# EFFECT OF ADDING RESVERATROL TO SOYBEAN-LECITHIN EXTENDER ON SOME SEMEN ATTRIBUTES OF BUFFALO BULLS

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**ABSTRACT**

**This study was conducted to explain the effect of adding different resveratrol (Res) concentrations to soybean-lecithin extender on some post-cryopreserved (PC) semen characteristics of the Iraqi buffalo bulls for different preservation periods. Eight mature bulls were used, and polled semen was equally divided into four groups within one trial. Three Res concentrations (100, 150, and 200 µM) were added to the soybean-lecithin extender, and comparisons in the response were made with the control group (soybean-lecithin extender, Res-0). The differences among groups in sperm cell individual motility percentages at different preservation periods lacked significance. Greater (P≤0.01) sperm acrosome integrity percentage was noticed for the Res-150 group compared with the C and Res-200 groups at cooling, 48 hrs., and 2 months PC. Lesser (P≤0.01) malondialdehyde concentrations and sperm DNA damage percentage were observed for the Res-150 group as compared with the other groups, 2 months PC. Higher (P≤0.01) total antioxidant activity was shown in Res-150 and Res-200 groups as compared with the control and Res-100 groups, 2 months PC. In conclusion, adding Res to the soybean-lecithin extender enhanced some of the PC semen characteristics of Iraqi buffalo bulls at different preservation periods.**

**Keywords: Sperm quality, antioxidants, DNA damage, buffalo.**



## تأثير اضافة الريسفيراترول الى مخفف soybean-lecithin في بعض صفات السائل المنوي لثيران الجاموس

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**المستخلص**

**اجريت هذه الدراسة لبيان تأثير اضافة تراكيز مختلفة من مركب الريسفيراترول الى مخفف Soybean–lecithin في بعض صفات السائل المنوي لثيران الجاموس العراقي بعد الحفظ لمدد زمنية مختلفة. تم استعمال ثمانية من ثيران الجاموس العراقي البالغة وجمع السائل المنوي وتجميعه منها ومن ثم تقسيمه الى اربعة اجزاء باستعمال مخفف Soybean–lecithin. تم اضافة مركب الريسفيراترول بثلاث تراكيز (100 و150 و200 مايكرومول) ومقارنته مع مجموعة السيطرة (المخفف فقط). انعدمت الفروق المعنوية في النسبة المئوية لحركة النطف الفردية بين المجاميع كافة اثناء مدد الحفظ المختلفة. حققت مجموعة الريسفيراترول بتركيز 150 مايكروليتر زيادة معنوية (P≥0.01) في النسبة المئوية لسلامة الاكروسوم مقارنة بمجموعتي السيطرة و200 مايكروليتر عند الحفظ بالتبريد و48 ساعة وشهرين من الحفظ بالتجميد. سجلت مجموعة 150 مايكروليتر اقل (P≥0.01) تركيز لمركب المالون ثنائي الألديهايد ونسبة مئوية لضرر المادة الوراثية للنطف مقارنةً ببقية المجاميع بعد شهرين من الحفظ بالتجميد. كما اظهرت مجموعتي 150 و200 مايكروليتر ريسفيراترول زيادة معنوية (P≥0.01) في فعالية مضادات الاكسدة الكلية مقارنةً بمجموعتي السيطرة و100 مايكروليتر بعد شهرين من الحفظ بالتجميد. يمكن الاستنتاج ان اضافة مركب الريسفيراترول بتركيز 150 مايكروليتر الى مخفف soybean–lecithin ادى الى المحافظة على بعض صفات السائل المنوي لثيران الجاموس العراقي بعد مدد مختلفة من الحفظ.**

