AMELIORATING POST-THAWED SEMEN OF BUFFALO BULLS USING A MILK-BASED EXTENDER SUPPLEMENTED WITH RESVERATROL

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ABSTRACT

The objective of the current study was to investigate the influence of adding resveratrol (Res) to a milk-based extender on some post-thawed semen quality of Iraqi buffalo bulls for different preservation periods (cooling at 5˚C, 48 h, 1, and 2 months post-cryopreservation; PC). Four bulls, 3.5 to 4 years old, were used from 11th November 2020 to 1st June 2021. Semen samples were collected, pooled, and divided into four groups using different Res concentrations (0, 100, 150, and 200 µM) added to the milk extender. The Res-150 µM group exhibited higher (P≤0.01) plasma membrane, acrosome integrity percentages, and total antioxidants concentration (TAC) compared to the other groups. Sperm abnormalities and DNA damage percentages, as well as malondialdehyde (MDA) concentration, were lesser (P≤0.01) in the Res-150 µM group. Finally, Res-150 µM added to a milk-based extender increased the percentage of post-thawed live sperm and TAC in seminal plasma and minimized abnormal sperm percentage, DNA damage percentage, and MDA concentration in the seminal plasma of Iraqi buffalo bulls.

Keywords: Antioxidants, sperm morphology, DNA damage, buffalo.

مجلة العلوم الزراعية العراقية 2024 -:55(عدد خاص):186-194 الهلال وعبدالكريم

تحسين السائل المنوي لثيران الجاموس بعد الحفظ بالتجميد باستعمال مخفف الحليب المضاف اليه الريسفيراترول

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المستخلص

اجريت هذه الدراسة لبيان تأثير اضافة مركب الريسفيراترول كمضاد اكسدة الى مخفف الحليب في بعض صفات السائل المنوي لثيران الجاموس العراقي بعد الحفظ لمدد زمنية مختلفة (التبريد عند 5 م˚ والتجميد لمدة 24 ساعة وشهر وشهرين بعد الحفظ بالتجميد). تم استعمال اربعة ثيران جاموس عراقي بعمر 3.5–4 سنة للمدة من 1 تشرين الثاني 2020 ولغاية 1 حزيران 2021. تم جمع السائل المنوي وتجميعه وتقسيمه الى اربعة مجاميع تضمنت تراكيز مختلفة (0، 100، 150، 200 مايكرومول) من مركب الريسفيراترول تم اضافتها الى مخفف الحليب. اظهرت مجموعة الريسفيراترول بتركيز 150 مايكرومول زيادة عالية المعنوية (P≤0.01) في النسبة المئوية لسلامة اكروسوم والغشاء البلازمي للنطف وتركيز مضادات الاكسدة الكلية في البلازما المنوية، فضلاً عن انخفاض عالي المعنوية (P≤0.01) في النسبة المئوية لتشوهات النطف الكلية وضرر المادة الوراثية وتركيز المالون ثنائي الألديهايد في البلازما المنوية لدى مجموعة الريسفيراترول بتركيز 150 مايكرومول. يمكن الاستنتاج ان اضافة مركب الريسفيراترول بتركيز 150 مايكرومول الى مخفف الحليب قد زاد من النسبة المئوية للنطف الحية وتركيز مضادات الأكسدة الكلية في البلازما المنوية وقلل من النسبة المئوية للنطف المشوهة وضرر المادة الوراثية وتركيز المالون ثنائي الألديهايد في البلازما المنوية للسائل المنوي لثيران الجاموس العراقي.

كلمات مفتاحية: مضادات أكسدة، شكل النطف، ضرر المادة الوراثية، الجاموس.

