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THE EFFECT OF ADDING NANO CONCENTRATIONS OF HYDROGEN PEROXIDE TO THE DILUTED SEMEN OF IRAQI LOCAL ROOSTERS ON SOME LABORATORY CHARACTERISTICS AFTER COOLING AND CRYOPRESERVATION

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Abstract

This study was conducted at the poultry farm located in the College of Agricultural Engineering Sciences, University of Baghdad, Abu Gharib (the old site), and laboratories of the Animal Production Department, Jadriya, to investigate the effect of adding hydrogen peroxide H_2O_2 at nanoscale levels to semen diluents of local roosters sperm in a number of semen characteristics. In this study, 80 roosters local Iraqi chickens were used, the roosters were trained three times a week, to collect semen, until the largest number of them responded. Then the best 40 of the roosters were elected for the purpose of collecting the semen with a pooled sample, and then the samples were diluted and divided equally into four parts. The concentrations of 0, 1, 10, 100, nM of H_2O_2 were added to each part of the diluted semen, then kept cool until the temperature reached 5 °C for three periods (0, 24, 48 hours), and cryopreservation (48 hours) for all four addition levels. A number of laboratory characteristics were studied including percentages of individual motility, dead sperm, mitochondrial efficacy, and DNA Fragmentation at the end of each repetition (10 repetitions). A variation based on the concentration was observed in the results of hydrogen peroxide, as it ranged from the non-affectivity of the two treatments 1, 10 nM H_2O_2, to the deterioration in some laboratory characteristics for the treatment of 100 nM H_2O_2, and according to the interactions between the addition concentrations and the cooling and cryopreservation periods. From this experiment, it can be concluded the inefficiency of the hydrogen peroxide concentrations used to semen preservation.

Keywords: Hydrogen Peroxide, Diluted Semen, Iraqi Local Roosters

Introduction

Despite the great development that has accompanied the poultry industry in recent decades, artificial insemination has not been exploited in a commercial application but is limited only in the research field (Asano and Tajima, 2017). This is due to the chemical characteristics of the poultry semen, especially roosters, which limits the process of preservation and use in artificial insemination after the creation of hereditary banks that contain the semen of highly productive varieties (Speake *et al*., 2003). As the lack of cytoplasm in the semen, and the production of free radicals in the process of oxidation, is one of the most important problems in preserving the semen of the roosters using cryopreservation techniques (Donoghue and Wishart, 2000; Woelders *et al*., 2006). As well as, the materials used to preserve the semen for mammals are not appropriate for the characteristics of the rooster's semen, the use of cholesterol and egg yolk resulted in a sharp decline in fertility when the semen was frozen and then dissolved (Tarvis, 2013). The fertility of thawed semen is also weak due to reduced motility and vitality of male semen after freezing and thawing freeze-thawing (Hou *et al*., 2008). These obstacles are often associated with mechanical, biochemical damages, and altered sperm structures (Mazur, 1984), which negatively affect the quality of semen and the fertility potential of the thawed semen. Therefore, new strategies are required to minimize this damage (Vishwanath and Shannon, 2000). In recent years, new methods have been applied to improve semen indices, which it relies on exposing semen to slight (sub-lethal) stress before the preserve by cooling or cryopreservation, including hydrostatic stress (Huang *et al*., 2009), otherwise, osmotic (Lin *et al*., 2009), oxidative stress (Sharafi *et al*., 2015). Accordingly, (Feyzi *et al*., 2018) studied the effect of preconditioning semen of roosters with sub-lethal oxidative stress induced by nitric oxide before cryopreservation of roosters semen, where a significant improvement was observed in indices of semen quality and fertility potential. Finally, due to the lack of studies on the effect of slight concentrations of hydrogen peroxide on the rooster's semen, and because it has many important effects in mammal semen (Maia *et al*., 2014). The current study aims to investigate the effect of adding hydrogen peroxide (in Nano concentrations) to dilute roosters semen during cooling or cryopreservation in some laboratory characteristics of semen,