**الكلمات مفتاحية: نوعية النطف، مضادات أكسدة، ضرر المادة الوراثية، الجاموس.**

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## INTRODUCTION

Nowadays, sperm cryopreservation of buffalo bulls (*Bubalus bubalis*) is an effective method for protecting their genetic resources. Cryopreservation techniques are now the reason for cryo-damages in dead spermatozoa, despite the benefits of frozen-thawed semen (21). Increased generation of reactive oxygen species (ROS) by abnormal, immature, and mortal spermatozoa might be the reason behind sperm mortality. Therefore, oxidative damage decreases the fertilization capacity of spermatozoa used for artificial insemination (28, 29). Cryopreservation decreases the natural semen antioxidant, causing oxidative stress to the spermatozoa. These are the utmost reasons for inefficient sperm function due to the high membrane content of polyunsaturated fatty acids and restricted repair mechanisms (34). Buffalo sperm have a greater lipid peroxidation rate when compared to cattle (11). Cytoplasmic is one of the primary protecting antioxidant systems in spermatozoa. Sperm dismisses all of its cytoplasms during the final stage of differentiation. Consequently, lake the significant cytoplasmic combination antioxidants that neutralize the detrimental effect of ROS and lipid peroxidation (31). The antioxidant in semen extender reduce oxidants damage and improve post-thaw semen quality by neutralizing oxidative stress (14, 27). There is some antioxidant system in spermatozoa and seminal plasma that scavenge reactive oxygen species (ROS) and prevent internal cellular detriment (2, 22, 42). The first ROS scavengers in buffalo semen are glutathione peroxidase, catalase, and superoxide dismutase (40). One way to enhance sperm quality, and fertilization capacity, is by adding an antioxidant to the cryopreservation extender (36). Resveratrol (Res) is one of the most crucial non-flavonoid polyphenols (25), existing in peanuts, the skin of red grapes, and red wine. It plays important roles as an antioxidant and has several biological activities, like anti-apoptotic and anti-inflammation properties (19). Moreover, Res has an effectual role in scavenging several ROS like, hydroxyl and superoxide radicals (24). Adding Res to human semen extender can decrease DNA damage in spermatozoa (20). Furthermore, Res protects acrosome integrity percentage in bull sperm after the freeze-thaw process (7). On the other hand, Res decreases malondialdehyde (MDA) concentration in ram semen stored at cooling conditions for up to 168 hrs. (5). Adding 20 mg/ml Res to cryopreservation media did not affect sperm's cell motility and vigor of frozen ram sperm (35). Also, adding Res to a cryopreservation extender can decrease DNA damage in bulls (10) and human (20) sperms. It is still unclear whether the effect of Res on buffalo spermatozoa variable is dose-depended and if adding resveratrol to a semen extender can impact sperm function when using in commercial buffalo bull semen extender. The influence of adding different concentrations of Res to soybean-lecithin extender on the semen characteristics of Iraqi buffalo bulls did not been previously investigated. Therefore, this study aimed to explore these effects.

## MATERIALS AND METHODS

### Animals and semen collection

The study was carried out at the Artificial Insemination Department (AID) belonging to the Directorate of Animal Resource, Ministry of Agriculture in the Abu Ghraib region (25 km west of Baghdad), Iraq during the period from October 2020 to September 2021. Eight Iraqi buffalo bulls (*Bubalus bubalis*) were trained to collect semen using the artificial vagina method, with ages ranging between 4.5-5 years and a body weight ranging between 500-700 kg/bull. All bulls were in good health, free of disease, and under continuous veterinary supervision. All animals fed 5 kg of concentrate diet/animal/day consisted of 35% barley, 33% wheat bran, 10% yellow corn, 20% soybean meal, 0.5% limestone, and 1% vitamins and minerals. The crude protein percentage and total energy amount of the diet were 18% and 2164 kilocalories/kg, respectively. Concomitantly, the buffalo bulls provided 7-9 kg of hay and 40-50 kg of alfalfa per animal/day. Salt blocks and water were freely available for bulls throughout the experimental period.Ejaculates were collected from each bull using an artificial vagina once a week. The ejaculates were pooled to increase the semen volume for replication and to eliminate variability among the samples. The pooled semen samples were

immersed in a water bath at 36 $^0$C until they could be assessed for progressive motility of the sperm cells. This study was replicated five times for each group.

