higher for VCL, VSL and VAP and lower for LIN, STR and WOB in cooled semen at 4 °C for 6 h

Table 1
Sperm motility of diluted semen stored at 4 °C for 6 h.

Extender	Total motile, %	Progressive motile, %	Non-progressive motile, %	Static sperm cells, %	Rapid sperm cells, %	Medium sperm cells, %	Slow Sperm cells %
Salin solution	97.20 ± 1.7	17.88 ± 1.7	79.33 ± 1.2	2.83 ± 1.7	3.65 ± 0.6	58.00 ± 8.3	35.53 ± 7.3
IMV Canadyl	98.75 ± 0.7	17.95 ± 0.3	80.80 ± 0.4	1.25 ± 0.7	2.55 ± 0.3	68.15 ± 1.4	28.00 ± 0.9
HIA-1	98.85 ± 0.7	18.70 ± 0.6	80.27 ± 0.3	1.18 ± 0.7	3.50 ± 1.8	64.83 ± 7.7	30.47 ± 9.1
AU	96.80 ± 1.5	16.98 ± 0.6	79.83 ± 1.0	3.23 ± 1.5	3.53 ± 1.0	63.93 ± 6.8	29.30 ± 5.6
SEM	0.66	0.52	0.44	0.66	0.45	3.46	3.13
P-Value	0.65	0.76	0.73	0.64	0.87	0.51	0.64

Table 2
Velocity sperm parameters of diluted semen stored at 4 °C for 6 h.

Extender	VCL, μm/s	VSL, μm/s	VAP, μm/s	LIN, %	STR, %	WOB, %
Salin solution	110.25 ± 10.6	31.98 ± 1.4	58.58 ± 3.8	29.55 ± 2.1	54.83 ± 1.7	53.70 ± 2.1
IMV Canadyl	115.10 ± 3.8	31.20 ± 0.9	56.55 ± 3.1	30.20 ± 1.9	55.40 ± 1.4	54.40 ± 2.0
HIA-1	112.87 ± 13.6	32.33 ± 2.0	58.10 ± 5.8	31.37 ± 2.6	56.17 ± 2.2	55.60 ± 2.6
AU	105.98 ± 9.3	30.80 ± 1.0	56.80 ± 3.3	29.60 ± 2.0	54.60 ± 1.7	54.05 ± 1.9
SEM	4.54	0.62	1.78	0.97	0.80	0.97
P-Value	0.93	0.85	0.98	0.93	0.93	0.94

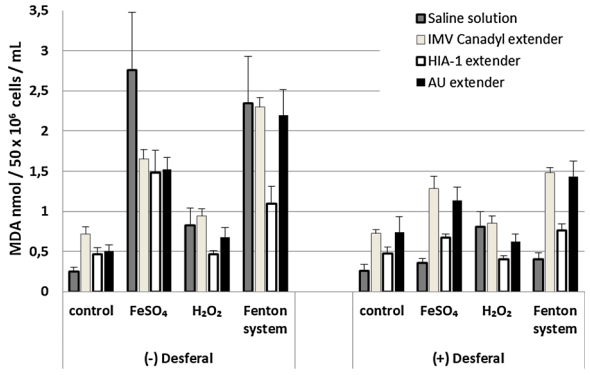

Fig. 1. Effect of prooxidants and Desferal on the lipid peroxidation of diluted semen in saline solution, IMV Canadyl extender, HIA-1 extender and AU extender. The control groups contained diluted semen samples without prooxidants.

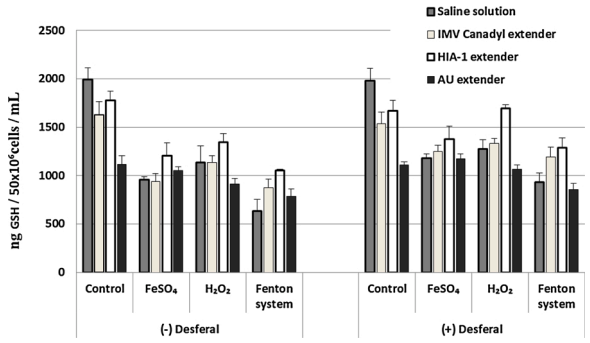

Fig. 2. Effect of prooxidants and Desferal on the total glutathione of diluted semen in saline solution, IMV Canadyl extender, HIA-1 extender and AU extender. The control groups are diluted semen samples without prooxidants.