Materials and Methods

Study site, harboring and managing birds

This study was conducted in the poultry farm located in the College of Agricultural Engineering Sciences / University of Baghdad - Abu Gharib. As 80 roosters and 288 chickens were used from the Iraqi, local chicken prepared from the Agricultural Research Department / Ministry of Agriculture at the age of 34 weeks, in a hall equipped with cages (one roosters/cage). A lighting system of 14-light: 8 dark hours/days, was used, and the water was provided freely, the diet was used twice a day (120 and 90 g / day for males and females, respectively, on two types of diets. The first one contained 1716.71 kcal/kg, 15.5% crude protein, 1.15% calcium, and 0.41 available phosphorous.

Training roosters and semen collection

Roosters were trained using dorso-abdominal massage as described by (Burrow and Quinn 1937). This process was applied until most of the study males responded and reached the stage of ejaculation by simply passing the hand over the back area down to the vent, since then the tail is high, erection and direct ejaculation take place. The well-trained

roosters were isolated and numbered with annular plastic numbers (it was fixed to one of the legs). Then, the semen was collected from each male to evaluate and select the best 40 male to create the best quality of semen, with a high concentration of semen. Besides, free from pollution, by assessing the volume and concentration of the pH of each ejaculate, taking into account the avoidance of males from contaminating the semen with clear liquid secretions or stool.

Semen preservation and experiment design

After selecting the top 40 males, a pooled sample of semen was collected and diluted to 1 billion semen/ml using diluted (Lake and Ravie 1984) adjusted by the researcher. The diluted semen was divided into 4 parts equally and was added concentrations (0, 1, 10, and 100 nM) by a concentration/part of hydrogen peroxide. Subsequently, the four parts or treatments were kept cool until the temperature reached 5 °C, which is considered the first period of preservation, as the laboratory tests begin at this degree. The second period represents 24 hours after evaluating the indices of semen for the first period of preservation, as well as the preservation period of 48 hours. However, at period zero, a part of the semen was taken from each treatment and 5% dimethylacetamide was added to it, where the samples were then left for one hour to make an equilibration between the water and the anti-freeze protective material. Furthermore, samples were packed with Polyvinyl French straws 0.25 ml, then the straws were exposed to nitrogen vapor (-120 °C) for 10 minutes, after placing the straws horizontally about 5 cm from the liquid nitrogen. The samples were flooded with liquid nitrogen at -196 °C, and the straws were removed from the liquid nitrogen 48 hours after preservation, and turn it into a liquid by immersing them in a water bath at 37 °C for 30 seconds. Finally, this process repeated 10 times, laboratory tests were performed for each repetition, and then the data were analyzed statistically.

Semen evaluation (microscopic tests)

1) Individual motility

The individual motility of sperm was estimated as a percentage according to (Wheeler and Andrews 1943) method. A 10 µl of diluted semen was placed on the heated glass slide (37 °C), the pre-heated slide cover was then placed on the sample and tested under a microscope with the objective lens with a magnification of 400x.

2) Dead sperm percentage

The percentage of dead sperm was estimated using the Eosin-Nigrosine stain, which was prepared according to (Al-Daraji and Al-Janabi 2005) procedure, after which a drop of diluted semen was placed on a glass slide and mixed with one drop of the Eosin-Nigrosine stain mixture. A smear was done by the tip of a second glass slide, then left to dry for one minute, then the slide was read using an optical microscope at a magnification force x 1000, and the number of sperm was calculated in 3-4 micro-fields, the number of sperm should not be less than 200 sperm. Dead sperm are diagnosed by pigmentation in red or pink color, while the live sperm is white transparent because the Eosin pigment is not able to penetrate its plasma membrane, then the percentage of dead sperm was calculated.

3) DNA Fragmentation test

The percentage of DNA Fragmentation in sperm was tested by preparation of Acridine Orange Stain, Tyrode's Solution, and Carnoy's Fixative, and the pigmentation was performed according to (Tejada *et al*., 1984) technique. The slides were tested on the day of pigmentation using a fluorescent microscope (40x lens) by a wavelength of 460 - 490 nm. At least 300 sperm were counted, and all the colors that appeared (green, orange, yellow, and red) were recorded. The green color of the sperm's head indicates the integrity of the genetic material. Then, the percentage of DNA Fragmentation was calculated.

