Polish Journal of Veterinary Sciences Vol. 26, No. 3 (2023), 497–509

DOI 10.24425/pjvs.2023.145050

Review paper

Lipid peroxidation in avian semen

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Abstract

The main cause of sperm chromatin damage is oxidative stress related to embryo development failure and adult infertility in mammals and also avian. Oxidative stress results in lipid peroxidation (LPO) causing cell damage. Lipid peroxidation is the oxidation of polyunsaturated fatty acids (PUFAs) in biological systems and causes changes in the physical structure and characteristics of the cell membrane. Due to the high amounts of PUFAs in the avian sperm membrane, its sperm seem susceptible to pe-roxidative damage and is a substantial factor in the fertilization capacity of sperm. The most commonly used methods for measuring LPO or its by-products, such as malondialdehyde (MDA) and 4-hydroksy-2-nonenal (4-HNE), in bird semen are based on the colorimetric method TBARS (thiobarbituric acid reactive substances) and on the use of a fluorescence probe (C_{11}-BODIPY$^{581/591}$) as a marker to evaluate membrane lipid peroxidation. This review aims first to introduce LPO in avian semen and its effects on avian sperm and second to summarize the commonly applied methods of evaluating LPO and its damage in fresh and stored avian semen.

Keywords: avian semen, lipid peroxidation, oxidative stress, semen cryopreservation

Introduction

Controlled production of reactive oxygen species (ROS) is required for the proper course of physiological processes in spermatozoa and fertilization (de Lamirande et al. 1997). The presence of physiological levels of ROS supports major spermatozoa functions such as motility, capacitation, acrosomal reaction, attachment to the oocyte's zona pellucida, and fusion with the oocyte (Dutta et al. 2019) (Fig.1). However, even a slight imbalance between ROS production and antioxidant (for example, antioxidant enzymes) protection causes oxidative stress (Hamilton et al. 2016). Reactive oxygen species are reactive molecules produced during oxygen reduction (Partyka et al. 2012a). Oxidative stress can cause major disorders in cell metabolism, including DNA-strand breakage, intracellular 'free' Ca2+ increase, damage to membrane ion transporters or other specific proteins, and lipid peroxidation (LPO) (Halliwell and Chirico 1993). Although protein and DNA damage are more significant and LPO often occurs later in the process (Halliwell and Chirico

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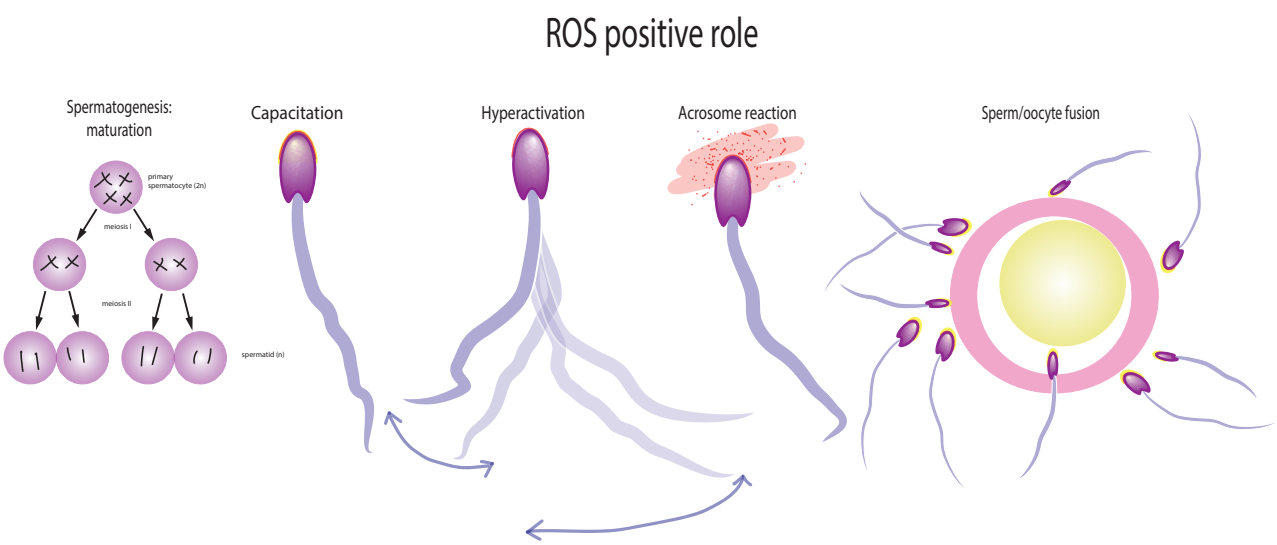

Fig. 1. The positive role of reactive oxygen species (ROS) in spermatozoa maturation and function. A primary spermatocyte (2n) turns into four spermatids (n) through meiosis (meiosis I & II) division. A spermatid must undergo some changes to be called spermatozoa. Sperm capacitation includes some physiological changes that make the sperm capable of penetrating and fertilizing an egg. Hyperactivation is a motility pattern seen during fertilization in mammals which may be critical to fertilization success. Acrosome reaction allows the spermatozoa to penetrate the zona pellucida and facilitate sperm/oocyte fusion in all species.

1993), it does not reduce the importance of occurring LPO as a result of oxidative stress. Therefore, this review will focus on the LPO process in spermatozoa cells and its impact on the gamete's function. In addition, we will describe the assays mostly used for its evaluation in avian semen.

Oxidative stress

The current definition of oxidative stress is "A situation with transient or chronically increased ROS concentration that can alter the cell's metabolism and damage its components" (Lushchak 2011, Lushchak 2014). Oxidative stress can be induced by antioxidant defense weakness, ROS production stimulating compounds, and externally added oxidants (Lushchak 2014). Oxidative stress in spermatozoa is associated with a high production of ROS and an increased rate of oxidation of cellular components (Alvarez and Storey 1982, Aitken et al. 1996, Partyka et al. 2012b). Although a low level of oxidative stress can have positive effects on cells (de Lamirande and Gagnon 1993), extensive oxidative stress can result in the impairment of mitochondrial activity, destruction of nucleic acids, proteins, and even carbohydrates and lipids, which can lead to cell death (Zini et al. 2000, Park et al, 2003, Agarwal et al. 2005, Peris et al. 2007, Partyka et al. 2012b) (Fig. 2).