**Semen processing and treatments**

The soybean-lecithin extender contained 2.42g Tris, 1.34g citric acid, 1g fructose, 6.4 ml glycerol (6.4%), 1g soybean-lecithin, 0.4 IU gentamycin, and 0.08 IU Tylosin in 87.36 ml distilled water (18). The extender was mixed with the pooled semen samples and divided into four parts. Three Res concentrations (100, 150, and 200 µM) were added to the soybean-lecithin extender within Res-100, Res-150, and Res-200 groups, and the soybean-lecithin extender served as the control (Res-0). Semen characteristics were investigated for different preservation periods (cooling at 5 ◦C, 48 h, 1 and 2 months post-cryopreservation; PC). Semen in terms of sperm cell individual motility (39), acrosome integrity (6), and DNA damage percentage (37) were evaluated. The concentrations of malondialdehyde and total antioxidants in seminal plasma were also assessed according to the Abdulkareem et al (1) and Eidan et al (15) methods.

**Statistical analyses**

Statistical computations were carried out using the complete random design (CRD) to study the influence of different concentrations of resveratrol on the studied traits in the SAS program (33). The statistical model for the analysis of variance was as follows:

$$Y_{ij} = \mu + T_i + P_j + e_{ij}$$

Where:

$Y_{ij}$ = dependent variable (semen characteristics).

µ= Overall mean

$T_i$= Effect of resveratrol concentrations (Res-0, Res-100, Res-150, and Res-200).

$P_j$ = Effect of preservation period (48 h, 1 and 2 months PC).

$e_{ij}$= error term

Differences among means were utilizing Duncan's multiple range test (13). The Chi-square test was used to compare different percentages of studied attributes.

## RESULTS AND DISCUSSION

**Sperm cell individual motility**

Adding different concentrations of Res to soybean-lecithin extender did not significantly affect the percentage of sperm cell individual motility (SCIM) of Iraqi buffalo bulls in all groups during the different preservation periods. When examining the effect of various preservation periods within one group, non-significant differences were observed among all periods in the Res-0 group, except for a decrease (P≤ 0.05) in this trait, two months PC compared to the cooling period (Table 1). It also noticed a decrease (P≤ 0.05) in this trait among the preservation periods at the first and second month PC compared to the cooling period for the Res-100, Res-150, and Res-200 groups (Table 1). Concomitantly, the differences between the 48 h PC and cooling period, on the one hand, and one and two months PC, on the other hand, in these groups for a similar trait (Table 1).

**Table 1. Effect of adding different resveratrol concentrations to soybean-lecithin extender on sperm's cells motility percentage of Iraqi buffalo bulls for different preservation periods (Mean ± SE).**

| Period / Group | Cooling 5° C | 48 hr. PC | 1 Month PC | 2 Month PC | Level of significance |
|---|---|---|---|---|---|
| Res-0 | 42.50±2.50 A | 35.00±2.88 AB | 33.75±3.75 AB | 31.25±3.14 B | P≤0.05 |
| Res-100 | 48.75±1.25 A | 42.50±1.44 AB | 37.50±3.22 B | 36.25±4.26 B | P≤0.05 |
| Res-150 | 48.75±1.25 A | 42.50±1.44 AB | 37.50±3.22 B | 37.50±3.22 B | P≤0.05 |
| Res-200 | 43.75±2.39 A | 36.25±3.14 AB | 33.75±2.39 B | 32.50±3.22 B | P≤0.05 |
| Level of significance | NS | NS | NS | NS | ------- |

**Small superscripts within a similar column indicate differences, and large superscripts within a similar row indicate differences. Res-0= control; Res-100= Resveratrol 100 µM; Res-150= Resveratrol 150 µM; Res-200= Resveratrol 200 µM; PC= post cryopreservation.**

**Sperm acrosome integrity percentage**

Greater (P≤0.01) sperm acrosome integrity percentage (SAIP) was observed for the Res-150 (82.50±0.86%) group as compared with the Res-0 (77.50±0.64%), Res-100 (78.50±1.19%) and Res-200 (77.50±1.04%) groups, while, the differences among the latter three groups lacked significance at cooling period (Table 2). Similarity, highly significant (P≤0.01) SAI was noticed in Res-150 at 48hrs. (75.75±0.75%) and two-months PC (66.50±1.29%) as compared with the Res-0 and Res-200 (Table2). Concomitantly, Res-150 exhibited greater (P≤0.01) SAIP (69.25±1.49%) as compared with Res-0 (61.50±1.93%), one-month PC (Table 2). Concerning the effect of preservation periods within each group, the cooling period exhibited higher (P≤0.01) SAIP in comparison with all PC periods for all groups (Table 2). Excluding data of Res-200, the SAIP decreased (P≤0.01) at one-month PC compared with the 48hrs. PC at all other groups (Table 2).