(Table 2) *versus* incubated semen at 37 °C for 30 min. The addition of Desferal did not affect the sperm velocity parameters ($P > 0.05$) – Table 4.

The kinetic parameters of the diluted semen stored at 4 °C for 6 h before incubation with the prooxidative agents were similar to those obtained in our previous study, which shows that these are probably the normal values for this avian species [39].

4. Discussion

A large number of studies have shown that poultry sperm is vulnerable to OS, as the latter can be responsible for sperm damage and reproductive failure. The degree of sperm OS sensitivity depends on the animal species. Higher levels of LPO as a marker of OS have been found in semen of ganders than in partridge roosters [10]. These higher levels of LPO in ganders have been also accompanied by higher percent of spermatozoa with damaged cell membrane, while in partridge roosters along with the lower levels of LPO there was also lower percent of spermatozoa with impaired membranes [9,10]. Muscovy duck semen is susceptible to OS and antioxidant supplementation maintain the quantity and quality of semen and the fertility rate *in vivo* [27]. The storage of Muscovy drake's semen at low temperature induces significant LPO, along with decrease in sperm motility and membrane damage [39–42]. In present study a pilot investigation of the effect of various prooxidants on Muscovy duck semen was performed aiming to reveal the mechanisms of OS induction and seek prevention. Our results showed that the application of various prooxidants led to different degrees of oxidative and functional damage. Hydrogen peroxide treatment showed the weakest effect on sperm LPO (Fig.1) and a significant adverse effect on sperm motility (Table 3). Similar results for the lack of a significant effect of H_2O_2 on LPO levels, but with a negative effect on motility parameters, were also observed in roosters [22], boars [43], rams [44,45], equines [46], even in men [47]. The most significant damages occur after application the combination of iron ions and H_2O_2 (Fenton system) (Fig.1, Table3). As expected, the hydroxyl radicals (O˙H) generated in the medium by the Fenton reaction have the strongest damaging effect. It is well known that O˙H is extremely reactive, attacks and oxidizes every molecule in their immediate vicinity that makes them the most damaging agents within cells [29]. They are able to induce LPO in the membrane structures of sperm, which in turn leads to an impairment of their fluidity and permeability with negative consequences for fertilization. It has been demonstrated that among various prooxidants (superoxide anion radical, hydrogen peroxide, hydroxyl radicals), the incubation of rooster semen in medium, generating O˙H, lead to impair the plasma membrane and acrosome and DNA integrity, and also mitochondrial activity [22]. Furthermore, these semen aliquots, used for artificial insemination, demonstrated sharply reduced fertility.

Another important marker of cellular OS is glutathione (GSH). In this study, all prooxidants led to decrease of GSH concentration in Muscovy duck semen, as the combination of iron ions and hydrogen peroxide (Fenton system) had the strongest effect (Fig. 2). Glutathione is endogenous non-enzymatic antioxidant, which plays a significant role in intracellular defense system against OS in avian sperm [48]. As direct interaction with ROS [49] or as a cofactor of glutathione peroxidase [50] it neutralizes reactive oxygen species (ROS), including H_2O_2 and lipid peroxides. It has been found that avian semen extenders enriched with GSH, preserved the viability of sperm cells, plasma membrane integrity, functional, kinetic and fertility parameters, suppressed OS in diluted

Table 3
Effect of extenders, prooxidants and Desferal on the sperm motility after induction oxidative stress, in %.

