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EFFECT OF ADDING DIFFERENT LEVELS OF INSULIN HORMONE IN ATPASE ENZYME AND MITOCHONDRIAL ACTIVITY AND GENETIC MATERIAL DAMAGES IN DILUTED SEMEN OF LOCAL COCKS THAT CRYOPRESERVED FOR DIFFERENT STORAGE PERIODS

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ABSTRACT

This study was conducted to examine the impact of different levels of Insulin hormone in some some characteristics of local cocks' semen after being cooling for four different periods. this study was executed at the Poultry Research Station/ Agricultural Research Department/ Ministry of Agriculture, for the period of 12th/Feb to 21th/ Dec/2022. cocks' semen was pooled, diluted with extender, and divided equally in to five groups: control treatment (C_1) 0 insulin, (C_2) contained 4 IU/insulin, (C_3) contained 5 IU/insulin., (C_4) contained 6 IU/insulin., and (C_5) contained 7 IU/insulin. The effect of these addition was studied for different cooling periods at 0 ,24 ,48 and 72 hr on ATPase, mitochondrial activity and DNA damage. The results revealed that the addition of insulin hormone were not significant differences in mitochondrial activity, A significant differences were observed on ATPase and DNAdamage when insulin hormone added with high levels within all of the cryopreservation periods.

Keywords: Fertility, Metabolism, Sperm, Phosphorylation, Purine.

تأثيرا ضافة مستويات مختلفة من هورمون الأنسولين في نشاط انزيم الفوسفاتيز القاعدي ATPase و المايتوكوندريا وضرر المادة الوراثية في السائل المنوي المخفف للديكة المحلية والمحفوظ بالتبريد لمدد خزن مختلفة

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الخلاصة

هدفت هذه الدراسة إلى بيان تأثير مستويات مختلفة من الأنسولين في بعض صفات نطف الديوك المحلية المحفوظة بالتبريد لأربع مدد مختلفة .أجريت هذه الدراسة في محطة أبحاث الدواجن/ دائرة البحوث الزراعية/ وزارة الزراعة، للمدة من 12/شباط/2022 ولغاية21/كانون الاول/2022، جمع السائل المنوي للديكة وخفف ووزع عشوائياً على خمس مجموعات حفظت بالتبريد: C_1 0/الأنسولين؛ C_2 4/ وحدة دولية/أنسولين؛ C_3 5/ وحدة دولية/أنسولين؛ C_4 6/ وحدة دولية/أنسولين؛ و C_5 7/ وحدة دولية/ أنسولين. بعد 0 و24 و48 و72 ساعة، تم تقييم ATPase ونشاط الميتوكوندريا وتلف الحمض النووي. اشارت النتائج الى ان اضافة هرمون الانسولين عدم وجود فروق معنوية في نشاط الميتوكوندريا، كما لوحظت فروق معنوية في نشاط انزيم ATPase والنسبة المئوية لضرر المادة الوراثية عند إضافة هرمون الأنسولين بمستويات عالية إلى السائل المنوي المخفف بعد أي من فترات الحفظ بالتبريد.

الكلمات المفتاحية: البيورين ، التمثيل الغذائي ، الخصوبة، الفسفرة ،النطف

*The article is taken from the doctoral thesis of the first researcher.

INTRODUCTION

Many studies have aimed to preserve the sperm of mammals (**Moreira *et al*., 2022**), birds (**Nizam & Selcuk, 2021; Mehaisen *et al*., 2022**) and fish (**Torres & Tiersch, 2016**), which is one of the most important steps in developing reproductive biotechnology. As livestock breeding depends mainly on the use of genetically distinct male semen (**Al-Hayani & Al. Al-Daraji, 2013; Al-Maksousi *et al*., 2019; Najafi *et al*., 2020; Oldenhof *et al*., 2021; Al-Hatheel & Ibrahim 2022**). this technology is also considered important in preserving endangered animals. Although the use of the technique of preserving semen by freezing in mammals such as pigs, cows, and sheep has reached very good levels, it cannot be adopted as an applied reference for preserving semen in chickens, which is in dire need of developing this technique for preserving semen due to its physiological properties that... It is difficult to treat (**Al-Daraji, 2005**). Research indicates that eggs or embryos can be preserved, but preserving rooster semen through cryopreservation is an indispensable strategy for managing genetic diversity programs and reducing the risk of extinction for some bird breeds. However, it is still a relatively poor technology, as the fertilization rate of cryogenically stored semen is not at the desired level (**Blesbois, 2007; Al-Hamdani & Al-Hayani., 2019**).