**Table 2. Effect of adding different resveratrol concentrations to soybean-lecithin extender on sperm acrosome integrity percentage of Iraqi buffalo bulls for different preservation periods (Mean ± SE).**

| Period / Group | Cooling 5° C | 48 hrs. PC | 1 Month PC | 2 Month PC | Level of significance |
|---|---|---|---|---|---|
| Res-0 | 77.50±0.64 A b | 67.25±1.31 B b | 61.50±1.93 C b | 58.50±1.44 C b | P≤0.01 |
| Res-100 | 78.50±1.19 A b | 71.75±1.70 B ab | 66.50±1.70 C ab | 62.25±1.60 C ab | P≤0.01 |
| Res-150 | 82.50±0.86 A a | 75.75±0.75 B a | 69.25±1.49 C a | 66.00±1.29 C a | P≤0.01 |
| Res-200 | 77.50±1.04 A b | 69.25±1.93 B b | 65.00±1.40 BC ab | 61.50±0.95 C b | P≤0.01 |
| Level of significance | P≤0.01 | P≤0.01 | P≤0.01 | P≤0.01 | ------- |

**Small superscripts within a similar column indicate differences, and large superscripts within a similar row indicate differences. Res-0= control; Res-100= Resveratrol 100 µM; Res-150= Resveratrol 150 µM; Res-200= Resveratrol 200 µM; PC= post cryopreservation**

**Malondialdehyde concentration in seminal plasma :** Leaser (P≤0.01) malondialdehyde (MDA) concentration was observed for the Res-150 group (0.88µM /109 sperm), followed by Res-200 (1.07 µM /109 sperm) and Res-100 groups (1.28 µM /109 sperm), while, the higher value registered for the Res-0 group namely 1.70µM /109 sperm (Figure 1).