4) Mitochondrial activity

This test was performed using a (3', 3; - Diaminobenzidine; DAB) dye, depending on the method of preparation and diagnosis that described by (Hrudka, 1987). where (10 µl) smears were prepared on glass slides and then dried. The slides were fixed using 10% formaldehyde for 10 minutes, then washed and dried with air again. At least 200 sperm were calculated using an optical microscope at a magnification power (1000 X), and the percentage of mitochondrial activity was estimated. The middle region of normal sperm appears in a dark brown color, while the damaged ones appear in a light white color.

- **Statistical analysis**

The Statistical Analysis System (SAS) was used to analyze the two experiment data, as two-way (4 × 4) analysis of variance was applied according to a Completely Randomized Design (CRD), and the significant differences between the averages were compared using Duncan's New Multiple Range Test (1955).

Results

Percentage of sperm motility

Table 1 (average concentration) showed a significant decrease ($P < 0.01$) in the percentage of individual motility during the use of concentration B_4 of hydrogen peroxide in semen diluents and their cooling and cryopreservation preserving, compared to the other concentrations (B_1, B_2, and B_3). However, it was evident that the individual motility increased significantly ($P < 0.01$) during the H_0 period, compared to the other periods. Besides, the period H_{24} recorded a significant increase ($P < 0.01$) in the individual motility percentage compared to the periods H_{48} and F_{48}, with a significant priority ($P < 0.01$) for period H_{48} compared to F_{48} (Table 1; average period). The interaction between concentration and periods of preservation resulted in a significant increase ($P < 0.01$) in individual motility percentage for the interactions $B1H_0$, B_2H_0, and B_3H_0, compared to the B_4H_0, and the other interactions. As interaction B_4H_{24} was recorded a significant decrease compared to the interactions $B1H_{24}$, B_2H_{24}, and B_3H_{24}, where the individual motility percentage for the interaction B_4H_{48} decreased compared to the interactions B_1H_{48}, B_2H_{48}, and B_3H_{48}. Furthermore, the interaction B_4F_{48} recorded a significant decrease in the individual motility compared to the B_1F_{48}, B_2F_{48}, B_3F_{48}, and other interactions as shown in Table 1. Moreover, no significant differences were observed between the interactions B_4H_0, B_1H_{24}, B_2H_{24}, and B_3H_{24}, neither between interactions B_4H_{24}, B_1H_{48}, B_2H_{48}, B_3H_{48}, nor B_4H_{48}, B_1F_{48}, B_2F_{48}, and B_3F_{48} in the percentage of individual motility of the local rooster's semen under the conditions of cooling and cryopreservation as shown in Table 1.

Percentage of dead sperm

Table 2 showed a significant increase (P <0.01) in the percentage of dead sperm in concentration B_4 of hydrogen peroxide compared to the other concentrations, where no significant differences were observed between the other concentrations. As the same Table indicated, a significant increase (P <0.01) in the percentage of dead sperm with the progression of the preservation period, the period F_{48} recorded the highest percentages, followed by period H_{48} and then H_{24} with a significant difference (P <0.01) between each period and another. The period H_0 reported a significant decrease (P <0.01) in the percentages of dead sperm compared to the other three periods. It was found that the interaction B_4F_{48} recorded a significant increase (P <0.01) in the percentage of dead sperm compared to the rest of the interactions, but that it did not differ significantly from the interaction B_4H_{48} which recorded a significant increase (P <0.01) compared to other interactions, except the interaction of the four concentrations with period F_{48}. The interaction B_4H_{24} also showed a significant increase (P <0.01) when compared to the interactions B_1H_{24}, $B_2H_{24,}$ and B_3H_{24}, whereas the same interaction did not differ significantly from the B_1H_{48}, $B_2H_{48,}$ and B_3H_{48}. Similarly, the interaction B_4H_0, it recorded a significant increase (P <0.01) compared to the three concentrations with the period H_0, and it did not differ significantly from the interactions B_1H_{24}, $B_2H_{24,}$ and B_3H_{24}. Furthermore that the interactions B_1H_0, $B_2H_{0,}$ and B_3H_0 recorded a significant decrease (P <0.01) in the percentage of dead sperm compared to the other interactions.

