# Impact of Different Levels of Insulin on Cryopreservation Local Roosters Stored Semen Biomarkers

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**Abstract.** The aim of this research to examine the impact of different level of Insulin certain biomarkers of local roosters semen after being cooling for varying lengths of periods, roosters semen pooled, diluted with extender, and distributed randomly among five groups: Diluted semen put through the semen cryopreservation technique; the control treatment (C1) 0 insulin ; (C2) contained 4 IU/insulin ; (C3) contained 5 IU/insulin; (C4) contained 6 IU/insulin; and (C5) contained 7 IU/insulin. After 0 ,24 ,48 and 72 hours, cooling semen were evaluated for plasma membrane integrity(MI), acrosome integrity(AI), , malondialdehyde (MDA) and total antioxidant capacity (TCA). The result showed significant differences between the treatments in terms of MI , AI and TCA in sperm with C5 , C3,C2 respectively , but there were no significant differences in terms of MDA. TCA were found to be significantly improved in the fourth storage period.

**Keywords.** Insulin, Cooling, Semen biomarkers, Local roosters.

## 1.Introduction

Poultry meat is the main source of dietary protein for humans which now can be notice that number of poultry has increased by about 11% worldwide, due to physiological speed and relatively low feed costs [1-3]. Artificial insemination of avian and semen cryopreservation is restricted practical application , partly due to the low efficiency of existing methods of cryopreservation, is a constraint on the development of the poultry industry [4,5].

A sharp decrease in physiological characteristics of spermatozoa is observed In the process of cryopreservation of avian semen[6,7,8] that is explained by a high level of damage of plasma membranes of sexual [9,10,11]. for the past decades, despite the fact that it is still not possible to preserve more than 50% of full-grown germ cells, researchers at Cryobiology feild have established certain success in cryopreservation of avian semen [12,13].

Increasing rate of oxidation of cellular components and the excessive production of reactive oxygen species (ROS) in sperm is a phenomenon called oxidative stress. Low levels of oxidative stress may have a beneficial effect on cells, while high levels can cause cell death through destroying nucleic acids, proteins, fats, and carbohydrates, .the influence of oxidative stress on sperm is particularly

important during semen storage and semen cryopreservation because of an increased production of reactive oxygen species (ROS) during these processes [14,15]. The main sites of ROS formation are the mitochondria [16] , rapid temperature changes are particularly vulnerable to damage of sperm cell membrane. [17,18] dilution, cooling, freezing, exposure to cryoprotectants, and thawing, all handling procedures related to semen preparation for storage, induce a higher concentration of malondialdehyde (MDA) and evoke osmotic stress which leads to oxidative stress in the semen [19,20].
Partially, this effect is related to the initial extension of the semen, as semen plasma has been shown to inhibit spontaneous lipid peroxidation. Interestingly enough, turkey seminal plasma has higher free radical trapping activity than chicken seminal plasma [21]. the analysis of cryopreserved semen of many mammalian species showed that during cryopreservation, the production of ROS is increased [22], and this has been confirmed in avian semen [23,24].
Significantly, concentration of the final product of lipid peroxidation in chicken semen cryopreservation are increase in both seminal plasma and sperm cells [25]. Peroxidation of lipids has critical consequences For spermatozoa . Oxidation reactions in plasma membranes lead to expansion the (ROS), disturbance of ion-gradients, loss of compartmentalization and plasma-membrane integrity ,changes in membrane fluidity, and modification of proteins and DNA [26].
This study aimed to explore the effect of insulin physiologically increasing the sperm survivability under cryopreservation conditions and its impact on its vital functions.

## 2.Materials and Methods

The study conducted at the poultry research station / Agricultural Research Directorate / Ministry of Agriculture in the Abu Ghraib region and operation with laboratories in Mosul university Ethical approval No. um.VET.2021.5. during the period from (12th feb to 22th dec in 2022). Humalog Mix50 100 units/ml KwikPen, suspension for injection in pre-filled pen(United kingdom) used, tests were conducted in the research station laboratory, acrosome integrity (AI ) according to Al-Daraji [10,27] membrane integrity (MI) according to Askarianzadeh[26], malondialdehyde (MDA) according to Kumaresan [28],total antioxidant capacity (TCA)according to Cohen [29].

### *2.1. Training Roosters and Semen Collection*

Roosters were trained using dorsa-abdominal massage as described by Burrow and Quin [30]. 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, the well-trained roosters were isolated and numbered with annular plastic numbers (fixed to leg), the semen was collected from each male to evaluate and select the best 40 male to obtain the best quality of semen, with a high concentration of semen taking into account the avoidance of males from contaminating the semen with clear liquid secretions or stool.

### *2.2. Semen Preservation and Experiment Design*

A pooled sample of semen was collected and diluted 1:3 using diluted Abdel-Khalek.et.al,2019 [31]. 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. 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.

### *2.3. Statistical Analysis*

The Statistical Analysis System (SAS) [32] 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 (1955) [33].