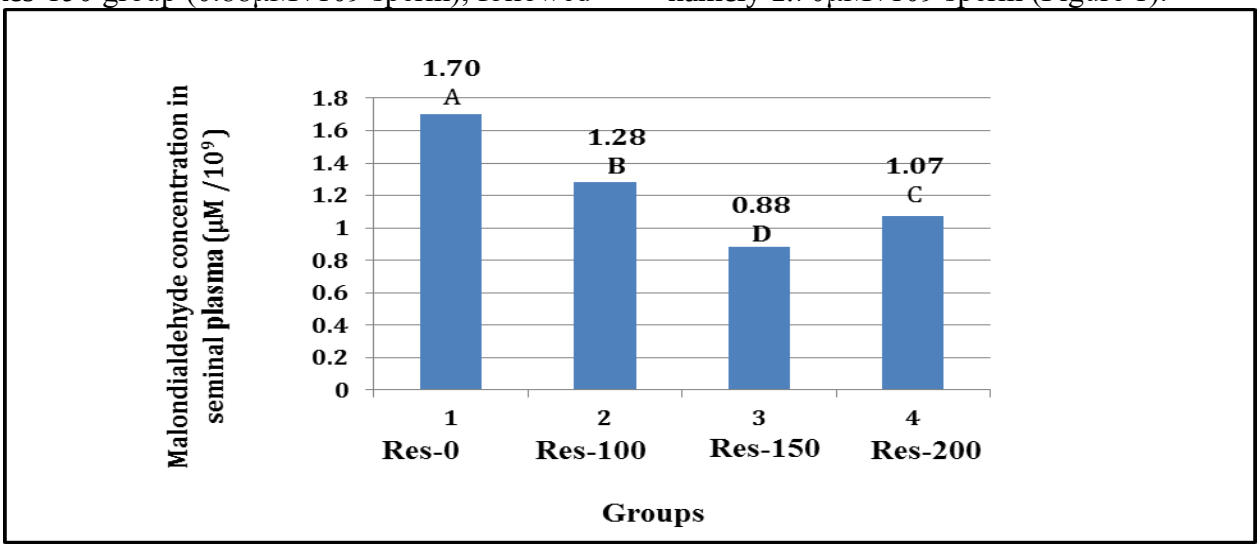


**Figure 1. Effect of adding different resveratrol concentrations to soybean-lecithin extender on malondialdehyde concentrations in seminal plasma (µM /$10^9$) of Iraqi buffalo bulls two months' post-cryopreservation**

**Different letters indicate significant differences (P≤0.01) among groups. Res-0= control group; Res-100= Resveratrol 100 µM; Res-150= Resveratrol 150 µM; Res-200= Resveratrol 200 Mm**

**Total antioxidants concentration in seminal plasma :** The Res-150 and Res-200 groups recorded higher (P≤0.01) total antioxidants concentration (0.13 ± 0.03 and 0.12 ± 0.01 mg/dl, respectively) compared to the Res-0 and Res-100 groups, which in turn excelled the Res-100 group two months PC (Figure 2).

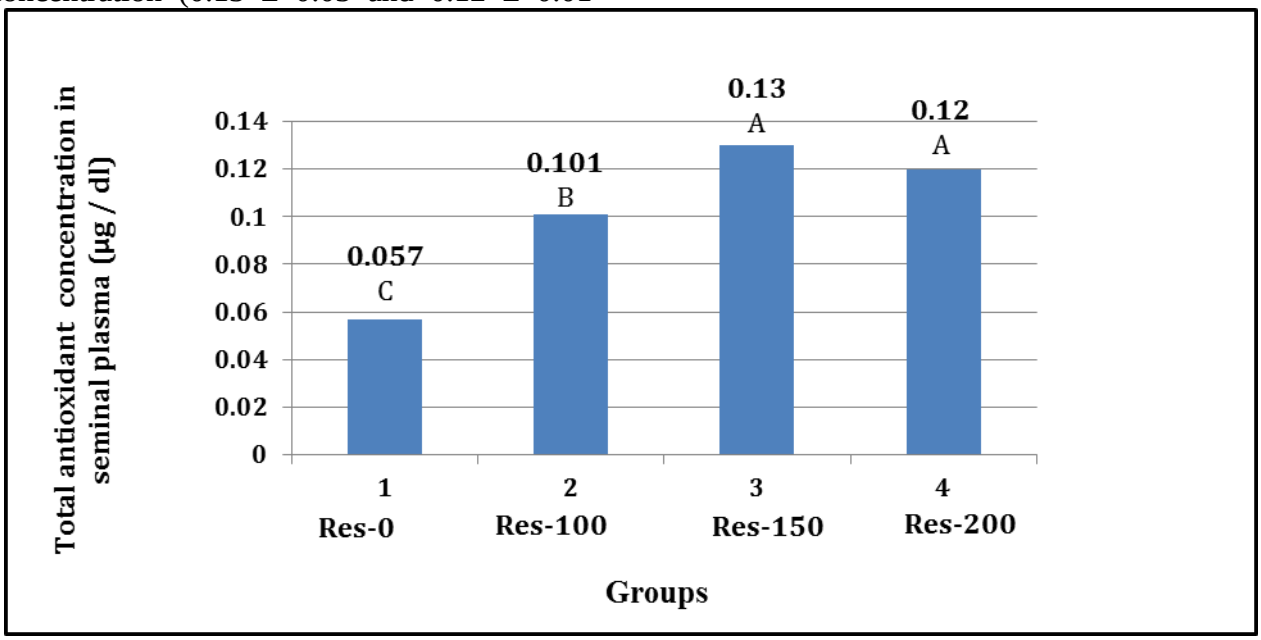


**Figure 2. Effect of adding different resveratrol concentrations to soybean-lecithin extender on total antioxidant concentrations in seminal plasma (µM /dl) of Iraqi buffalo bulls two months' post-cryopreservation**

**Different letters indicate significant differences (P≤0.01) among groups. Res-0= control group; Res-100= Resveratrol 100 µM; Res-150= Resveratrol 150 µM; Res-200= Resveratrol 200 µM**

**Sperm DNA damage percentage**

Highly significant (P≤0.01) decrease in the percentage of sperm DNA damage was observed for the Res-150 (3.68 %) compared to the Res-0 (7.05 %), Res-100 (6.37 %), and Res-200 (6.13 %) groups. Differences among Res-0, Res-100, and Res-200 in sperm DNA damage percentage lacked significance (Figure 3).