The effect of different concentrations on the DNA Fragmentation

It was observed from Table 3 that the concentrations of hydrogen peroxide did not significantly affect the percentage of DNA Fragmentation, as no significant differences were observed between the four concentrations (B_1, B_2, $B_{3,}$ and B_4). A significant decrease (P <0.01) was observed in the percentage of DNA Fragmentation during the period H0 compared to the other three periods, and the period H_{24} recorded a significant decrease (P <0.01) compared with the periods H_{48} and F_{48}. While H_{48} recorded a significant decrease (P <0.01) in the percentage of DNA Fragmentation compared to the period F_{48}, which recorded the highest percentage of DNA Fragmentation with significant differences (P <0.01) from the other three periods as shown in Table 3. Table 3 showed that the interactions B_1H_0, B_2H_0, $B_3H_{0,}$ and B_4H_0 resulted in a significant decrease in the percentage of DNA Fragmentation in sperm undercooling and cryopreservation conditions, compared to other experimental interactions. While the interactions B_1F_{48}, B_2F_{48}, $B_3F_{48,}$ and B_4F_{48} recorded a significant increase in DNA Fragmentation percentage compared to other interactions. A significant decrease in DNA Fragmentation percentage was observed in the interactions B_1H_{24}, B_2H_{24}, $B_3H_{24,}$ and B_4H_{24} compared to interactions B_1H_{48}, B_2H_{48}, B_3H_{48}, B_4H_{48} and interactions B_1F_{48}, B_2F_{48}, $B_3F_{48,}$ and B_4F_{48}. Additionally, no significant differences were observed between the four concentrations within each preservation period.

The mitochondria activity

The results of Table 4 indicated that the concentrations of hydrogen peroxide (0, 1, 10, 100 nM) in semen diluents did not significantly affect the percentage of mitochondria activity, while the preservation periods affected a significant effect (P <0.01) on the mitochondria activity. As the period H_0 recorded the highest activity percentage for the mitochondria, then H_{24} and then H_{48} and finally F_{48} with significant differences (P <0.01) between each period to another. The interactions B_1H_0, B_2H_0, $B_3H_{0,}$ and B_4H_0 also led to an increase in the mitochondria activity compared to the other interactions, followed by B_1H_{24}, B_2H_{24}, B_3H_0 and B_4H_{24} with significant differences from the B_1H_{48}, B_2H_{48}, B_3H_{48} and B_4H_{48}, which increased significantly compared to the B_1F_{48}, B_2F_{48}, $B_3F_{48,}$ and B_4F_{48}. Finally, no significant differences were observed between peroxide concentrations and the interactions within the same period as shown in Table 4.

Table 1 : Effect of adding different concentrations of hydrogen peroxide on the percentage of individual motility (%; mean ± standard error) of the local rooster's semen at different periods of cooling and cryopreservation

The concentration of hydrogen peroxide	Periods of cooling (hour; H)			Periods of cryopreservation (48hour; F)	Average concentration
B_1	^{A} 1.14 ±82.50	^{B} 1.46 ± 69.50	^{C} 1.80 ± 57.30	^{E} 1.36 ± 38.30	^{A} 2.69 ± 61.90
B_2	^{A} 2.53 ± 80.80	^{B} 1.16 ± 69.40	^{C} 2.33 ± 56.30	^{E} 1.11 ± 38.10	^{A} 2.72 ± 61.15
B_3	^{A} 2.02 ± 81.30	^{B} 0.94 ± 68.80	^{C} 1.82 ± 56.70	^{E} 1.18 ± 38.40	^{A} 2.64 ± 61.30
B_4	^{B} 1.65 ± 67.20	^{C} 1.63 ± 57.30	^{D} 2.18 ± 46.90	^{F} 2.06 ± 29.50	^{B} 2.41 ± 50.23
Average period	^{A} 1.35 ± 77.95	^{B} 1.04 ± 66.25	^{C} 1.20 ± 54.30	^{D} 0.93 ± 36.08	
Significance level					
Concentration	**0.01**				
Period	**0.01**				
Concentration× period	**0.01**				
B_1; B_2; B_3; B_4: Addition of 0, 1, 10, 100 nM of hydrogen peroxide successively to semen diluents.					