Oxidative stress has been shown to be the main cause of sperm chromatin damage (Sakkas and Alvarez 2010, Hamilton et al. 2016). Since oxidative stress is consistently associated with abnormalities in spermatozoa, it can result in infertility (Agarwal et al. 2014, Hamilton et al. 2016). Moreover, it has previously been reported as the main reason for male infertility (Aitken and Baker 2002, Agarwal et al. 2003, Agarwal et al. 2008, Agarwal et al, 2014, Hamilton et al. 2016). Lushchak (2014) classified oxidative stress as basal oxidative stress (BOS), low-intensity oxidative stress (LOS), intermediate-intensity oxidative stress (IOS), and high-intensity oxidative stress (HOS). Other classifications are based on potential interest, such as mild oxidative stress (MOS), temperate oxidative stress (TOS), and finally, severe (strong) oxidative stress (SOS) (Lushchak 2014). Furthermore, it was stated that under mild or intermediate oxidative stress, organisms usually block their general life cycle programs, such as reproduction or extensive biosynthesis, to prevent or neutralize the negative effects of ROS (Lushchak 2014). Under stress conditions, the level of ROS may increase further than the normal range causing acute or chronic oxidative stress, and under some conditions, ROS levels may not return to their initial range and stabilize at a new quasi-stationary level (Lushchak 2014).

Lipid peroxidation

Lipid peroxidation is the oxidation of polyunsaturated fatty acids (PUFAs) in biological systems (Gutteridge 1995). Polyunsaturated fatty acids are long-chain fatty acids with more than one double bond (for example, linoleic acid, arachidonic acid, and docosahexaenoic acid) (Gaschler and Stockwell 2017). Actually, the presence of double bonds in fatty acids makes the C-H bond weaker. Therefore, PUFAs are particularly sensitive to peroxidation (Gutteridge 1995). In summary, we can say that oxidative stress results

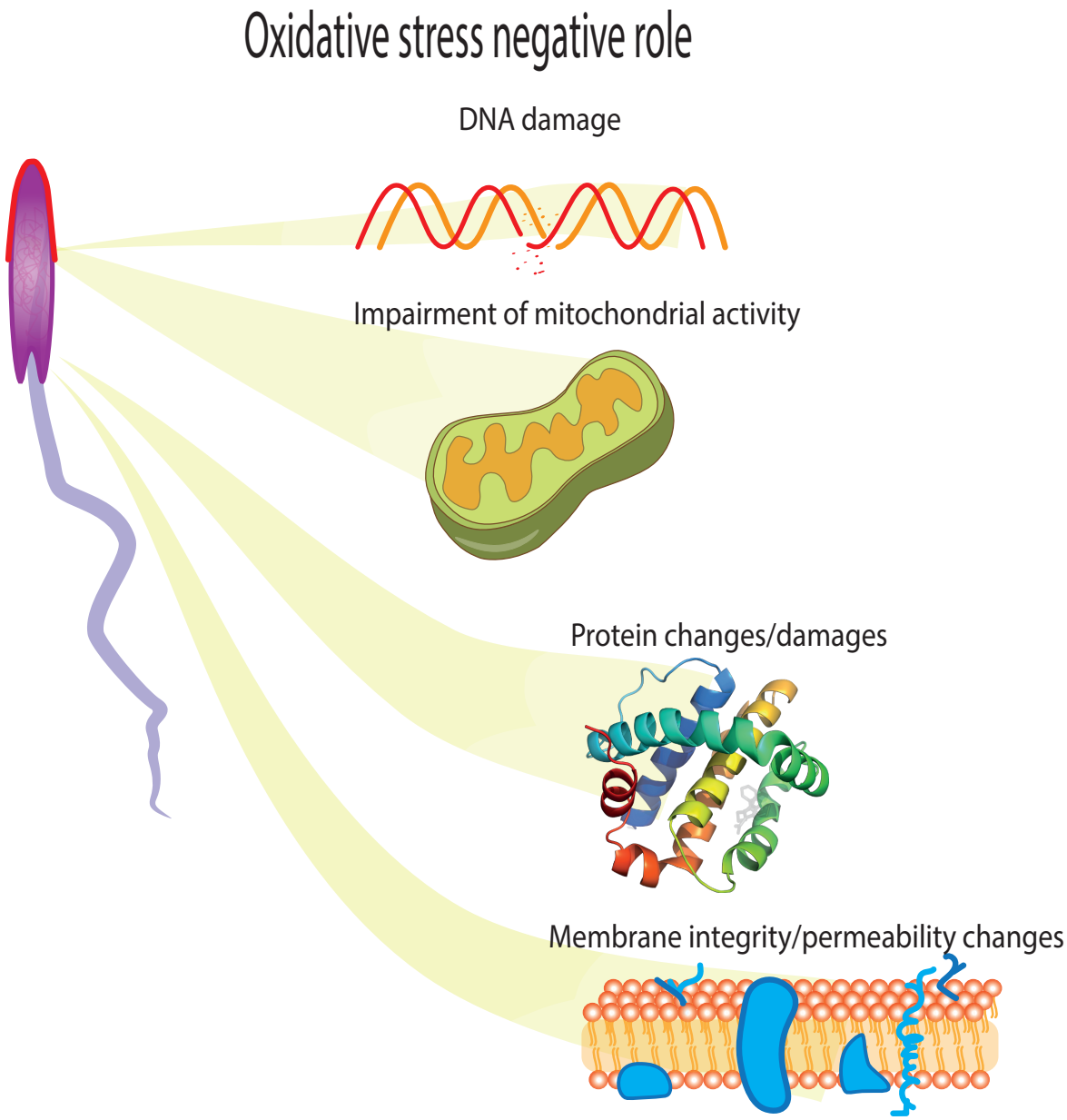

Fig. 2. The negative effect of oxidative stress on spermatozoa leads to cell damage. DNA damage, impairment of mitochondrial activity, changes or damages to protein structures, and changes in permeability/integrity of the spermatozoa membrane could be some of the negative results of oxidative stress on spermatozoa.

in LPO, which causes cell damage. But it can be more common that oxidative stress causes cell damage and results in secondary increased LPO of damaged cells (Halliwell and Chirico 1993).

There are two types of biological LPO, including:

– Non-enzymatic LPO, and

– Enzymatic peroxidation (Gutteridge 1995).

The process of non-enzymatic LPO, which is mediated by oxygen-centered and carbon-centered radicals, as well as all other radical reactions, can be categorized into three distinct stages: initiation, propagation, and termination (Fig. 3) (Gutteridge 1995, Gaschler and Stockwell 2017). Although the term "initiation" is typically used to describe any reaction that accelerates LPO, Gutteridge (1995) argued that it should only be used to refer to the reaction that initiates the first chain. LPO's propagation stage in a biological membrane does not progress until it reaches a protein. Thus, in vivo LPO can cause significant damage to membrane proteins, which can be more severe than damage to membrane lipids (Wolff and Dean 1986, Davies 1987, Gutteridge 1995).