The first fertilization of a female chicken with chilled semen in 1947(**Polge *et al*., 1949**) and currently there is continuous work to preserve the semen of other birds such as turkeys and ducks (**Woelders, 2021**) but it is still far from the commercial applications of this technique due to the low fertility after thawing and other important factors (**Hamdia *et al*., 2021; Svoradova *et al*., 2021; Yánez-Ortiz *et al*., 2021**).the process of preserving sperm results in a decrease in their viability(**Abdul-Hassan & Razuki, 2006**) due to several factors, the most important of which is the deterioration of the plasma membrane and acrosome integrity in addition to mitochondria functions during this process(**Al-Daraji, 2006; Karakus *et al*., 2021; Kumar *et al*., 2021**).Compared to some types of mammals, chicken sperm contains less mitochondria and cytoplasm, which corresponds to a higher amount of unsaturated fatty acids in its plasma membrane (**Najafi *et al*., 2020**).

When the insulin hormone molecule binds to its receptor, this enzyme works to independently add a molecule of phosphorus to the inner part of the receptor. This process, in turn, launches a number of physiological processes that arise after insulin binds to its receptor, which initiates energy consumption by converting the Adenosine Triphosphate (ATP) molecule to Adenosine Diphosphate (ADP), so that the phosphorylated molecule binds to the internal substrate of the receptor, IRS-Insulin Receptor Substrate, which In turn, it releases (PI3k-Phosphatidylinositol 3-kinase) as a concentrated signal for insulin. (**Petersen & Shulman, 2018**). this type of signals contributes to the formation of multiple types of internal proteins, such as (AKT/PKB-protein kinase B) and PIPD1 & 2 (PKC-Protein kinase C). Together, these types directly affect mechanisms that include glucose to activate and stimulate a number of important glucose transporters, such as (GLUT4-Glucose Transport protein 2) (**Simon, 1989., Seki *et al*., 2003; lee *et al*., 2022**)

Compared to mammalian sperm, which contain 50-70 mitochondria, bird sperm has half the number (about 30) (**Dzeja *et al*., 2002**; **Al-Daraji, 2012**; **Johnson, 2015**) functional in their ability to convert adenosine diphosphate (ADP) into adenosine triphosphate (ATP), which causes decrease in sperm energy supply and thus decrease in their ability to motility (**Kasai *et al*., 2002; Panda *et al*., 2016**) and this phenomenon may be attributed to the existence of a direct relationship between programmed cell death and the mitochondria functions in sperm. Mitochondria play a primary role in the process of programmed cell death

(apoptosis) after opening the mitochondrial pores, which leads to Release of pro-apoptotic factors. In addition, ATP synthesis is controlled by mitochondrial activity, and mitochondrial damage leads to loss sperm motility (**Feyzi *et al.*, 2018**).the significant decrease in semen pH is due anaerobic hydrolysis of glucose producing pyruvate acid, which is the main product of the glycolytic process that reduced to lactate acid, which is transported to the mitochondria to participate in the reaction in Krebs cycle (Brooks, 2018).As it produces about 38 ATP molecules per glucose molecule from the oxidative phosphorylation process than other metabolic pathways produce (**Tourmente *et al.*, 2015**).