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INTRODUCTION

Buffalo sperms are highly sensitive to the different stresses of cryopreservation due to their unique biological and physiological characteristics (2, 25). The plasma membrane of the buffalo bull's spermatozoa is rich in polyunsaturated fatty acids (PUFA). This PUFA makes the membrane detrimental to lipid peroxidation (34). Complementing exogenous antioxidants may be a practical strategy as opposed to all cryogenic damage that occurs through cryopreservation (6, 16, 17, 30). They play a significant role in scavenging reactive oxygen species (ROS) during cryopreservation (5, 18, 23, 24, 29). Resveratrol (Res) is a non-flavonoid polyphenol found in grapes, red wine, and peanuts (20). It has pleiotropic effects, including antioxidant, anti-aging, anticancer, anti-inflammatory, neuroprotective, and cardioprotective properties (22). The effect of resveratrol during cryopreservation of sperm has been reported and achieved different outcomes. During the freezing of bull sperm, Res treatment in the cryopreservation medium could protect sperm motility, DNA integrity, and elevated mitochondrial activity (14). However, during the freezing of human sperm, the result showed that Res supplementation could prevent lipid damage (21) and DNA deterioration (7, 13) induced by sperm cryopreservation. In rams, the Res supplementation did not prevent the negative effect on post-thawed percentages of acrosome and plasma membrane integrity along with sperm motility (31). The milk-based extender was successfully used for improving Holstein bull's semen supplemented with *Melissa officinalis* leaf extract (1). The possible effect of Res added to the milk-based extender on post-thawed semen characteristics of Iraqi buffalo bulls has not been previously reported. Therefore, this study aimed to explore this effect.

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 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 milk-based extender was intended according to Paulenz *et al* (32). This extender contained 90.52 ml skim milk, 5 ml glycerol, 4 ml egg yolk, 0.4 ml gentamicin, and 0.08 tylosin. 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 milk-based extender within Res-100, Res-150, and Res-200 groups, and the milk-based 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 plasma membrane integrity (27), sperm acrosome integrity (8), sperm abnormalities (22), and DNA damage percentage (8, 37) were evaluated. The concentrations of malondialdehyde and total antioxidants in seminal plasma were also assessed according to the Abdulkareem et al (4) and Eidan and Khudhir (19) 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 (3, 15). The Chi-square test was used to compare different percentages of studied attributes.

RESULTS AND DISCUSSION

Percentage of plasma membrane integrity

The Res-150 exhibited a higher (P≤0.05) percentage of plasma membrane integrity (PMI) as compared with the Res-0 at cooling (75.60 ± 1.56%), 48 hr. (69.60 ± 0.50%), and a month post-cryopreservation (PC; 64.20 ± 1.11%) compared with the Res-0 group. Concomitantly, the differences among the Res-0 group and Res-100 group on one hand, and Res-150 and Res-200 groups, on the other hand, lacked significance at similar trait and preservation periods (Table 1). Two months PC, the Res-150 group recorded a high (P≤0.01) percentage of PMI compared to the Res-0, Res-100, and Res-200 groups, which did not differ significantly among them (Table 1). A higher (P≤0.0001) PMI was noticed in all supplemented groups at the cooling period as compared with the other preservation periods, except for the Res-200 group, which did not differ significantly between the two mentioned periods (Table 1). Non-significant differences were observed in PMI between one and two months of PC for Res-150 and Res-200 groups. Lesser (P≤0.0001) PMI was shown two months of PC compared with the one-month PC in the Res-0 and Res-100 groups (Table 1).

Table 1. Effect of adding resveratrol to milk-based extender on sperm plasma membrane integrity percentage of Iraqi buffalo bulls for different preservation periods (Mean ± SE).

Period / Group	Cooling 5° C	48h PC	1 Month PC	2 Month PC	Level of significance
Res -0	70.00±1.22 A b	56.60±1.16 B b	59.00±0.89 C b	55.40±1.07 D b	P≤0.0001
Res -100	74.20±0.86 A ab	67.60±0.74 B ab	61.00±0.83 C ab	57.60±1.50 D b	P≤0.0001
Res -150	75.60±1.56 A a	69.60±0.50 A a	64.20±1.11 C a	61.60±0.50 C a	P≤0.0001
Res -200	71.60±2.15 A ab	66.00±1.78 B ab	61.40±1.53 BC ab	57.20±1.56 C b	P≤0.0001
Level of significance	P≤0.05	P≤0.05	P≤0.05	P≤0.01	-----