Lipids are a primary component of the cell membrane, and they play a crucial role in maintaining the structural integrity of the cell. As a result, peroxidation of membrane lipids leads to alterations in the physical structure and properties of the cell membrane (Gaschler and Stockwell 2017). This peroxidation causes a reduc-

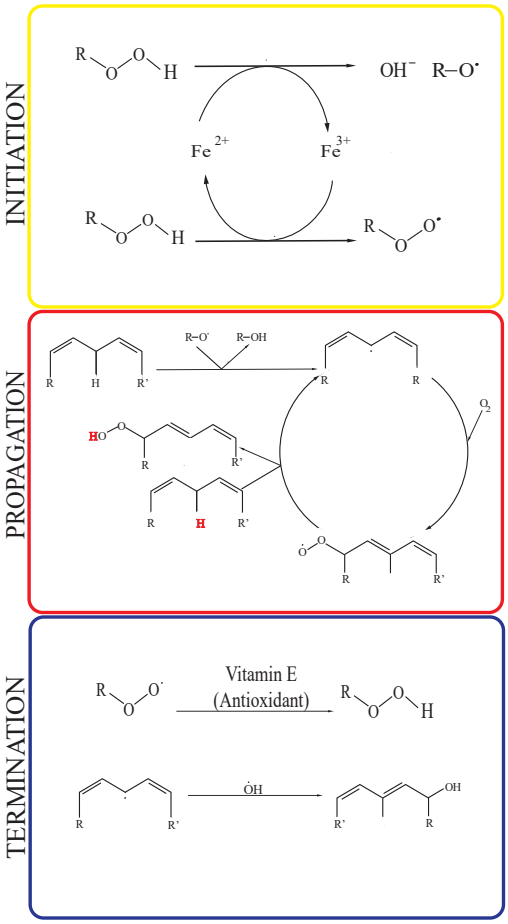

Fig. 3. The three steps of non-enzymatic lipid peroxidation: initiation, propagation and termination. Fe2+ ions facilitate the non-enzymatic lipid peroxidation initiation in the presence of molecular oxygen, and the first radicals are generated. During propagation, radicals can create new radicals by reacting with new substrates. In the termination step, antioxidants quench radicals or react with another radical.

tion in fluidity, decreased membrane potential, heightened permeability to ions, rupture, and release of cell and organelle contents, which are all outcomes of LPO in biological membranes (Esterbauer et al. 1991, Gutteridge 1995). Additionally, evidence suggests that LPO can occur in plasma and nuclear membranes, meaning oxygen radicals can interact with chromatin and DNA (Higuchi 2004, Barrera et al. 2008).

In addition, LPO has been identified as the initial driver of ferroptosis occurrence (Gaschler and Stockwell 2017). In fact, ferroptosis is a non-apoptotic form of regulated cell death that can be identified by the mass of lipid peroxides (Gaschler and Stockwell 2017). Furthermore, an increase in LPO was observed in ferroptotic cells without an increase in other ROS (such as superoxide) (Gaschler and Stockwell 2017).

Lipid peroxides and their by-products

Lipid peroxides are toxic and can damage most cells in the body (Halliwell and Chirico 1993). Lipid peroxides destroy phospholipid structures such as lipid membranes and subcellular structures (mitochondria and lysosomes), causing enzyme inactivation, protein degradation, and dysfunction. Due to their significant role in various diseases and even death, such as inflammation, cancer, and neurodegenerative diseases, it is crucial to identify and mitigate the toxic effects of lipid peroxides (Gaschler and Stockwell 2017). Lipid peroxides are typically categorized into lipid endoperoxides and lipid hydroperoxides, which are recognized as crucial mediators of cell disease and death (Gaschler and Stockwell 2017).

Lipid peroxide is degraded into malondialdehyde (MDA) and 4-hydroxy-2-noneal (4 HNE) (Fig. 4), among others. Both react with other lipids and amplify the damaging effect of LPO. Detection of products such as MDA and 4HNE (described in Section 7) can generally reflect the degree of LPO in the body, helping to determine/predict the occurrence and prognosis of related diseases. Esterbauer et al. (1991) discovered many aldehydes produced during LPO. The aldehyde products of LPO are toxic to cells; both MDA and 4-HNE, which are in this class, are also highly reactive molecules (Gaschler and Stockwell 2017).

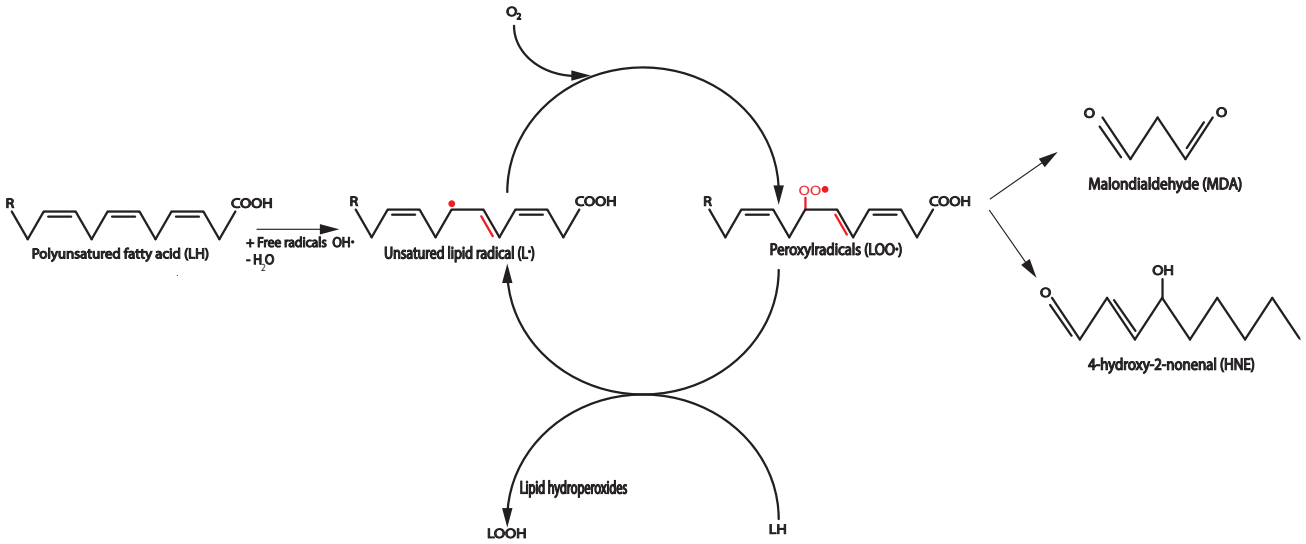

Fig. 4. Products of lipid peroxidation. Includes: malondialdehyde (MDA) and 4-hydroxy-2-noneal (4-HNE).