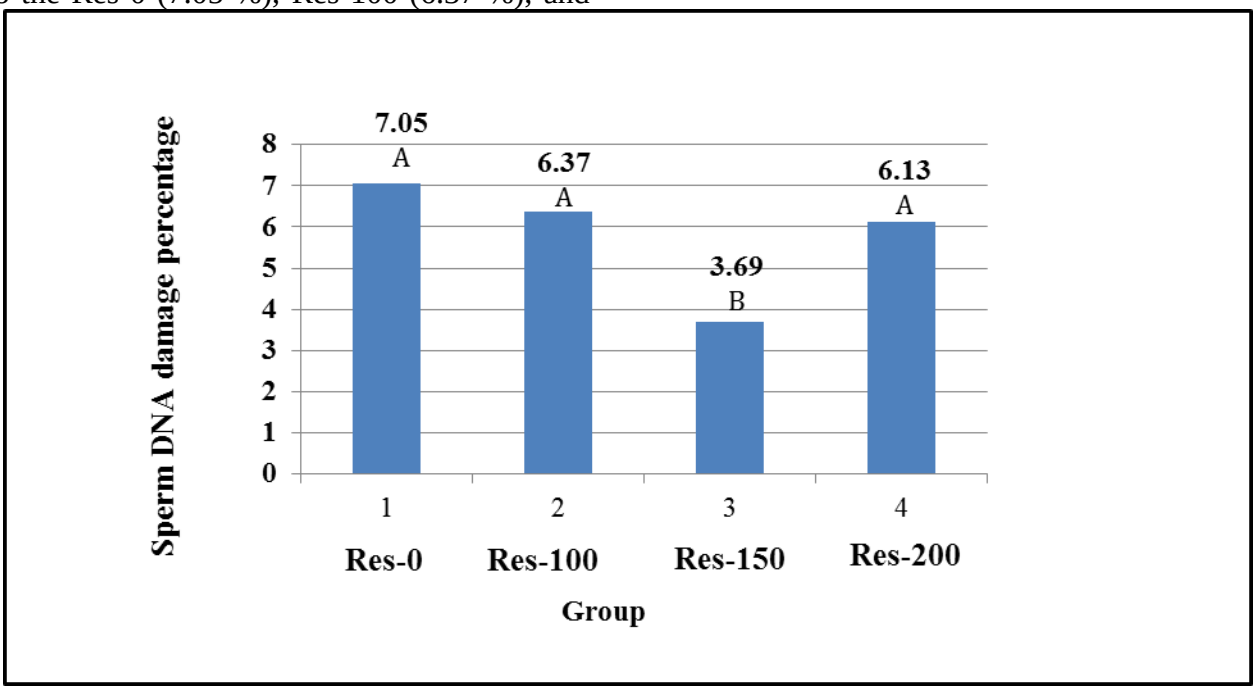


**Figure 3. Effect of adding different resveratrol concentrations to soybean-lecithin extender on sperm DNA damage percentage of Iraqi buffalo bulls two months' post-cryopreservation**

**Different letters indicate significant differences (P≤0.01) among groups. Res-0= control group; Res-100= Resveratrol 100 µM; Res-150= Resveratrol 150 µM; Res-200= Resveratrol 200 µM.**