## 3.Results and Discussion

Essential metabolic substrate like glucose transport into animal cells by the family of proteins called glucose transporter (GLUT) by insulin stimulates glucose uptake into skeletal muscle and adipose tissue by starting a stimulation process, insulin responsive glucose uptake on molecular level. GLUT4 is well known as a major insulin-responsive transporter in mammals which is working distribute glucose through plasma membrane to the intracellular storage vesicles [33]. In chickens, GLUT1 and GLUT12 is among GLUT isoforms suggested to be insulin-responsive GLUTs [35].

When insulin reaches the cells, it usually begins to search for its receptors, which encircle the insulin molecule and attach to it in order to distribute it to most surfaces of the cells of the body and spread it as widely as possible on the fat surfaces , muscle and liver cells. Therefore, it is considered the link between the outer and inner cells. these receptors consist of two main parts, where the molecular weight of the largest part, which is called alpha, reaches 130,000 Daltons, and this part extends to the outer part of the cell, while the smaller part of these receptors is called Beta, which weighs 90,000 Daltons which extends into the cell [36,37]

Associated with an enzyme that becomes effective when the insulin molecule is attached to its receptor, as this enzyme works to add a molecule of phosphorus independently to the inner part of the receptor. This process, in turn, launches a number of physiological processes that arise from insulin binding to its receptor, which begins to cause energy consumption by converting the molecule of Adenosine Triphosphate (ATP) into Adenosine Diphosphate (ADP) to bind the phosphorylated molecule with the inner substrate of the receptor, IRS-Insulin Receptor Substrate, which It, in turn, releases PI3k-Phosphatidylinositol 3-kinase (PI3k-Phosphatidylinositol 3-kinase) as a signal that concentrates insulin [38,39].

This type of signal contributes to the formation of multiple types of endogenous proteins such as (AKT / PKB-protein kinase B) and PIPD1 & 2 (PKC-Protein kinase C). Together, these types directly influence mechanisms involving glucose to activate and stimulate a number of important glucose transporters such as (GLUT1 and GLUT12 in avian ) -Glucose Transport protein [40] and indirectly influence the formation of glycogen and protein formation pathways, anti-lipid degrading enzymes and gene expression, which occur under the influence of active genetic factors such as MAP Kinase-Mitogen activated protein kinase and Mitogenic effects within the nucleus [41,42].

From the insulin mechanism of action, we can clearly notice the effect of insulin concentration C3 (Table 1) on the percentage of the plasma membrane integrity (MI) and the concentration C5 (Table 2) on the percentage of acrosome integrity (AI) [43].

**Table 1.** Effect of different level of insulin on plasma membrane integrity of local rooster after different cryopreservation periods (%, Mean±Std Error).

| Concentration | Time | | | | Significance |
|---|---|---|---|---|---|
| | $H_{0.00}$ | $H_{24}$ | $H_{48}$ | $H_{72}$ | |
| $C_1$ | 80.04±1.91 d | 81.43±2.59 bcd | 81.57±3.91 abcd | 81.22±1.91 cd | * |
| $C_2$ | 81.81±1.22 abcd | 82.31±1.54 abcd | 84.55±1.46 abcd | 83.93±1.51 abcd | * |
| $C_3$ | 85.23±1.12 abcd | 86.68±1.84 abcd | 87.72±1.06 a | 85.52±1.22 abcd | * |
| $C_4$ | 87.58±1.06 abcd | 86.03±1.90 abcd | 85.08±1.68 abcd | 83.72±1.47 abcd | * |
| $C_5$ | 86.24±1.51 abcd | 86.11±2.02 abcd | 85.79±1.71 abcd | 82.70±1.62 abcd | * |
| Significance | N.S | N.S | N.S | N.S | |

*(P≤0.05) for Concentration

Capital letters to compare interaction among Concentration and periods

$C_1$=0 UI/insulin

$C_2$=4 UI/insulin

$C_3$=5 UI/insulin

$C_4$=6 UI/insulin

$C_5$=7 UI/insulin

**Table 2.** Effect of different level of insulin on acrosome integrity of local rooster after different cryopreservation periods (%, Mean±Std Error).

| Concentration | Time | | | | Significance |
|---|---|---|---|---|---|
| | $H_{0.00}$ | $H_{24}$ | $H_{48}$ | $H_{72}$ | |
| $C_1$ | 84.53±3.51 abcd | 80.69±1.98 bcd | 78.15±3.09 cd | 81.04±1.32 bcd | * |
| $C_2$ | 86.80±2.84 ab | 83.16±2.59 abcd | 80.98±3.42 bcd | 80.88±2.86 bcd | * |
| $C_3$ | 88.68±1.79 ab | 83.38±1.92 abcd | 76.80±3.57 d | 82.50±1.78 abcd | * |
| $C_4$ | 85.56±0.96 abc | 81.59±1.71 bcd | 81.95±1.71 bcd | 82.60±2.05 abcd | * |
| $C_5$ | 89.81±1.53 a | 84.09±1.73 abcd | 88.20±0.99 ab | 81.45±2.43 bcd | * |
| Significance | * | * | * | * | |

*(P≤0.01) for periods
Capital letters to compare interaction among Concentration and periods
$C_1$=0 UI/insulin
$C_2$=4 UI/insulin
$C_3$=5 UI/insulin
$C_4$=6 UI/insulin
$C_5$=7 UI/insulin

Unsaturated fat oxidation occurs when the sperm is exposed to oxygen immediately after ejaculation, which has a very harmful effect on live sperm, which extends to constitute a major obstacle during the preservation of bird semen in vitro[44]. Therefore, lipid peroxidation can lead to damage Plasmal membrane [45] and may trigger an early acrosomal reaction [46,47].