Table 2 : Effect of adding different concentrations of hydrogen peroxide on the percentage of dead sperm (%; mean ± standard error) of the local roosters at different periods of cooling and cryopreservation.

The concentration of hydrogen peroxide	Periods of cooling (hour; H)			Periods of cryopreservation (48hour; F)	Average concentration
B_1	^{F} 1.39 ± 17.64	^{D} 0.32 ± 29.07	^{C} 1.36 ± 38.95	^{B} 1.10 ± 51.70	^{B} 3.81 ± 34.34
B_2	^{F} 2.35 ± 18.49	^{D} 1.68 ± 28.83	^{C} 0.88 ± 39.18	^{B} 1.35 ± 52.06	^{B} 3.82 ± 34.64
B_3	^{F} 1.45 ± 18.20	^{D} 2.23 ± 29.89	^{C} 1.68 ± 40.08	^{B} 2.16 ± 52.23	^{B} 3.87 ± 35.10
B_4	^{E} 0.95 ± 24.67	^{C} 1.48 ± 38.54	^{AB} 0.97 ± 56.57	^{A} 1.92 ± 61.79	^{A} 4.49 ± 45.39
Average period	^{D} 1.10 ± 19.75	^{C} 1.39 ± 31.58	^{B} 2.31 ± 43.69	^{A} 1.47 ± 54.44	
Significance level					
Concentration	**0.05**				
Period	**0.01**				
Concentration× period	**0.01**				
B_1; B_2; B_3; B_4: Addition of 0, 1, 10, 100 nM of hydrogen peroxide successively to semen diluents.					

Table 3 : Effect of adding different concentrations of hydrogen peroxide on the percentage of DNA Fragmentation (%; mean ± standard error) of the local rooster's semen at different periods of cooling and cryopreservation

The concentration of hydrogen peroxide	Periods of cooling (hour; H)			Periods of cryopreservation (48hour; F)	Average concentration
B_1	^{E} 0.33 ± 2.37	^{C} 0.40 ± 4.50	^{B} 0.25 ± 6.20	^{A} 0.42 ± 11.37	1.01 ± 6.11
B_2	^{E} 0.32 ± 2.33	^{C} 0.31 ± 4.70	^{B} 0.18 ± 6.13	^{A} 0.83 ± 11.80	1.07 ± 6.24
B_3	^{E} 0.12 ± 2.10	^{C} 0.54 ± 4.97	^{B} 0.09 ± 5.88	^{A} 0.55 ± 11.60	1.05 ± 6.14
B_4	^{E} 0.12 ± 2.60	^{C} 0.55 ± 4.93	^{B} 0.12 ± 6.00	^{A} 0.50 ± 11.93	1.05 ± 6.37
Average period	^{D} 0.12 ± 2.35	^{C} 0.20 ± 4.78	^{B} 0.08 ± 6.05	^{A} 0.26 ± 11.68	
Significance level					
Concentration	N.S				
Period	**0.01**				
Concentration× period	**0.01**				
B_1; B_2; B_3; B_4: Addition of 0, 1, 10, 100 nM of hydrogen peroxide successively to semen diluents.					