Small superscripts within a similar column indicate differences and large superscripts within a similar row indicate differences. Res-0= Milk-based extender (control); Res-100= Resveratrol 100 µM; Res-150= Resveratrol 150 µM; Res-200= Resveratrol 200 µM; PC= post cryopreservation

Percentage of sperm acrosome integrity

The differences in the percentage of acrosome integrity (AI) among groups lacked significance during the cooling period. However, the Res-150 recorded the highest numerical AI value (77.80 ± 1.90%) during the same period (Table 2). During 48 h PC, the Res-150 was superior (P≤0.02) among groups in AI percentage (73.60 ± 0.50%) as compared with the remaining groups. Non-significant differences were observed among Res-0, Res-100, and Res-200 in AI value at a similar period (Table 2). The AI percentage increased (P≤0.0248) in the Res-150 group (68.30 ± 1.15%) compared to the Res-0 during a month PC. Non-significant differences were noticed between Res-150 and Res-200 groups and between Res-0 and Res-100 groups for the same preservation period (Table 2). At two months PC, a higher (P≤0.0070) AI percentage was observed for Res-150 and Res-100 groups compared to the Res-0 group. Moreover, the differences between the Res-100 group compared with the Res-150 and Res-200

groups during similar preservation periods (Table 2). Concerning the effect of the preservation periods within one group, a significant decrease ($P \leq 0.0001$) was observed for the AI percentage during 48 h PC compared to the cooling period in all groups (Table 2). Further, the AI percentage decreased significantly ($P \leq 0.0001$) a month PC for all groups compared to the cooling and 48 h PC periods. This percentage decreased significantly ($P \leq 0.0001$), two months PC in the Res-0 and Res-100 groups compared to a month PC period (Table 2).

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

Period / Group	Cooling 5° C	48 h PC	1 Month PC	2 Month PC	Level of significance
Res-0	73.80±0.80 A	68.60±1.16 B b	62.40±1.02 C b	59.00±1.14 D c	P≤0.0001
Res-100	76.20±0.73 A	70.20±0.73 B b	65.40±1.6 C ab	63.00±1.30 D ab	P≤0.0001
Res-150	77.80±1.90 A	73.60±0.50 B a	68.30±1.15 C a	56.80±1.15 C a	P≤0.0001
Res-200	75.00±1.11 A	69.40±1.53 B b	64.60±1.43 C ab	61.80±1.06 C bc	P≤0.0001
Level of significance	NS	P≤0.0213	P≤0.0248	P≤0.0070	-----

Small superscripts within a similar column indicate differences and large superscripts within a similar row indicate differences. Res-0= Milk-based extender (control); Res-100= Resveratrol 100 µM; Res-150= Resveratrol 150 µM; Res-200= Resveratrol 200 µM; PC= post cryopreservation. NS: Non-significant.

Sperm total abnormalities percentage

The percentage of total sperm abnormalities (TSA) decreased significantly ($P \leq 0.01$) in the Res-150 group during cooling (9.80 ± 0.48%) and 48 h PC (11.10 ± 0.50%) compared to the Res-0 and Res-200 groups. Concomitantly, this percentage decreased ($P \leq 0.01$) in the Res-150 group (12.20 ± 0.58%) as compared with the other groups, a month, and two months PC (Table 3). The percentage of TSA increased obviously ($P \leq 0.0001$) a month and two months PC compared with cooling and 48 h PC in both Res-0 and Res-100 groups. Moreover, the TSA percentage increased ($P \leq 0.0001$) a month and two months PC in comparison with the cooling period in Res-150 and Res-200 (Table 3).

Table 3. Effect of adding resveratrol to milk-based extender on total sperm abnormality percentage of Iraqi buffalo bulls for different preservation periods (Mean ± SE).