There are two classes of lipid peroxide inhibitors (Gaschler and Stockwell 2017), including:

– Peroxides formation preventor molecules

– Peroxides eliminator molecules

But the most common strategy to prevent the formation of lipid peroxides is the inhibition of their synthesizing enzymes (Gaschler and Stockwell 2017).

As mentioned above, MDA is the by-product of the oxidation of PUFAs, whose level corresponds with the grade of spermatozoa membrane integrity and sperm fertilization ability (John Aitken et al. 1989, Long and Kramer 2003, Partyka et al. 2007). Furthermore, Douard et al. (2003) and Wishart (1984) indicated a significant correlation between the increase in MDA level and the decrease in the fertility of birds. Therefore, knowledge of MDA production during the following steps of the semen preservation process may be useful, especially because the level of MDA production can be affected by the applied diluent, time, and storage temperature (Fujihara and Howarth 1978, Fujihara and Koga 1984, Long and Kramer 2003, Douard et al. 2004, Partyka et al. 2007).

4-hydroxynonenal is an aldehyde, and it is the most significant product of LPO that can influence the expression of various genes involved in regulating cell proliferation, differentiation, and apoptosis; as such, 4HNE acts as a signal to produce specific biological outcomes (Barrera et al. 2008). Moreover, 4-HNE can alter the structure and function of proteins and nucleic acids, responsible for their cytotoxicity (Gaschler and Stockwell 2017).

Lipid peroxidation in spermatozoa

Semen is exposed to atmospheric oxygen during *in vitro* storage, which can lead to LPO in sperm membranes (Fujihara and Koga 1984, Wishart 1984, Douard et al. 2003, Douard et al. 2004, Partyka et al. 2007). Furthermore, peroxidation of PUFAs in the sperm membranes decreases spermatozoa fertilization ability (Alvarez and Storey 1982, Partyka et al. 2012a). Dos Santos Hamilton et al. (2016) demonstrated that ram spermatozoa with high susceptibility to LPO also indicated high susceptibility to DNA damage (Hamilton et al. 2016). Generally, it is assumed that the main reason for sperm chromatin damage is oxidative stress (Sakkas and Alvarez 2010), which is related to failure in embryo development (Virro et al. 2004) and infertility in adults (Tesarik et al. 2002). However, it is substantial that even DNA-fragmented spermatozoa can fertilize the oocyte (Evenson and Wixon 2006). Although small-extension damage to sperm chromatin can be corrected by the oocyte after fertilization, we should consider that extensive damage causes embryonic fragmentation (Ahmadi and Ng 1999, Tamburrino et al. 2012, Hamilton et al. 2016).

The results mentioned above were confirmed by Simoes et al. (2013) who found that cattle semen with low susceptibility to LPO showed low susceptibility to DNA damage. They declared that the high susceptibility of spermatozoa to LPO can result in poor sperm quality (Simoes et al. 2013).

It is well established that LPO can occur in living cells under pathological and physiological conditions. Its aldehydic products can regulate cellular processes, such as proliferation, differentiation, and apoptosis (Barrera et al. 2008). The sperm membrane of birds contains high levels of PUFAs, making their spermatozoa vulnerable to peroxidative damage (Fujihara and Howarth 1978, Wishart 1984, Surai et al. 1998). This is significant because membrane PUFAs are crucial

in maintaining fluidity and flexibility during fertilization (Long and Kramer 2003) and can affect the sperm's fertilization capacity (Kelso et al. 1996, Blesbois et al. 1997, Partyka et al. 2011). Although LPO can occur in oxygen situations just after ejaculation in avian semen (Fujihara and Howarth 1978), different studies have indicated that there are clear species-specific differences in the intensity of LPO and antioxidant defense activity between different avian species (Partyka et al. 2012a) and even between breeds (Mavi et al. 2020). This may result from physiological differences and various metabolic requirements in avian spermatozoa (Long 2006). However, it should be noted that the applied method to determine the by-products of LPO might have had a considerable influence (Partyka et al. 2012a). Despite the important role of antioxidant enzymes in avian semen, information about them up to 10 years ago was scarce. Antioxidant enzymes include: glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT), and natural antioxidants include: vitamins A, C, E, uric acid, glutathione, carotenoids (Surai et al. 2001, Bréque et al. 2003, Cerolini et al. 2006, Partyka et al. 2012a,b). The main element of the primary antioxidant defense in semen is SOD (Surai et al. 2001, Partyka et al. 2012a). The presence of GPx and SOD in the spermatozoa and seminal plasma of geese, chickens, turkeys, ducks, and guinea fowls has been discovered by Surai et al. (1998). Additionally, Partyka et al. (2012a,b) detected the presence of CAT in chicken spermatozoa and seminal plasma. A detailed description of antioxidant defense in bird semen can be found in our previous article (Partyka and Niżański 2021).

In mammals, a positive correlation was demonstrated between LPO and oxidative DNA damage, plasma membrane damage, and acrosome damage. On the other hand, there was a negative correlation between LPO, sperm motility, and average mitochondrial potential (Trevizan et al. 2018). Interestingly, LPO was negatively correlated with average mitochondrial potential but not with high and low mitochondrial potential (Trevizan et al. 2018). Mavi et al. (2020) presented that decreased LPO results in enhanced motility, viability, membrane, acrosome, DNA integrity of spermatozoa, and ultimately high fertility of roosters. Furthermore, in turkey semen stored at 4°C, both motility and osmotic resistance showed a significant negative correlation with the level of MDA (Rosato et al. 2012).

High susceptibility to LPO increased the percentage of total defects in spermatozoa (Hamilton et al. 2016). Furthermore, despite the absence of oxidative stress, a higher percentage of sperm acrosome and plasma membrane injuries were observed, indicating the direct or indirect relationship between oxidative stress and sperm abnormalities, damage to the acrosome, membrane, and DNA fragmentation (Hamilton et al. 2016).