Table 4 : Effect of adding different concentrations of hydrogen peroxide on the percentage of mitochondria activity (%; mean ± standard error) of the local rooster's semen at different periods of cooling and cryopreservation

Concentration of hydrogen peroxide	Periods of cooling (hour; H)			Periods of cryopreservation (48hour; F)	Average concentration
B_1	^{A} 1.76 ± 81.84	^{B} 0.72 ± 62.05	^{C} 2.33 ± 51.95	^{D} 1.38 ± 38.87	4.78 ± 58.68
B_2	^{A} 1.92 ± 78.96	^{B} 2.93 ± 63.10	^{C} 1.23 ± 52.93	^{D} 2.87 ± 39.62	4.45 ± 58.66
B_3	^{A} 2.81 ± 80.26	^{B} 2.24 ± 61.48	^{C} 1.99 ± 51.69	^{D} 4.06 ± 39.98	4.62 ± 58.35
B_4	^{A} 3.20 ± 79.38	^{B} 2.23 ± 61.81	^{C} 1.30 ± 52.06	^{D} 1.20 ± 39.24	4.51 ± 58.12
Average period	^{A} 1.12 ± 80.11	^{B} 0.95 ± 62.11	^{C} 0.77 ± 52.16	^{D} 1.14 ± 39.43	
Significance level					
Concentration	N.S				
Period	**0.01**				
Concentration× period	**0.01**				
B_1; B_2; B_3; B_4: Addition of 0, 1, 10, 100 nM of hydrogen peroxide successively to semen diluents.					

Discussion

This study is concerned with investigating the role of sub-lethal concentrations of hydrogen peroxide (specifically Nano concentrations) in the quality of semen in cooling and cryopreservation. This study expected to have positive effects on semen characteristics. As there may be an educational or cautionary role for these slight concentrations in inducing or producing slight oxidative stress, through which certain mechanisms can be stimulated in the rooster's sperm that enable them to resist or reduce the damage resulting from cooling or cryopreservation. Especially that the physiological concentrations of hydrogen peroxide have important effects in the semen of mammals, where several studies have shown that the effect of hydrogen peroxide depends mainly on the concentration (Maia *et al*., 2014). Furthermore, hydrogen peroxide contributes to the process of adaptation of semen (Yanagimachi, 1994), which enables semen to occur in the reaction of acrosome before fertilization and penetration of the zona pellucida (ZP). Several changes occur during capacitation, including cholesterol flow from the plasma membrane (Langlais and Roberts, 1985; Visconti *et al*., 1999) and increase free calcium ion inside the cells (Handrow *et al*., 1989; Ruknudin and Silver, 1990; Baldi *et al*., 1991). In addition to cAMP (Parrish *et al*., 1994; Parinaud and Milhet, 1996; Zhang *et al*., 1991), pH (Vredenburgh-Wilberg and Parrish, 1995), the