Period / Group	Cooling 5° C	48 h PC	1 Month PC	2 Month PC	Level of significance
Res -0	13.30±0.81 B a	14.80±0.88 B a	18.70±0.86 A a	20.80±1.17 A a	P≤0.0001
Res -100	10.90±0.50 B ab	12.70±0.73 B ab	15.20±0.68 A c	17.70±0.52 A b	P≤0.0001
Res -150	9.80±0.48 C b	11.10±0.50 BC b	12.20±0.58 AB b	14.00±0.94 A c	P≤0.001
Res -200	12.80±1.39 C a	15.00±1.11 BC a	17.70±1.10 AB a	19.20±1.16 A ab	P≤0.001
Level of significance	P≤0.01	P≤0.01	P≤0.001	P≤0.001	-----

Small superscripts within a similar column indicate differences and large superscripts within a similar row indicate differences. Res-0= Milk-based extender (control); Res-100= Resveratrol 100 µM; Res-150= Resveratrol 150 µM; Res-200= Resveratrol 200 µM; PC= post cryopreservation.

Sperm DNA damage percentage

The Res-150 group exhibited a lesser ($P \leq 0.0005$) sperm DNA damage percentage (2.77 %) compared to the remaining groups. The Res-100 group ranked second for the lowest percent (4.50 %), while the Res-0 and Res-100 groups did not differ significantly between them (Figure 1). Moreover, the

differences between Res-100 and Res-200 lacked significance, noting the numerical decrease of the percent in the Res-100 group (Figure 1).

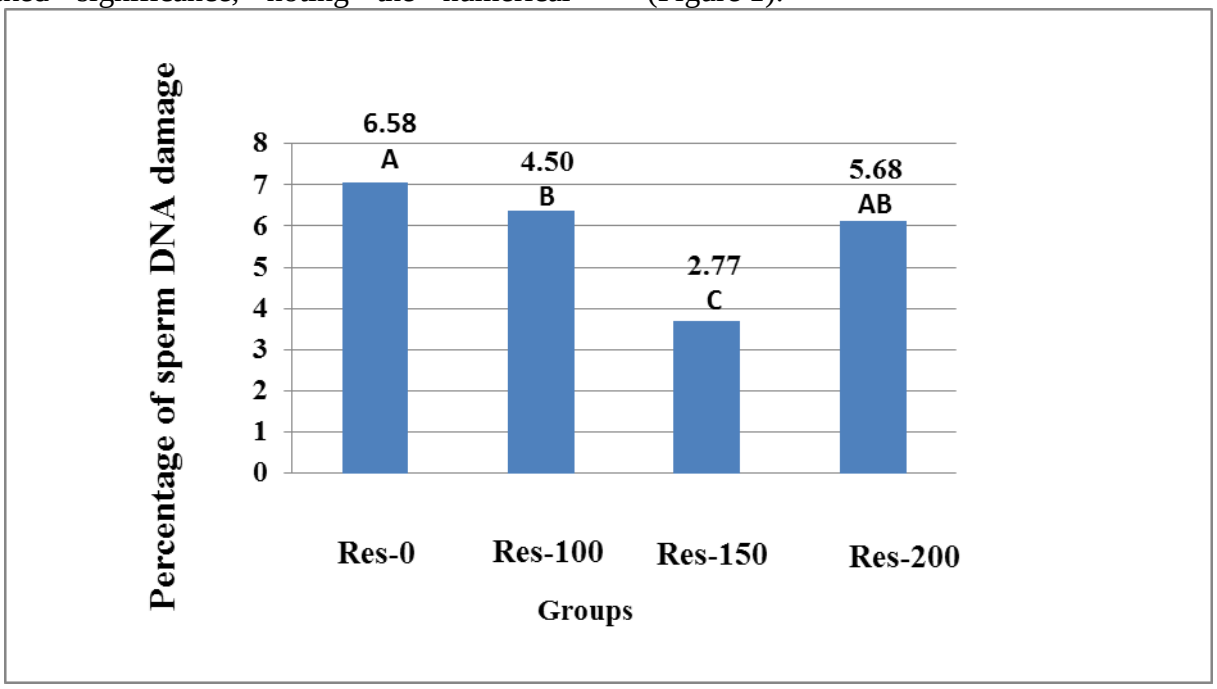

Figure 1. Effect of adding different resveratrol concentrations to milk-based extender on sperm DNA damage percentage of Iraqi buffalo bulls two months' post-cryopreservation