Effect of								
Extender	Effect of Pro-oxidant	Effect of Desferal	Progres-sive motile, %	Non-progressive motile, %	Static, %	Rapid, %	Medium, %	Slow, %
Saline solution	Control	(-)	7.80 ± 5.1	53.08 ± 2.1^{ab}	39.10 ± 4.9^{abc}	2.40 ± 2.3	9.68 ± 4.1	48.78 ± 3.3
	$FeSO_4$		0.58 ± 0.4	18.28 ± 3.1^{b}	81.18 ± 3.43^{ab}	0.05 ± 0.0	0.98 ± 0.4	17.80 ± 3.1
	H_2O_2		0.28 ± 0.2	24.20 ± 7.3^{ab}	75.55 ± 7.4^{abc}	0.13 ± 0.1	0.38 ± 0.3	24.00 ± 7.3
	Fenton		0.18 ± 0.1	20.83 ± 6.6^{ab}	79.10 ± 6.6^{abc}	0.00	0.30 ± 0.1	20.60 ± 6.6
	Control	(+)	1.75 ± 1.1	34.60 ± 6.8^{ab}	63.63 ± 7.4^{abc}	0.55 ± 0.5	1.88 ± 0.7	33.95 ± 6.7
	$FeSO_4$		4.90 ± 2.1	51.50 ± 10.7^{ab}	43.60 ± 11.4^{abc}	0.20 ± 0.1	7.43 ± 2.8	48.80 ± 10.0
	H_2O_2		0.65 ± 0.4	18.53 ± 5.7^{b}	80.83 ± 6.1^{ab}	0.08 ± 0.0	1.03 ± 0.3	18.05 ± 5.8
	Fenton		0.18 ± 0.1	18.33 ± 3.0^{b}	81.48 ± 3.0^{a}	0.08 ± 0.0	0.43 ± 0.2	18.03 ± 3.1
IMV	Control	(-)	12.5 ± 6.2	60.73 ± 4.9^{ab}	26.73 ± 5.9^{abc}	3.27 ± 3.0	14.40 ± 5.9	55.60 ± 4.9
	$FeSO_4$		2.90 ± 1.8	50.03 ± 12.5^{ab}	47.07 ± 13.9^{abc}	0.17 ± 0.1	4.63 ± 2.5	48.17 ± 11.7
	H_2O_2		0.33 ± 0.2	30.93 ± 11.9^{ab}	68.73 ± 12.0^{abc}	0.17 ± 0.1	1.00 ± 0.6	30.10 ± 11.7
	Fenton		0.80 ± 0.4	44.47 ± 5.4^{ab}	54.73 ± 5.8^{abc}	0.03 ± 0.0	2.43 ± 1.5	42.80 ± 4.2
	Control	(+)	9.40 ± 6.8	54.80 ± 7.3^{ab}	35.80 ± 11.2^{abc}	1.17 ± 0.6	10.90 ± 8.6	52.17 ± 6.5
	$FeSO_4$		4.57 ± 2.7	51.17 ± 20.9^{ab}	44.30 ± 22.9^{abc}	0.13 ± 0.1	8.10 ± 4.0	47.43 ± 19.2
	H_2O_2		0.23 ± 0.1	21.13 ± 9.4^{ab}	78.63 ± 9.5^{abc}	0.00	0.33 ± 0.0	21.00 ± 9.4
	Fenton		1.57 ± 0.8	37.63 ± 4.8^{ab}	60.77 ± 5.5^{abc}	0.17 ± 0.1	2.47 ± 1.5	36.57 ± 3.9
HIA-1	Control	(-)	0.80 ± 0.4	63.03 ± 11.4^{ab}	36.10 ± 11.6^{abc}	0.00	1.67 ± 0.5	62.20 ± 11.4