Changes in the plasma membrane and its functions are also associated with increase the ROS factor (**Al-Daraji *et al.*, 2007**), which leads to change the activity of mitochondrial enzymes necessary for the production of energy units (ATP) and damage to mitochondrial DNA (**Shahin *et al.*, 2020; Nesci *et al.*,2020**). It was also found there was significant decrease in DNA integrity and the concentration of H3K9 acetyl and H3K49 methylation enzymes, and this may be due to several physiological mechanisms that work to raise the sensitivity of sperm to preservation and increase the complexity of the preservation process (**Masoumeh *et al.*, 2019**), Therefore, this study aimed to estimate the effect of adding different levels of insulin on mitochondrial activity, ATPase levels, and DNA damage in diluted cock semen under different periods of cryopreservation.

MATERIALS AND METHODS

The study was conducted at the poultry research station / Agricultural Research Directorate / Ministry of Agriculture in the Abu Ghraib region during the period from (12 fab to 22 dec 2022). One hundred units of the hormone per mililiter (Humalog Mix50) KwikPen, suspension for injection in pre-filled pen (UK) used.

Training Roosters and Semen Collection

According to **Burroughs and Quinn** (**1937**) roosters received back and abdominal massages as part of their training. This process was applied until most of the males in the study responded and reached the ejaculation stage by simply passing the hand over the back area down to the ventilation hole. The well-trained cocks were isolated and numbered with ring plastic numbers. Semen was collected from them, and the best of 40 males were evaluated and selected to obtain the best semen quality with a high concentration of semen. In addition to being free of contamination, this is done by estimating the size, taking into account that males avoid contaminating the semen with clear liquid secretions or feces.

Semen Preservation and Experiment Design

After selecting the top 40 males, a pooled sample of semen was collected and diluted 1:3 using diluted (**Khaeruddin&Amir, 2019**). The diluted semen was divided into 5 parts equally and was added concentrations (0, 4, 5, 6 and 7 IU/insulin), the five treatments were kept cool until the temperature reached 5 °C, as the laboratory tests begin at this degree. The second period represents 24 hours after evaluating the indices of semen for the second period of preservation, as well as the preservation period of 48 and 72 hours as third and fourth period respectively. Finally, this process repeated 7 times, laboratory tests were performed for each repetition, and then the data were analysed statistically.

Study tests

Tests were conducted in the research station laboratory, mitochondrial activity estimate by oxidation of 3,3-diaminobenzidine(DAB)by the cytochrome c complex (including cytochrome c oxidase), in a chain reaction in which the reagent is polymerized and deposited at the reaction sites (**Blumer *et al.*, 2012**), ATPase according to Perform ATP Hydrolysis Reaction with Purified Protein as nmol pi/umol protein (**Rule *et al.*, 2016**), DNAdamage according to (**Tejada *et al.*, 1984**)by fixing sample slides in Carnoys solution (3parts methanolll part glacial acetic acid).

Statistical Analysis

The Statistical Analysis System (**SAS, 2012**) used to analyse the experiment data, as two-way (5 × 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 (**Duncan, 1955).**

RESULTS AND DISCUSSION

There were no significant differences for the effect of insulin concentrations used on the mitochondrial activity (Table 1) in presence of glucose as energy source in diluted Chilled semen was one of the exciting and scientifically interesting results in this study.

Table (1): Effect of different level of insulin on mitochondrial activity of local rooster after different cryopreservation periods.

Concentration	Time				Significance
	$H_{0.00}$	H_{24}	H_{48}	H_{72}	
C_1	67.76±3.36 abc	64.55±6.28 abcd	51.21±2.94 cdef	66.34±2.63 CD	N.S
C_2	62.31±5.33 abcde	66.33±4.07 abc	53.86±14.01 cdef	78.97±2.71 ABCD	N.S
C_3	45.44±6.04 ef	62.23±1.83 abcde	55.99±2.51 bcdef	63.00±1.89 ABCD	N.S
C_4	58.54±7.81 bcde	63.32±4.23 abcde	38.00±5.63 f	73.08±4.09 ABCD	N.S
C_5	66.66±1.78 abc	65.42±5.96 abcd	46.87±8.58 def	52.34±0.52 ABCD	N.S
Significance	*	*	*	*	

*(P≤0.01) for Concentration
%, Mean±Std Error
C_1=0 UI/insulin, C_2=4 UI/insulin, C_3=5 UI/insulin, C_4=6 UI/insulin, C_5=7 UI/insulin

Despite the significant increase in ATPase enzyme activity (Table 2) under the influence of concentrations C_4 and C_5 with C_1, mitochondrial activity was not significant under the influence of any insulin concentrations used. The improvement in any traits under the effect of insulin concentrations used is due to other metabolic pathways that affected the metabolic activity of sperm, and this is consist with what was indicated by (**Tourmente *et al.*, 2015**).