phosphorylation of tyrosine proteins (Visconti *et al*., 1995; Galantino-Homer *et al*., 1997) and the actin polymerization (Brener *et al*., 2003). It has been suggested that Reactive Oxygen Species (ROS) such as hydrogen peroxide and superoxide anion are involved in regulating human sperm adaptation and phosphorylation of tyrosine proteins (Aitken *et al*., 1996; de Lamirande *et al*., 1997; Leclerc *et al*., 1997). It was observed that hydrogen peroxide is also involved in the polymerization of actin in cattle sperm (Brener *et al*., 2003), and in increasing levels of calcium ion inside the cells and the possibility of fertilization in the semen of mice (Cohen *et al*., 1998). CAMP and protein kinase A (PKA) are also involved in regulating protein tyrosine phosphorylation (Visconti *et al*., 1995; Galantino-Homer *et al*., 1997; Leclerc *et al*., 1996). Many researchers have also indicated a need for low concentrations of ROS, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) for sperm capacitation, hyperactivity of motility, acrosome interaction and sperm-oocyte fusion (De Lamirande and Gagnon, 1993; Aitken, 1995; Baumber *et al*., 2003; O'Flaherty *et al*., 2003). These positive effects of slight concentrations of hydrogen peroxide in several species of mammals support the hypothesis of the current experience, which led to the belief that it may be possible to obtain close effects during the using hydrogen peroxide in the sperm of roosters. It may occur through the educational or cautionary role of sperm resulting from the use of slight sub-lethal concentrations of hydrogen peroxide that may help induce or produce slight oxidative stress, through which certain mechanisms can be stimulated in the rooster's sperm that enable them to resist or reduce the damage resulting from cooling or cryopreservation, but these did not happen. As the results of the current study indicate that there was no significant difference in all the laboratory characteristics of roosters semen during the using concentrations of 1 and 10 nM of hydrogen peroxide. Besides that the use of the concentration 100 nM resulted in a significant decrease in both the percentage of individual motility as shown in Table 1 and the percentage of dead sperm as shown in Table 2, compared to semen that was not treated with hydrogen peroxide in the different preservation conditions used in this study (cooling and cryopreservation periods). The reason for this deterioration may be attributed to the direct influence of hydrogen peroxide (100 nM) on the sperm motility. Many studies have shown that H_2O_2, which is added or produced by sperm, is toxic to sperm in mammals which causing sperm cell damage, including motility inhibition and a decrease in energy metabolism (Armstrong *et al*. 1999; O'Flaherty *et al*. 1999; Baumber *et al*. 2000, 2002; Bilodeau *et al*., 2001, 2002; Garg *et al*., 2009). These studies related to excessive concentrations of hydrogen peroxide in mammals, despite the different concentrations that used from the current study, support the interpretation of the hydrogen peroxide influence (concentration of 100 nM) on sperm motility. However, this may not be dependent on energy metabolism, especially since it did not observe the presence of a significant difference in the percentage of mitochondrial activity as shown in Table 4 in the present study. As the results indicate that, the mitochondria continue with their physiological roles despite the low percentage of individual sperm motility as shown in Table 1. Therefore, the current study indicates that the inhibitory effects of hydrogen peroxide on the sperm motility of chickens (when cooling and cryopreservation) forming independently of the injuries that appear on the DNA and mitochondria as shown in Tables 3 and 4. In general, this study is not the only one that failed to detect changes accompanying treatment with hydrogen peroxide, where similar results have already been described in studies conducted on human sperm, bull, male boar, and stallion, which have failed to discover changes in sperm viability. As well as, lipid peroxidation and mitochondrial membrane potential despite a decrease in motility that caused by hydrogen peroxide (Baumber *et al*., 2000; Armstrong *et al*., 1999; Bilodeau *et al*., 2002; Guthrie *et al*. 2008 Guthrie and Welch 2012). Also, similar results were observed by (Rui *et al*., 2016) when studying the effect of hydrogen peroxide at a concentration of 20 mM after incubated with the rooster's semen at a temperature of 37 °C for 30 minutes. It was observed a severe decrease in motility and fertility for semen treated with a concentration above without significant changes occurred in the percentage of plasma membrane safety, acrosome safety, malondialdehyde concentration, mitochondrial activity, and DNA Fragmentation. There is evidence to suggest that hydrogen peroxide inhibits sperm motility by damaging the axoneme by affecting the synthesis/consumption of ATP or on contractile flagellum apparatus (Delamirande *et al*., 1992a and b; Guthrie *et al*., 2008; Guthrie and Welch, 2012). Thus, based on the fact that the DAB measurement (an indirect measure of respiration by mitochondrial) showed no effect of hydrogen peroxide as in Table 4. Therefore, it can believe that the effect of hydrogen peroxide used in this study (100 nM) on motility is due to its interaction with axoneme function, either by reducing dynein-ATPase activity or by inhibiting glycolytic enzymes in the f fibrous sheath of the tail or both. Furthermore**,** it seems unlikely that hydrogen peroxide will weaken the axoneme of chicken sperm by attaching aldehydes deriving from lipid peroxidation-derived aldehydes to the major proteins in the flagellum, as (Aitken *et al*., 2012) previously reported because semen incubation with malondialdehyde was not able to influence motility (Rui *et al*., 2016). This is the first report on the effects of sublethal oxidative stress caused by Nano concentrations of hydrogen peroxide on roosters semen before cooling or cryopreservation, which showed that there is no necessity to use hydrogen peroxide in cooling and cryopreservation processes.

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