	$FeSO_4$		0.50 ± 0.1	50.00 ± 6.9^{ab}	49.50 ± 6.8^{abc}	0.03 ± 0.0	1.83 ± 0.6	48.70 ± 7.3
	H_2O_2		0.17 ± 0.1	24.80 ± 9.6^{ab}	75.03 ± 9.6^{abc}	0.00	0.70 ± 0.2	24.23 ± 9.5
	Fenton		0.73 ± 0.4	30.07 ± 13.0^{ab}	69.23 ± 13.4^{abc}	0.03 ± 0.0	2.07 ± 1.7	28.70 ± 11.7
	Control	(+)	1.23 ± 1.0	59.50 ± 8.7^{ab}	39.27 ± 9.6^{abc}	0.03 ± 0.0	3.13 ± 2.3	57.57 ± 7.7
	$FeSO_4$		0.27 ± 0.2	33.33 ± 19.3^{ab}	66.43 ± 19.5^{abc}	0.03 ± 0.0	0.50 ± 0.3	33.03 ± 19.2
	H_2O_2		0.33 ± 0.1	17.70 ± 3.8^{b}	82.00 ± 3.7^{a}	0.00	0.43 ± 0.1	17.53 ± 3.7
	Fenton		0.70 ± 0.0	31.43 ± 2.3^{ab}	68.50 ± 2.3^{abc}	0.00	0.37 ± 0.1	31.17 ± 2.2
AU	Control	(-)	6.35 ± 4.2	63.38 ± 5.5^{ab}	30.30 ± 8.3^{abc}	0.20 ± 0.1	12.58 ± 4.4	56.88 ± 4.3
	$FeSO_4$		6.08 ± 2.0	69.98 ± 9.7^{ab}	23.95 ± 11.3^{bc}	0.90 ± 0.3	17.95 ± 5.8	57.18 ± 5.9
	H_2O_2		2.28 ± 1.4	41.98 ± 13.4^{ab}	55.78 ± 14.5^{abc}	0.25 ± 0.2	6.73 ± 4.7	37.28 ± 10.8
	Fenton		1.03 ± 0.7	29.25 ± 14.3^{ab}	69.73 ± 15.0^{abc}	0.00	3.88 ± 3.2	26.38 ± 11.9
	Control	(+)	8.18 ± 3.5	68.38 ± 5.9^{ab}	23.43 ± 6.5^{c}	0.50 ± 0.3	11.85 ± 3.5	64.28 ± 6.7
	$FeSO_4$		5.70 ± 1.1	72.20 ± 3.1^{a}	22.15 ± 4.0^{c}	0.55 ± 0.3	12.90 ± 2.3	64.43 ± 5.3
	H_2O_2		0.98 ± 0.6	50.15 ± 12.1^{ab}	48.88 ± 12.4^{abc}	0.08 ± 0.0	4.48 ± 3.4	46.60 ± 10.8
	Fenton		1.48 ± 1.2	40.53 ± 11.9^{ab}	58.00 ± 13.1^{abc}	0.38 ± 0.3	6.53 ± 5.8	35.13 ± 7.3
SEM			0.578	3.012	3.452	0.126	0.909	2.687
Extender			0.018	0.000	0.000	0.408	0.000	0.001
Prooxidant			0.000	0.000	0.000	0.022	0.000	0.000
Desferal			0.908	0.970	0.965	0.357	0.683	0.892
Extender × Prooxidant			0.000	0.000	0.000	0.071	0.000	0.000
Extender × Desferal			0.190	0.003	0.003	0.691	0.001	0.010
Prooxidant × Desferal			0.000	0.000	0.000	0.068	0.007	0.000
Extender × Prooxidant × Desferal			0.060	0.000	0.000	0.060	0.228	0.063