According to Partyka et al. (2012a), the level of LPO in the fresh semen of geese and chicken differs greatly. Goose spermatozoa are characterized by a higher concentration of MDA than chicken cells. The same relationship was confirmed by checking LPO in the sperm plasma membranes in both species (Partyka et al. 2011). Moreover, a correlation was found between MDA and antioxidant enzyme activity, such as CAT and GPx, in the goose. The increased activity of antioxidant enzymes and the greater amount of LPO can indicate higher oxidative stress without using enzymes for cell protection (Partyka et al. 2012a). On the other hand, in chickens, there is a negative correlation between MDA level and sperm plasma membrane integrity, SOD, and GPx activity (Partyka et al. 2012a). Hence, if the semen quality is good despite a lower lipid peroxidation (LPO) level and lower activity of antioxidant enzymes, it may indicate that the semen was exposed to oxidative stress and the antioxidant enzymes were utilized to preserve the se-men quality (Partyka et al. 2012a).

The level of MDA (malondialdehyde) has been found to determine the severity of sperm membrane permeability and ultimately affect sperm fertilization capacity (John Aitken et al. 1989, Long and Kramer 2003). Several studies have demonstrated a significant correlation between MDA levels and a decrease in fertility (Wishart 1984, Douard et al. 2003). Additionally, individual variations in MDA concentrations have been reported among roosters (Wishart 1984), and the impact of individual turkeys on LPO levels has been distinguished by Long and Kramer (Long and Kramer 2003). Therefore, the MDA level is an important factor in assessing the quality and fertilization capacity of sperm.

Lipid peroxidation in semen preservation

In vitro semen storage has deleterious effects on sperm motility, viability, and morphology in poultries (Fujihara and Koga 1984, Łukaszewicz 1988, Blesbois et al. 1993, Douard et al. 2003, Douard et al. 2004, Blesbois et al. 2005). Although early studies declared that the peroxidation process appeared within semen *in vitro* storage (Wishart 1984, Cecil and Bakst 1993), later studies demonstrated that LPO begins during ejaculation (Blesbois et al. 1993, Blesbois et al. 2005) and even in the male reproductive tract (Long and Kramer 2003). Previous studies showed that the intensity of LPO depends on situations in which semen is subjected, such as semen diluents (Fujihara and Koga 1984, Douard et al. 2004) and storage temperatures

(Fujihara and Howarth 1978, Long and Kramer 2003, Partyka et al. 2007).

Since LPO is considered the main reason for the reduction in fertilization ability during *in vitro* storage of avian semen (Blesbois et al. 1997, Zaniboni and Cerolini 2009, Partyka et al. 2011), the result of oxidative stress is particularly substantial during this process (Partyka et al. 2012b). Furthermore, it was shown that decreased mitochondrial activity might be associated with increased ROS production, which can cause an increase in LPO (Guthrie et al. 2008, Partyka et al. 2012b). The susceptibility of cell membranes to injury during freezing and thawing has been well-established, and it has been shown that this loss of membrane integrity is due to LPO. Therefore, assessing semen quality after *in vitro* storage is crucial for successful artificial insemination. This importance became more evident when it was indicated that LPO increased in avian sperm membranes after cryopreservation, damaging the gamete viability (Fujihara and Howarth 1978). Then this statement was confirmed by many other scientists in different species and breeds of birds.

Generally, the MDA level depends on the applied diluent, time, and storage temperature (Fujihara and Howarth 1978, Fujihara and Koga 1984, Long and Kramer 2003, Douard et al. 2004, Partyka et al. 2007). Moreover, it was reported that LPO in sperm during *in vitro* storage is positively correlated with the age of males (Douard et al. 2003).

In the turkey, an increase in the MDA level was found with a simultaneous reduction in the total antioxidant activity after liquid storage for 72 h (Izanloo et al. 2021). Additionally, even a shorter period of semen storage in turkeys led to a three-fold increase in MDA, which increased reactive oxygen species (ROS) levels in sperm (Słowińska et al. 2018). Moreover, during the storage of turkey semen in the liquid state, increased MDA concentration was accompanied by decreased phospholipid-free cholesterol content in the sperm plasma membrane (Zaniboni and Cerolini 2009). In chickens, it is stated that the loss of phospholipids is related to the phenomenon of peroxidation (Blesbois et al. 1999). Several studies have demonstrated that storing chicken semen in a liquid state for 24 h significantly increases MDA concentration. This concentration tends to remain at the same level for the following 24 h (Rad et al. 2016, Fattah et al. 2017, Ghaniei et al. 2019). This promising phenomenon can practically extend the possibility of using insemination doses for up to 48 h if needed. However, it should be noted that not all studies have found an intensification of MDA production in fowl semen stored in the liquid state (Partyka et al. 2007).

When it comes to semen cryopreservation, the situation is more challenging. Cryopreservation can significantly damage various cell structures, including the plasma membrane, mitochondria, nucleus, and flagellum (Watson et al. 1995, Donoghue and Wishart 2000, Holt 2000, Blesbois et al. 2005, Partyka et al. 2010, Partyka et al. 2012b). Additionally, cryopreservation can decrease the cholesterol content in sperm membranes, which can cause membrane instability (Bailey et al. 2008). ROS production increases during the semen freezing and thawing processes (Ball et al. 2001, Chatterjee and Gagnon 2001, Park et al. 2003). The main ROS production site is mitochondria (Brouwers and Gadella 2003) and sperm cell membrane (Agarwal et al. 2005), which are particularly susceptible to cell damage from rapid temperature changes. The high percentage of active mitochondria and the low level of LPO after freezing-thawing in geese support the thesis on the association between LPO and mitochondrial function (Partyka et al. 2011). In contrast, in rooster sperm, LPO is greater after cryo-preservation (Partyka et al. 2010, Partyka et al. 2011). Therefore, it can be concluded that the susceptibility of avian spermatozoa to LPO is greater when antioxidant concentrations are sub-optimal, resulting in a lower ROS scavenging capacity, which disrupts redox homeostasis in sperm (Partyka et al. 2012a,b).

In avian species, an increase in reactive oxygen species (ROS) levels in sperm cells after the freezing--thawing process is observed, which is similar to what is seen in chicken semen (Słowińska et al. 2018). This suggests that the varying rates of oxidative metabolism in bird spermatozoa are significant factors in their susceptibility to peroxidation (Partyka et al. 2012b).