ATP is produced mainly via two functionally different metabolic pathways and in different part of the tail (**Setiawan *et al.*, 2020a**). Firstly, oxidative phosphorylation occurs in the tightly packed mitochondria in the mid-piece, and secondly, glycolysis, whose role is limited to the principal piece, which is the longest subpart of the flagellum, where anaerobic glycolysis occurs, which is sufficient to supply sperm with a large amount of ATP. The conversion of glucose to pyruvate is the main metabolic pathway for obtaining ATP in human sperm (**Williams & Ford, 2001**) and birds (**Setiawan *et al.*, 2020b**), as sperm derivate hexose sugar through specialized transporters such as Glucose transporters (GLUTs) (**Bucci *et al.*, 2011**). As soon as the glucose enters the sperm cytoplasm, the process of its phosphorylation begins to be used in the various metabolic pathways, including the pentose phosphate pathway (PPP) to manufacture glycogen, or to convert the extra glucose to produce pyruvate. The presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hexokinase I, essential for glucose degradation, in the mid-piece and principal piece indicates that the glucose hydrolysis pathway is active along the tail in chicken sperm (**Setiawan *et al.*, 2020a**). the presence of number of glycolytic enzymes, including hexokinase I and GAPDH, in capsid spermatozoa is directly or indirectly associated with the fibrous sheath, which is found particularly in the tail primaries (**Welch *et al.*, 1992**., **Westhoff&Kamp, 1997**). the enzyme hexokinase I plays role in the PPP pathway semi-independently and parallel to the glycolysis pathway (**Setiawan *et al.*, 2020a**))

The aforementioned PPP metabolic pathway is the main source of NADPH, which can result from the degradation of tricarboxylic acid cycle (TCA) products and from fatty acid oxidation (**Horecker, 2002; Pitia and *et al.*, 2017; Dick & Ralser, 2015; Cherkas *et al.*, 2020**).

PPP consists of two branches, the oxidative branch, which leads to the generation of NADPH and ribonucleotides, and the non-oxidative branch, which involves reversible reactions that consume glucose intermediates to be converted to pentose phosphate in a reverse way (**Patra&Hay, 2014**). In the oxidative branch, the first reaction is the dehydrogenation of glucose-6-phosphate by the action of the enzyme glucose-6-phosphate dehydrogenase (G6PD) to produce NADPH and 6-phosphogluconolactone, which is then hydrolyzed by phosphoglucosactonase to 6-phosphogluconate.

After this step, the catalyzed oxidative decarboxylation of 6-phosphogluconate dehydrogenase is catalyzed to produce NADPH again and convert ribulose-5-phosphate to ribose-5-phosphate (**Patra&Hay, 2014**). in this context, it is necessary to emphasize that the main function of the PPP pathway is relative inhibition of the the oxidized glutathione (GSSG) is converted to reduced glutathione (GSH) in sperm (**Urner&Sakkas, 1999; Miraglia *et al.*, 2010; Evdokimov *et al.*, 2015**).

The storage period had a significant effect in mitochondrial activity, as the level of this effect varied between the greater decrease in the level of oxidative phosphorylation activity for the period H48, and there were no significant differences between the period H_{24} and H_{72} compare to period $H_{0.00}$ (Tables 1), which was shown by the statistical analysis of ATPase activity under the influence of storage period, whose effect overlapped with insulin concentration in the cooled diluted semen (Tables 2) ,also varied, as the storage period had the greatest effect on mitochondrial and ATPase activity .

Table (2): Effect of different level of insulin on ATPase levels of local rooster after different cryopreservation periods.