Note: Values in the corresponding column with different superscripts are significantly different ($P < 0.05$).
In corresponding row (group).
Σ (% of progressive motile + % of non-progressive motile + % of static spermatozoa) = 100%.
Σ (% of rapid spermatozoa + % medium spermatozoa + % slow spermatozoa + % of static spermatozoa) = 100 %.

Table 4
Effect of extenders, prooxidants and Desferal on the sperm velocity parameters after induction oxidative stress, in %.

Effect of Extender	Effect of Pro-oxidant	Effect of Desfe ral	VCL, μm/s	VSL, μm/s	VAP, μm/s	LIN, %	STR, %	WOB, %
Saline solution	Control	(-)	4868 ± 6.6 ^{ab}	22.95 ± 5.2^{ab}	33.63 ± 5.5^{ab}	46.00 ± 4.3	66.88 ± 4.2	68.45 ± 2.0
	$FeSO_4$		38.93 ± 2.1^{ab}	15.05 ± 1.53^{ab}	23.83 ± 0.8^{ab}	39.45 ± 5.5	62.93 ± 4.4	61.03 ± 4.2
	H_2O_2		27.58 ± 3.5^{ab}	14.68 ± 1.23^{ab}	20.13 ± 1.8^{b}	54.85 ± 5.3	73.40 ± 3.6	74.20 ± 3.8
	Fenton		32.70 ± 4.2^{ab}	13.00 ± 0.69^{b}	20.00 ± 1.3^{b}	42.28 ± 6.7	65.85 ± 5.8	63.05 ± 4.9
	Control	(+)	35.73 ± 1.9^{ab}	17.48 ± 2.43^{ab}	24.33 ± 1.8^{ab}	49.08 ± 6.8	70.83 ± 4.6	68.43 ± 4.8
	$FeSO_4$		41.25 ± 5.9^{ab}	20.03 ± 2.63^{ab}	28.63 ± 3.6^{ab}	49.25 ± 2.6	70.08 ± 1.7	70.15 ± 2.3
	H_2O_2		31.85 ± 2.6^{ab}	15.25 ± 1.96^{ab}	21.88 ± 1.6^{ab}	48.90 ± 8.2	69.30 ± 6.0	69.28 ± 5.1
	Fenton		35.73 ± 2.8^{ab}	14.75 ± 0.75^{ab}	22.28 ± 1.4^{ab}	41.65 ± 2.1	66.53 ± 2.3	62.53 ± 1.3
IMV	Control	(-)	53.33 ± 9.2^{ab}	27.30 ± 5.80^{a}	37.47 ± 6.6^{a}	51.07 ± 4.5	72.17 ± 3.5	70.53 ± 3.2
	$FeSO_4$		36.60 ± 3.5^{ab}	20.00 ± 1.70^{ab}	26.87 ± 2.3^{ab}	54.83 ± 1.1	74.63 ± 0.4	73.47 ± 1.1
	H_2O_2		29.47 ± 2.8^{ab}	16.30 ± 0.61^{ab}	22.17 ± 0.9^{ab}	56.23 ± 5.6	73.70 ± 4.3	75.97 ± 3.6
	Fenton		33.43 ± 6.8^{ab}	14.67 ± 1.53^{ab}	22.43 ± 3.5^{ab}	46.03 ± 6.3	66.70 ± 5.2	68.50 ± 3.8
	Control	(+)	41.97 ± 10.4^{ab}	23.33 ± 5.68^{ab}	30.53 ± 6.9^{ab}	55.73 ± 6.6	75.50 ± 5.2	73.40 ± 3.8
	$FeSO_4$		45.47 ± 4.5ab	18.23 ± 3.00^{ab}	29.03 ± 3.7^{ab}	39.67 ± 3.4	62.20 ± 3.2	63.50 ± 2.2
	H_2O_2		29.80 ± 1.7ab	13.57 ± 0.62^{b}	19.70 ± 0.8^{b}	45.70 ± 2.7	68.70 ± 0.6	66.47 ± 3.5