Measurement of lipid peroxidation or its by-products

Measurement of LPO by-products is one of the most widely applied techniques to locate the oxidative status of semen (Hamilton et al. 2016). Due to the LPO increase in disease or toxicology (Halliwell and Gutteridge 1984), its measurement can be a great marker for tissue damage (Gutteridge 1995). There are three general methods to assess the extent of LPO: 1. losses of PUFAs, 2. contents of primary peroxidation products, and 3. contents of secondary peroxidation products (Halliwell and Chirico 1993). In general, many primary techniques to measure LPO are based on the reactivity of an aldehyde degradation product (Gaschler and Stockwell 2017). Since the chemical composition of the end products of LPO depends on the chemical composition of applied PUFA and the kind of metal ions present, selecting only one single test for the monitoring of LPO can misguide results (Halliwell

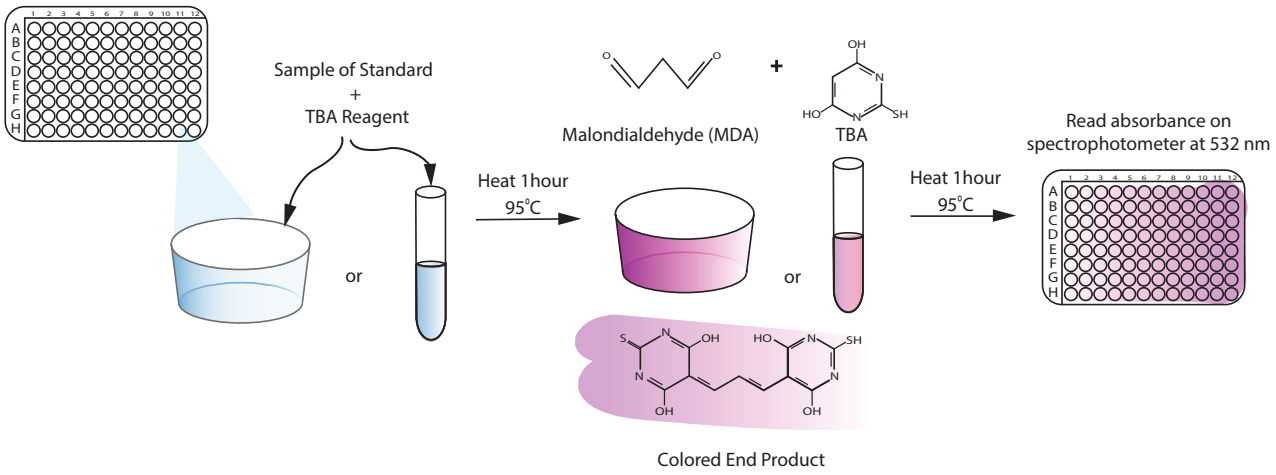

Fig. 5. Assay principle for the thiobarbituric acid reactive substances (TBARS) test. It measures the concentration of malondialdehyde (MDA) as a lipid peroxidation product. An MDA molecule reacts with two thiobarbituric acid molecules and makes a pink-colored product measured by spectrophotometry at 532 nm.

and Chirico 1993). Although until recently, LPO detection on the spermatozoa membrane was based on colorimetric methods such as TBARS (thiobarbituric acid reactive substances) and detection of end products of LPO such as MDA and 4-HNE (Aitken et al. 1993, Blesbois et al. 1993, Baumber et al. 2000, Long and Kramer 2003, Partyka et al. 2007), this does not distinguish between live and dead cells (Partyka et al. 2011). Furthermore, since this method measures very late by-products of LPO, it is not clear what exactly is happening at the membrane level (Partyka et al. 2011). Therefore, fluorescent membrane probes described below have been used as an alternative to trace lipid peroxide formation in mammalian spermatozoa (Brouwers and Gadella 2003, Almeida and Ball 2005, Brouwers et al. 2005, Neild et al. 2005, Aitken et al. 2007, Thuwanut et al. 2009, Partyka et al. 2011).

Malondialdehyde measurement by thiobarbituric acid reactive substances (TBARS) assay

The TBA test is one of the techniques for estimating sperm membrane damage (Partyka et al. 2007). It is the most common technique because it is cheap and simple to use (Halliwell and Chirico 1993). In birds, it is the most common test used among others by Partyka et al. (2007), Thananurak et al. (2019, 2020), Elsami et al. (2018), Moghbeli et al. (2016), Amini et al. (2015), Rosato et al. (2012), Rui et al. (2017), Fattah et al. (2017), Surai et al. (1998), and Rad et al. (2016). It measures the concentration of MDA produced in peroxidizing lipid systems (Halliwell and Chirico 1993) through the reaction between LPO products and TBARSs (Partyka et al. 2007). The major substance that reacts with thiobarbituric acid is MDA (Hamilton et al. 2016). Ohkawa et al. (1979) described this method in which two molecules of thiobarbituric acid react with an MDA molecule and produce a pink-colored complex, measured by spectrophotometry at 532 nm (Fig. 5). Ferrous ions and ascorbate are used as suitable promoter systems to enhance the rate of this reaction (Partyka et al. 2007).

Malondialdehyde measurement by commercial kits

MDA reacts not only with thiobarbituric acid but also with a chromogenic reagent, NMPI (N-methyl-2--phenylindole), at 45°C (Erdelmeier et al. 1998, Partyka et al. 2012b). In a commercial kit such as MDA-586 (OxisResearch, Portland, OR, USA), one molecule of MDA reacts with two molecules of NMPI, and the reaction leads to a stable carbocyanine dye which has maximum absorption at 586 nm (Partyka et al. 2012a). Although widely used in mammalian species, Long and Kramer (2003) and Partyka et al. (2012a) have used these tests in birds, specifically in turkeys and chickens.