Concentration	Time		Significance
	$H_{0.00}$	H_{72}	
C_1	2.29±0.54 ab	2.38±0.45 ab	*
C_2	2.47±0.56 ab	1.50±0.54 b	*
C_3	2.46±0.18 ab	2.23±0.59 ab	*
C_4	3.12±0.35 a	2.34±0.50 ab	*
C_5	1.49±0.24 b	1.87±0.13 ab	*
Significance	N.S	N.S	

*($P \leq 0.05$) for Concentration
Mean±Std Error
C_1=0 UI/insulin, C_2=4 UI/insulin, C_3=5 UI/insulin, C_4=6 UI/insulin, C_5=7 UI/insulin

The significant effect of adding insulin to the cooled semen increasing DNAdamage in concentrations C_4 and C_5 (Table 3) , is consist with a decrease in the activity of oxidative enzymes such as MDA, or at least the inability of the quantities produced from them to keep up with the high levels of oxidation that sperm are exposed during the period of cold storage, which makes them vulnerable to mechanical and oxidative damage, which includes the release of free radicals, which reduces the activity of sperm significantly, as they contain high concentrations of long-chain polyunsaturated fatty acids in phospholipids. Thus they are susceptible to damage by oxidative stress (**Blesbois *et al.*, 1997**).

The high level of reactive oxygen species (ROS) resulting from oxidative stress is associated with damage to the sperm DNAdamage, as free radicals oxidize the nitrogenous bases of purine and pyrimidines within the DNA structure of sperm and cause damage to it, as antioxidants represent the first line of defense to prevent free radicals that are produced of the oxidation process of polyunsaturated fatty acids within phospholipids (**Shafigh *et al.*, 2016**),

Table (3): Effect of different level of insulin on DNA damage of local rooster after different cryopreservation periods.

Concentration	Time				Significance
	$H_{0.00}$	H_{24}	H_{48}	H_{72}	
C_1	6.38±0.29 k	8.49±0.69 ij	9.48±0.28 fghij	10.66±0.52 defg	*
C_2	5.88±0.05 k	8.18±0.29 j	9.80±0.06 fghij	10.10±0.40 efghi	*
C_3	8.84±0.18 hu	9.29±0.61 ghij	11.07±0.26 cdef	12.31±0.52 bc	*
C_4	10.29±0.24 efgh	11.66±0.34 cd	12.43±0.33 bc	14.08±0.66 a	*
C_5	11.10±0.83 cdef	12.10±0.82 cd	13.73±0.60 ab	14.18±0.92 a	*
Significance	*	*	*	*	

*(P≤0.01) for Concentration
%, Mean±Std Error
C_1=0 UI/insulin,C_2=4 UI/insulin,C_3=5 UI/insulin,C_4=6 UI/insulin,C_5=7 UI/insulin

It is also interesting that the high DNAdamage in (Tables 3) was very clear under the influence of the storage period factor, especially the storage period H_{27}, compared to $H_{0.00}$. the effect of the interaction between the concentration of insulin and storage period was most significant in interactions C_4H_{72},C_5H_{72} , which the interactions with insulin concentrations were the highest, while the interactions $C_1H_{0.00}$,$C_2H_{0.00}$ is the least harmful, and this indicates that the higher concentrations of insulin increased the metabolic activity of the sperm, which is usually associated with increase in the ROS produced, as the activation of cell support mechanisms and their requirements of energy sources and cellular compounds required for their maintenance in conditions of oxidative stress leads to shifting cellular metabolism toward catabolism and glucose depletion to activate self-protective mechanisms, including stimulation of endogenous antioxidant production, including release of glucose from glycogen (**Stelmakh *et al*., 2016; Höhn *et al*., 2017; Anton *et al*, 2018**). This completely consist with the effect of the high concentration of insulin on sperm metabolism and glucose consumption at the level of the highest two concentrations C_4,C_5 (**Feinman & Fine, 2013**).

CONCLUSIONS

It can be concluded that adding Insulin to cryopreservation-stored semen for local cocks had no significant effect on mitochondrial activity, high levels of insulin in diluted semen significantly increase ATPase levels and DNAdamage after any of the cryopreservation periods.

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