	Fenton		31.50 ± 6.6ab	18.00 ± 2.23^{ab}	23.83 ± 3.2^{ab}	59.93 ± 8.4	76.07 ± 5.7	78.07 ± 6.1
HIA-1	Control	(-)	30.87 ± 3.1^{ab}	16.90 ± 1.76^{ab}	23.13 ± 2.3^{ab}	54.77 ± 1.8	73.13 ± 1.4	74.87 ± 1.1
	$FeSO_4$		33.37 ± 6.1^{ab}	16.23 ± 1.58^{ab}	22.97 ± 3.0^{ab}	50.73 ± 5.6	71.33 ± 3.2	70.73 ± 5.0
	H_2O_2		28.97 ± 1.6^{ab}	14.47 ± 0.52^{ab}	20.40 ± 0.3^{ab}	50.30 ± 4.7	70.63 ± 1.7	71.03 ± 5.0
	Fenton		35.57 ± 6.0^{ab}	15.37 ± 1.49^{ab}	23.53 ± 3.2^{ab}	44.33 ± 3.3	66.07 ± 2.7	67.00 ± 2.4
	Control	(+)	31.60 ± 7.3^{ab}	14.73 ± 2.41^{ab}	21.73 ± 4.1^{ab}	48.17 ± 3.7	68.53 ± 2.5	70.07 ± 2.9
	$FeSO_4$		29.30 ± 3.7^{ab}	12.77 ± 0.98b	19.60 ± 1.6^{b}	44.57 ± 5.3	65.33 ± 3.7	67.73 ± 4.5
	H_2O_2		32.67 ± 2.8 ^{ab}	15.93 ± 1.19^{ab}	21.87 ± 1.2^{ab}	49.27 ± 4.9	72.50 ± 2.1	67.67 ± 5.2
	Fenton		25.30 ± 2.5^{b}	14.43 ± 0.69^{ab}	19.37 ± 0.5^{b}	58.13 ± 6.1	74.47 ± 2.6	77.70 ± 5.9
AU	Control	(-)	55.33 ± 5.2^{ab}	21.15 ± 3.69^{ab}	33.08 ± 3.5^{ab}	38.28 ± 5.7	62.98 ± 5.2	59.88 ± 3.7
	$FeSO_4$		59.55 ± 7.1^{a}	20.38 ± 1.27^{ab}	34.33 ± 2.1^{ab}	35.98 ± 5.6	59.83 ± 4.4	59.15 ± 4.7
	H_2O_2		44.70 ± 10.4^{ab}	16.98 ± 1.93^{ab}	27.15 ± 4.3^{ab}	40.78 ± 4.0	63.80 ± 2.7	63.43 ± 3.9
	Fenton		43.78 ± 5.7^{ab}	16.73 ± 1.34^{ab}	26.40 ± 3.0^{ab}	39.80 ± 5.5	64.45 ± 4.4	61.23 ± 4.8
	Control	(+)	51.25 ± 1.8^{ab}	22.98 ± 3.63^{ab}	33.80 ± 3.0^{ab}	44.30 ± 5.5	66.78 ± 4.7	65.58 ± 3.7
	$FeSO_4$		57.78 ± 7.5^{ab}	21.48 ± 1.49^{ab}	34.55 ± 1.4^{ab}	40.23 ± 7.9	63.00 ± 6.6	61.95 ± 5.8
	H_2O_2		41.08 ± 9.9^{ab}	14.55 ± 1.50^{ab}	24.25 ± 3.8^{ab}	39.63 ± 7.6	61.58 ± 5.6	62.55 ± 6.9
	Fenton		39.55 ± 7.46^{b}	14.18 ± 0.84^{ab}	23.15 ± 2.6^{ab}	39.05 ± 6.9	62.40 ± 4.8	61.30 ± 6.2
SEM			1.649	0.627	0.921	1.159	0.819	0.948
Extender			0.000	0.028	0.000	0.000	0.000	0.000
Prooxidant			0.001	0.000	0.000	0.468	0.400	0.505
Desferal			0.434	0.568	0.409	0.752	0.763	0.806
Extender × Prooxidant			0.000	0.000	0.000	0.050	0.106	0.014
Extender × Desferal			0.000	0.196	0.004	0.005	0.011	0.002
Prooxidant × Desferal			0.012	0.000	0.000	0.577	0.631	0.557
Extender × Prooxidant × Desferal			0.000	0.005	0.000	0.167	0.305	0.083