Lipid peroxidation detection by C_{11} BODIPY $^{581/591}$

The C_{11}-BODIPY$^{581/591}$ is a fluorescence probe named 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a, 4a-diaza-s-indacene-3-undecanoic acid used as a marker for membrane LPO. It is a fluorescent analogue of fatty acids sensitive to oxidation (Drummen et al. 2002, Partyka et al. 2011) that can be inserted into the plasma membrane, and when LPO occurs, it will fluoresce from red to green (non-peroxidized: red; peroxidized: green) (Trevizan et al. 2018, Drummen et al. 2002). Changes are monitored by a fluorescent microscope or flow cytometric analysis (Fig. 6). Furthermore, C_{11}-BODIPY$^{581/591}$ is almost insensitive to environmen-

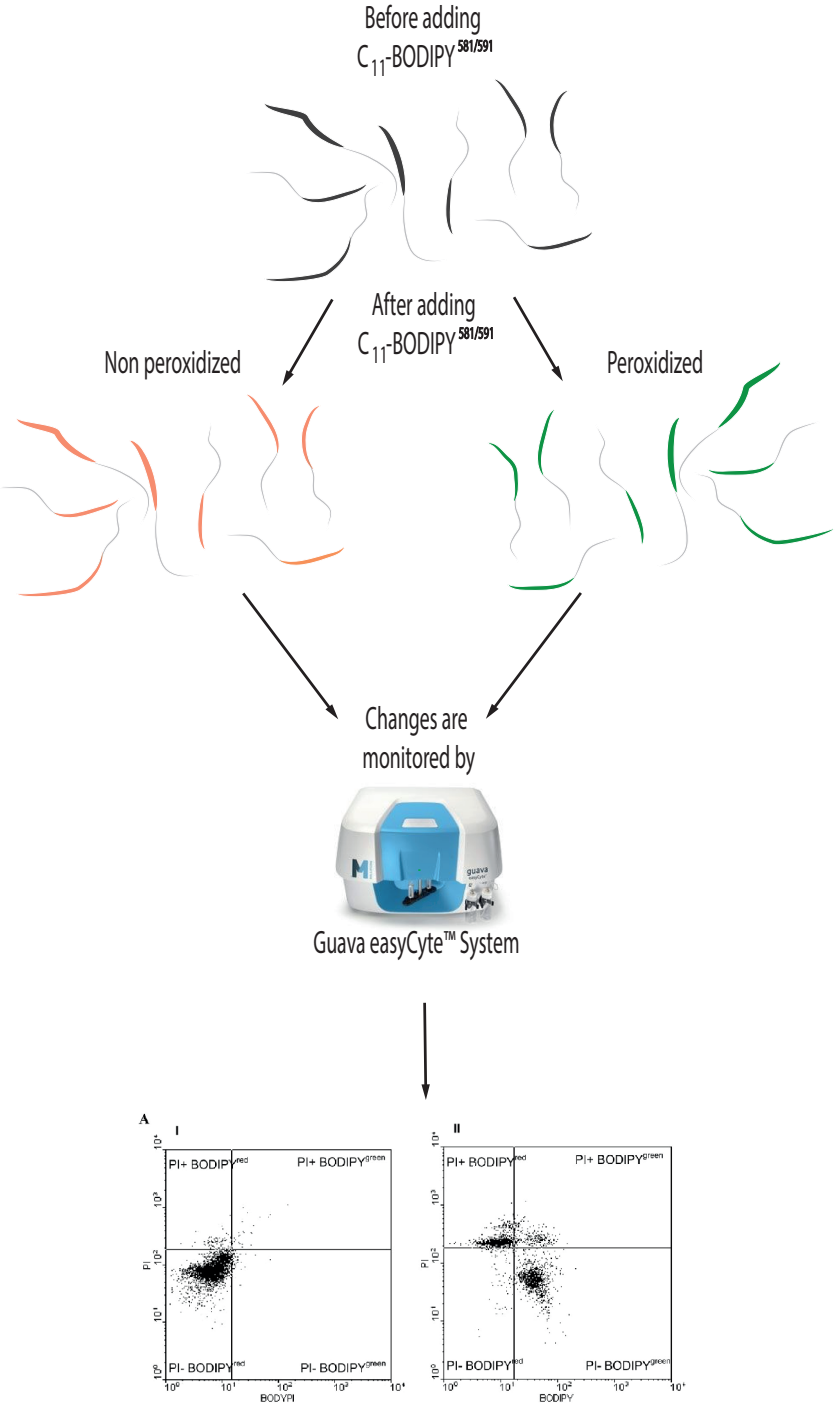

Fig. 6. Schematic of the sperm principle staining by C_{11}-BODIPY$^{581/591}$. Red fluorescence associated with the non-oxidized probe. Green fluorescence associated with the oxidized probe. On the dot plot of C_{11}-BODIPY$^{581/591}$/PI, spermatozoa can be divided into four subpopulations: dead without lipid peroxidation (LPO) (PI+ BODIPYred), dead with LPO (PI+ BODIPYgreen), live without LPO (PI- BODIPYred) and live with LPO (PI- BODIPYgreen).

tal changes and does not leave the lipid membrane after oxidation (Brouwers and Gadella 2003, Brouwers et al. 2005, Partyka et al. 2011). This dye has been used successfully for many years to evaluate LPO in the mammalian sperm plasma membrane, and since 2011 it has also been used for bird semen (Partyka et al. 2011, Mehaisen et al. 2020).

Intracellular ROS detection by fluorescent probes

The intracellular level of ROS, as some of the most prevalent oxidants in cells, can be detected using different fluorescent probes and flow cytometry. Hydrogen peroxide (H_2O_2) in sperm cells can be detected using the fluorescent stain 5-(and-6)-carboxy-20,70-dichlorodihydrofluoresceindiacetate (carboxy-H_2DCFDA, also