This study is the first in Iraq and the world to deal with the effect of adding Res as a natural antioxidant to soybean-lecithin on some semen characteristics of Iraqi buffalo bulls. The apparent improvement in the current semen attributes belonging to the Res-150 group may return to the crucial role of resveratrol as a powerful antioxidant and has anti-cancer, anti-aging, and anti-inflammatory effects as well as cardioprotective and neuroprotective agents (23). Resveratrol can maintain the cell viability of bovine sperm post-cryopreservation (7). Moreover, resveratrol contains flavonoids that are excellent scavengers for free radicals and several hydroxyl groups through the phenol ring that appears to enhance the antioxidant capacity of the polyphenol molecule (41, 44). It also found that adding Res to semen extenders of bovine sperm led to the protection of individual motility, mitochondrial activity, and the integrity of the sperm's DNA (10). The resveratrol works to increase the fluidity of the sperm lipid bilayer through its action in interacting efficiently with free radicals that disrupt the plasma membrane (12), as well as its work on modulating the lipid metabolism of sperm (44). In this regard, some studies confirmed that Res promotes spermatogenesis by stimulating the hypothalamic-pituitary-testicular axis (Hypothalamic-pituitary-testicular axis), as well as reducing apoptosis of spermatozoa and increasing their motility in mice and rats (33). Zhang et al. (44) confirmed the findings of Zhu et al. (45) regarding the possibility of using resveratrol as a powerful antioxidant against oxidative stress and its subsequent effects by its ability to inhibit or remove free radicals, as well as its ability to interact with other antioxidants (26). These notions were confirmed by the current increasing the post-cryopreserved total antioxidant concentration in the seminal plasma of buffalo bull semen in a Res-150 group (Figure 2) when added to the soybean-lecithin extender. The current results agreed with those reported by Zhang et al. (44), who observed the positive effect of Res in improving sperm quality in male giant pandas when added to semen extender at 50, 100, and 150 μM. A similar trend was noticed in male pigs at 25, 50, 75, 100, and 125 μM of resveratrol. (45). The current results are also in line with those observed by Bang et al. (8), who noted the possibility of the resveratrol to reduce the oxidative stress of the mitochondria and reduce the sperm DNA damage, increase the percentage of acrosome integrity and thus preserving the number of sperm and their motility and vitality (38). Similarly, the current results agreed with those found by Ahmed et al. (3) of an improvement in the SAIP and SCIM, a decrease in sperm DNA damage percentage, and MDA concentration in buffalo bulls post-freezing-thawing process when adding 50 and 100 micromoles of resveratrol to Tris extender. On the other hand, the significant improvements in SCIM, SAIP, and remarkable reduction in MDA concentration and sperm DNA damage percentage of Iraqi buffalo bulls belonging to the Res-150 may be attributed to the role of resveratrol in inhibiting the activity of phosphodiesterase enzyme and thus increasing the intracellular cAMP concentration by increasing the activity of protein kinase and phosphorylation of endogenous protein (30). Phosphodiesterase improves the percentage of SCIM by using the excess energy generated by accelerating glycolysis (16). In parallel, Silva et al. (35) showed that Res has a strong efficacy that exceeds the effectiveness of vitamins C and E, in addition to its low toxicity and works at the onset of oxidative stress, and increases the fluidity of the plasma membrane (10). It is produced naturally in plants when exposed to infection or danger from pathogens such as bacteria and fungi as a means of defense. This compound is called phytoalexin due to its functional performance (17). The remarkable decrease in the PC sperm DNA damage percentage of the Res-150 group compared to the Res-0 group may be because of the priority of resveratrol (as a natural antioxidant) to protect the nucleus and DNA before any other organelle in the cell, so as not to affect the protein and fat synthesis process of the cell (14). In other words, DNA damage stimulates transcription factors that modify the cellular proteins and fats synthesis and produce substances that stimulate the pro-inflammatory and anti-inflammatory cytokines (9). The reason behind the success of the soybean-lecithin extender in preserving the PC sperm

quality of Iraqi buffalo bulls as a result of adding Res (Res-150) may return to the nature of the nutrients present in it, such as phosphatidylcholine which is a source of sperm energy and protecting them from the risks of cryopreservation, as well as the decrease in its viscosity, compared to the Tris extender (4). The difference in the extender density, its viscosity, and the presence of large particles (macromolecules) in it resulting from inaccuracy during the preparation of the extender are all factors that affect the characteristics of sperm (43).

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