Note: Values in the corresponding column with different superscripts are significantly different ($P < 0.05$).

fresh and post-thawed semen [28,51–53].

Given the involvement of iron ions in the O'H generation and the induction of OS, we hypothesized that the addition of Desferal in the reaction medium would have a beneficial effect on the studied sperm parameters of Muscovy drakes. Moreover, Desferal possesses antioxidant activity independent of its iron binding capability [31]. Expected, Desferal used in this study, significantly reduced the LPO, induced by Fe^{2+} and the $Fe^{2+} + H_2O_2$ combination and raised the lowered by these prooxidants tGSH levels. Our results are consistent with previous observations on the effect of Desferal on bovine sperm, where its addition to the medium led to a decrease in LPO [54]. In the same study was observed that Desferal reduces the immotile spermatozoa quantity and the number of morphological abnormalities [54]. It has been demonstrated that *in vitro* supplementation of metal chelators to media used for sperm storage and processing diminishing oxidative injury and improving the quality of the semen. Such studies have been conducted with EDTA, deferoxamine mesylate, penicillamine, 2,3-dimercaptopropan-1 sulfonate, and meso-2,3-dimercapto-succinimic acid [55,56]. In this study, however, we did not find a significant improvement in sperm motility and velocity parameters after Desferal addition, except the motility in the group, where the sperm diluted with saline was incubated with $FeSO_4$. Thus, the extenders provide the necessary protection of sperm cells when are exposed to prooxidants.

Among extenders we used, HIA-1 showed the best protective effect on Muscovy duck semen against the OS impact. We found that the increased values of LPO, induced by H_2O_2 and Fenton system, were twice lower in sperm, diluted with HIA-1, than those in sperm, diluted with other extenders (Fig. 1). All three extenders, that we used (IMV Canadyl, HIA-1 and AU) promote the reduction of LPO induced by ferrous sulfate compared to saline. In regards to tGSH, HIA-1 maintained it amount higher than the other extenders regardless of the prooxidant applied (Fig.2). Although HIA-1 provided better protection against OS, its effect in terms of functional parameters was the weakest (Table 3 and 4). The addition of Desferal had no statistically significant effect on motility and velocity parameters of semen diluted with the tested extenders. Lack of beneficial effects of five antioxidants including and Desferal on sperm motility during storage in a chemically-defined diluent in rams was found [57]. However, in boar semen the application of Desferal in the incubation medium had a protective effect against $FeSO_4$+ H_2O_2induced LPO, increase of immotile spermatozoa and morphological abnormalities [43]. Obliviously, further studies are needed to determine the role of antioxidant chelators in maintaining sperm quality and successful reproduction.

5. Conclusion

Muscovy duck semen is susceptible to OS, as $FeSO_4$ and a combination $FeSO_4 + H_2O_2$ (Fenton system) demonstrated a higher prooxidant effect, inducing LPO to a greater extent. The metal chelator Desferal had a beneficial effect in the semen samples, incubated with $FeSO_4$ and Fenton system by significantly lowering ($P < 0.05$) the LPO and protecting the tGSH level in Muscovy duck spermatozoa. The studied

prooxidants significantly reduced total and progressive sperm motility, as well as the velocity parameters of sperm ($P < 0.05$). Although Desferal reduced lipid peroxidation of semen, it had no statistically significant effect on motility and velocity parameters of sperm diluted with the extenders used (HIA-1, IMV Canadyl and AU). The HIA-1 extender showed the best protective effect on Muscovy duck semen against the OS impact, however its effect on the motillity and velocity parameters was the weakest compared to those of IMV Canadyl and AU. This study is the first one that attempts to elucidate the role of Desferal for maintaining the avian sperm quality in oxidative stress conditions.

Author statement

We hope you would appreciate the improved revision of our manuscript (revision 2). In case, you have new remarks, we are ready to correct them. We would also answer any additional questions you may have.

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Author's contribution

V. Gerzilov and P. Petrov – conceived and designed the experiments, secured funding, overseen the work, collection of ejaculates, manipulation, dilution, and transport of semen samples.

A. Alexandrova, E. Tsvetanova, A. Georgieva and M. Andreeva – manipulation of semen samples with prooxidants and Desferal, determination of lipid peroxidation and tGSH level.

M. Andreeva, R. Stefanov and V. Gerzilov – computing motile and kinetic sperm analysis

V. Gerzilov A. Alexandrova and A. Georgieva - performed the statistical analysis, writing and editing of the manuscript. All other authors edited and proofread it.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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