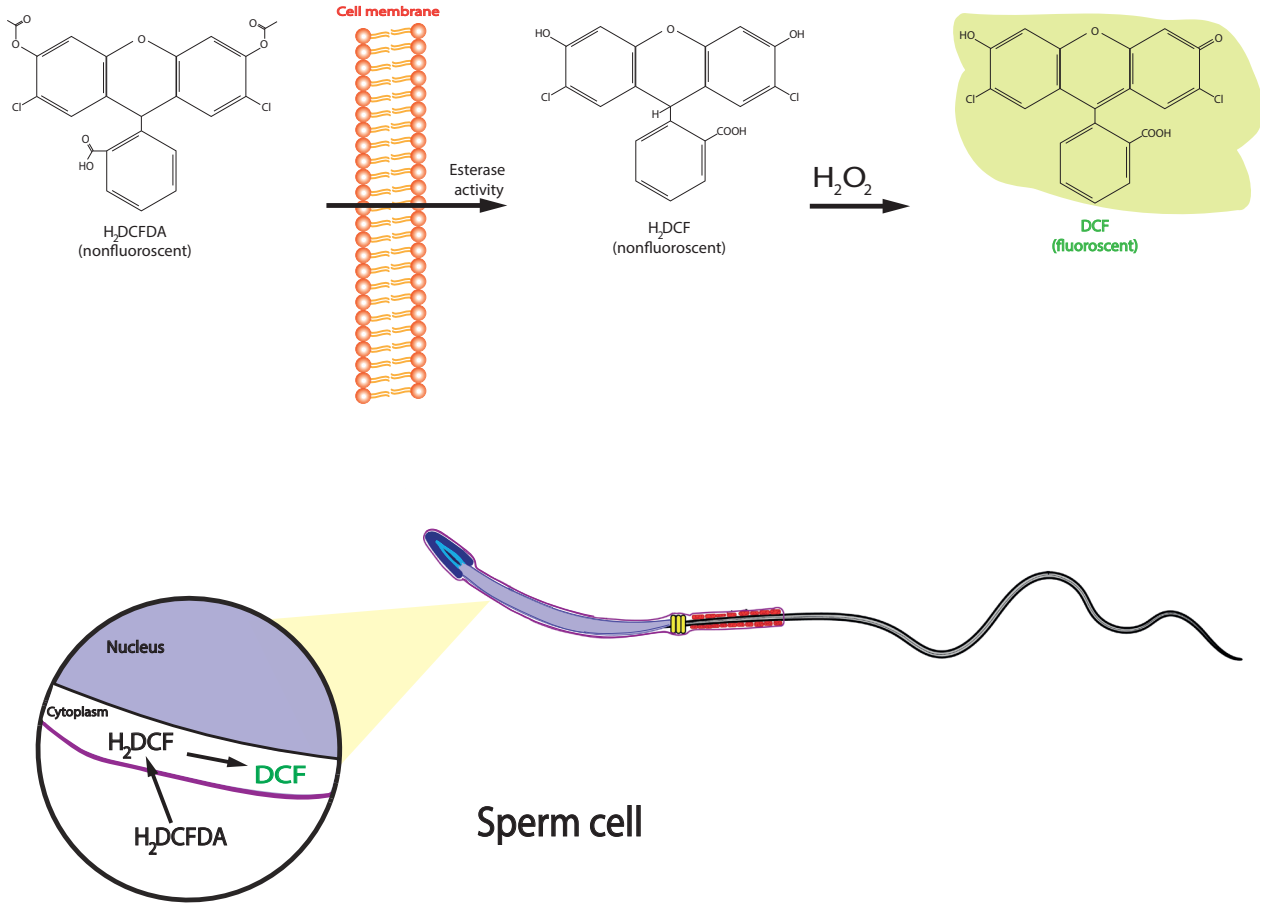

Fig. 7. Schematic of the sperm principle staining by 5-(and-6)-carboxy-20,70-dichlorodihydrofluoresceindiacetate (H_2DCFDA). Cell permeable H_2DCFDA is cleaved by intracellular esterases to non-fluorescent 2′,7′-dichlorodihydrofluorescein (H_2DCF), which is oxidized by various ROS to green fluorescent dichlorofluorescein (DCF) which can be detected by flow cytometry.

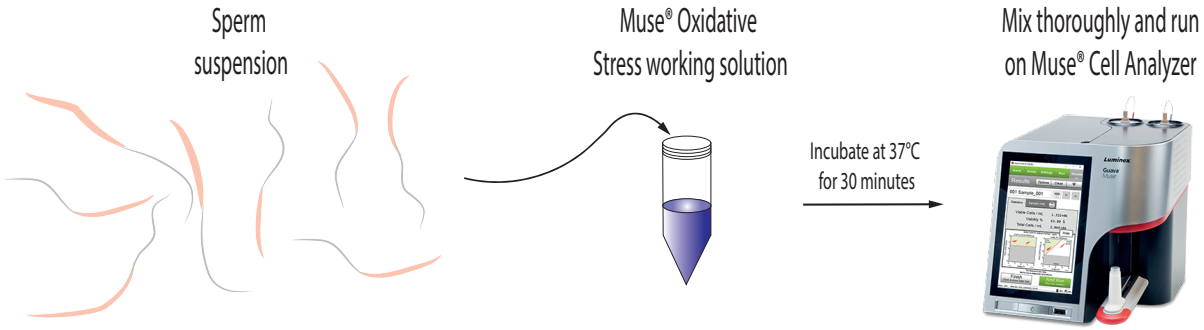

Fig. 8. Schematic of the sperm staining by Muse Oxidative Stress Kit. The ROS levels in spermatozoa are measured using a Muse Oxidative Stress Kit (EMD Millipore) based on dihydroethidium, that allows the determination of the count and percentage of cells undergoing oxidative stress according to the intracellular detection of superoxide radicals.

called dichlorofluorescin diacetate (DCFH-DA) (Fig. 7). Viable sperm are differentiated from dead cells by a counterstain – propidium iodide, and the subpopulations of sperm with a high H_2O_2 level (strong fluorescence) and those with a low H_2O_2 level (weak fluorescence) can be distinguished. In our opinion, this stain is also worth using for avian semen evaluation (personal experience, data not published). There are rare studies presenting results of DCFH-DA stained chicken sperm evaluated on a flow cytometer (Salehi et al. 2020, Masoudi et al. 2021).

Sperm ROS production can also be determined using commercial kits such as a Muse Oxidative Stress Kit (EMD Millipore) and flow cytometry (Fig. 8). The Muse Oxidative Stress Reagent is based on dihydroethidium (DHE), which upon reaction with superoxide anions, undergoes oxidation to form the DNA-binding fluorophore ethidium bromide or a struc-

turally similar product that intercalates with DNA, resulting in red fluorescence. Two populations of cells are visible in the assay: ROS–, live cells, and ROS+, cells exhibiting ROS. This kit was used for the evaluation of turkey semen (Słowińska et al. 2018).

Conclusions

In conclusion, we can say that, similar to mammals, oxidative stress, particularly LPO, is the main cause of damage and infertility in avian sperm. Measurement of its by-products is an important key to understanding their negative effects on spermatozoa characteristics, especially during the *in vitro* storage of the semen. Due to this, it is possible to introduce such strategies to improve the quality of bird semen described previously (Partyka and Niżański 2021). As we present here, in this group of animals, the TBARs test is the leading method of LPO measurement. However, presenting other techniques for evaluating oxidative stress biomarkers, we encourage the readers to use the new, more advanced palette of assays applied more frequently in mammals.

Acknowledgements

The publication was co-financed under the Leading Research Groups support project from the subsidy increase for the period 2020–2025 in the amount of 2% of the subsidy referred to Art. 387 (3) of the Law of 20 July 2018 on Higher Education and Science, obtained in 2019. The APC was founded by "Maintenance and development of the research potential of departments and institutes – subvention for Wroclaw University of Environmental and Life Sciences (Poland), 2023", no. B010/0031/23.

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