There were no significant differences in malondialdihyde levels (MDA), which results from lipid oxidation in the sperm plasma membrane, which is a sign of oxidative stress and antioxidant deficiency[48] between the concentrations of insulin used compared to the C1 concentration in diluent with glucose presence as an energy source (Tables 3 ) and the high levels of total oxidation capacity (TAC) with the variation effect of insulin concentration used in (Tables 4) correspond to the improvement in the percentage of plasma membrane integrity and the percentage of acrosome integrity in concentration C3 (Table 1) and C5 (Table 2), respectively, which indicates the role of insulin in increasing the ability of sperm to provide a greater amount of ATP energy molecules to resist the process of lipid oxidation that occurs to the plasma membrane during the storage period and increase the chances of an early acrosomal reaction [49].

**Table 3.** Effect of different level of insulin on malondialdehyde levels of local rooster after different cryopreservation periods (%, Mean±Std Error).

| Concentration | Time | | | | Significance |
|---|---|---|---|---|---|
| | $H_{0.00}$ | $H_{24}$ | $H_{48}$ | $H_{72}$ | |
| $C_1$ | 12.30±4.49 | 6.86±2.93 | 11.30±2.01 | 13.08±1.11 | N.S |
| $C_2$ | 8.85±2.37 | 9.18±1.14 | 14.49±1.30 | 10.01±1.80 | N.S |
| $C_3$ | 10.02±4.71 | 9.74±3.64 | 10.97±1.58 | 15.38±0.88 | N.S |
| $C_4$ | 12.75±8.74 | 5.46±2.89 | 15.38±0.62 | 14.04±2.28 | N.S |
| $C_5$ | 8.72±3.50 | 10.53±0.43 | 16.02±0.61 | 11.80±0.83 | N.S |
| Significance | * | * | * | * | |

*(P≤0.05) for periods
$C_1$=0 UI/insulin
$C_2$=4 UI/insulin
$C_3$=5 UI/insulin
$C_4$=6 UI/insulin
$C_5$=7 UI/insulin

**Table 4.** Effect of different level of insulin on total antioxidants capacity of local rooster after different cryopreservation periods (%, Mean±Std Error).

| Concentration | Time | | | | Significance |
|---|---|---|---|---|---|
| | $H_{0.00}$ | $H_{24}$ | $H_{48}$ | $H_{72}$ | |
| $C_1$ | 4.97±0.48 b | 3.73±0.75 cd | 3.63±0.32 cd | 4.23±0.48 bc | * |
| $C_2$ | 6.77±0.03 a | 3.60±0.23 cd | 3.00±0.15 c | 4.77±0.75 b | * |
| $C_3$ | 1.33±0.03 efgh | 1.90±0.42 ef | 1.13±0.18 efgh | 1.17±0.43 efgh | * |
| $C_4$ | 1.60±0.21 ef | 0.83±0.03 fgh | 0.73±0.09 gh | 2.03±0.09 de | * |
| $C_5$ | 0.87±0.12 fgh | 0.47±0.03 h | 0.23±0.04 h | 0.87±0.03 fgh | * |
| Significance | * | * | * | * | |

*(P≤0.01) for periods
Capital letters to compare interaction among Concentration and periods
$C_1$=0 UI/insulin
$C_2$=4 UI/insulin
$C_3$=5 UI/insulin
$C_4$=6 UI/insulin
$C_5$=7 UI/insulin

The best that can be concluded from the lack of significant differences for the effect of the storage period in the (MI) when using insulin with glucose (Table 1) with a decrease in the (AI) during periods ($H_{24}$, $H_{48}$ and $H_{72}$) (Table 2 ) with a decrease in the same indicators when using insulin and the rise in MDA levels (Tables 3 ) and the corresponding decrease in TAC levels (Table 4), that the effect of storage period tends to appear stronger move towards the longer storage period ($H_{72}$) [48][49][50] However, the results in the glucose-containing dilute "as an energy source" tended to be where the MI , AI , MDA and TAC levels were high during the periods $H_{48}$ and $H_{72}$ of cryopreservation process .

The significant differences (P<0.05) observed for the effect of the interaction between concentration and cryopreservation period were clearly showing the effect of insulin concentrations on the percentage of plasma membrane integrity (Tables 1) and acrosome integrity (Table 2) and on the levels of MDA (Tables 3) and TAC (Tables 4). Depending on the duration of storage, it was not far from the obvious negative effect of increasing the storage period [51,52].

## Conclusions

It concluded that insulin addition to diluted semen had a positive effect on raising the percentage of plasma membrane and acrosome integrity by reducing or slowing down the lipid peroxidation processes and raising the level of total antioxidants capacity that work to resist oxidative stress accompanies the preservation process.

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