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

Malondialdehyde concentration in seminal plasma: A significant decrease (P≤0.0001) in the malondialdehyde (MDA) concentration in the seminal plasma was observed in the Res-150 group (0.73 µM / 106 sperm) compared to the Res-0, Res-100, and Res-200 groups. Moreover, the Res-100 and Res-200 did not differ significantly in MDA concentration (Figure 2).

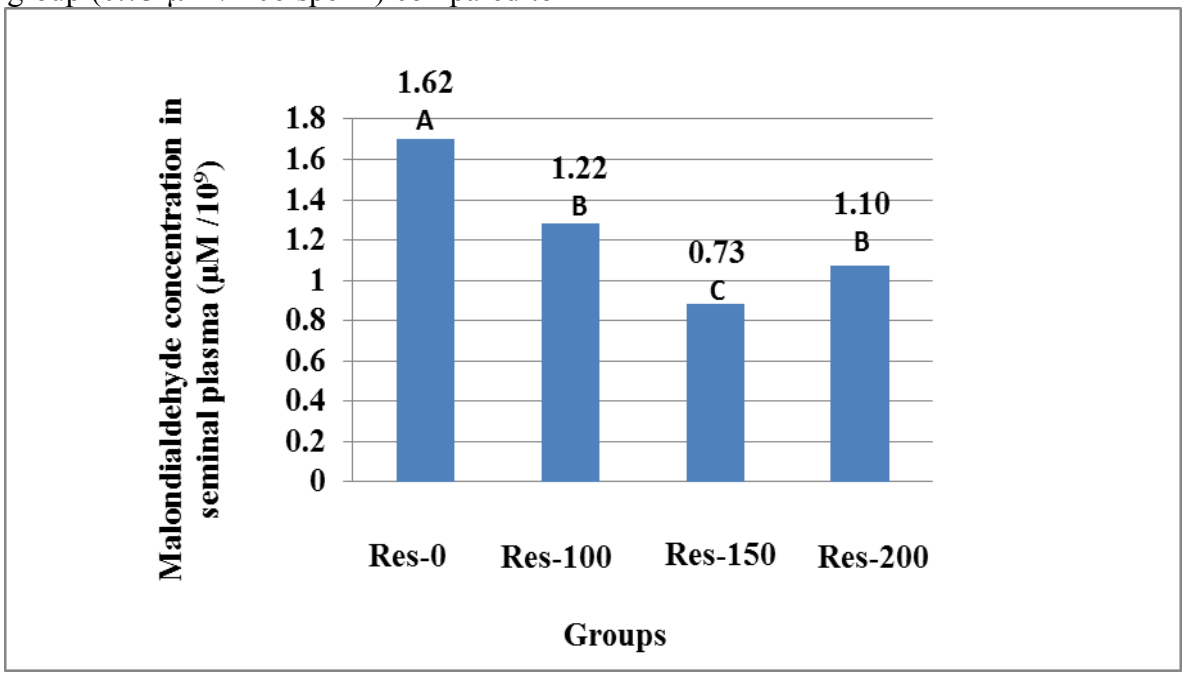

Figure 2. Effect of adding different resveratrol concentrations to milk-based extender on malondialdehyde concentration in the seminal plasma of Iraqi buffalo bulls two months' post-cryopreservation

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

Total antioxidant concentrations in seminal plasma: Adding resveratrol with different concentrations (100, 150, and 200 µM) had a pronounced (P≤0.0001) effect on the total antioxidant concentrations (TAC) in the seminal plasma of the buffalo bulls compared to the Res-0 group. Further, the Res-150 and Res-200 groups recorded a higher (P≤ 0.0001) TAC in comparison with the Res-100 group (Figure 3).

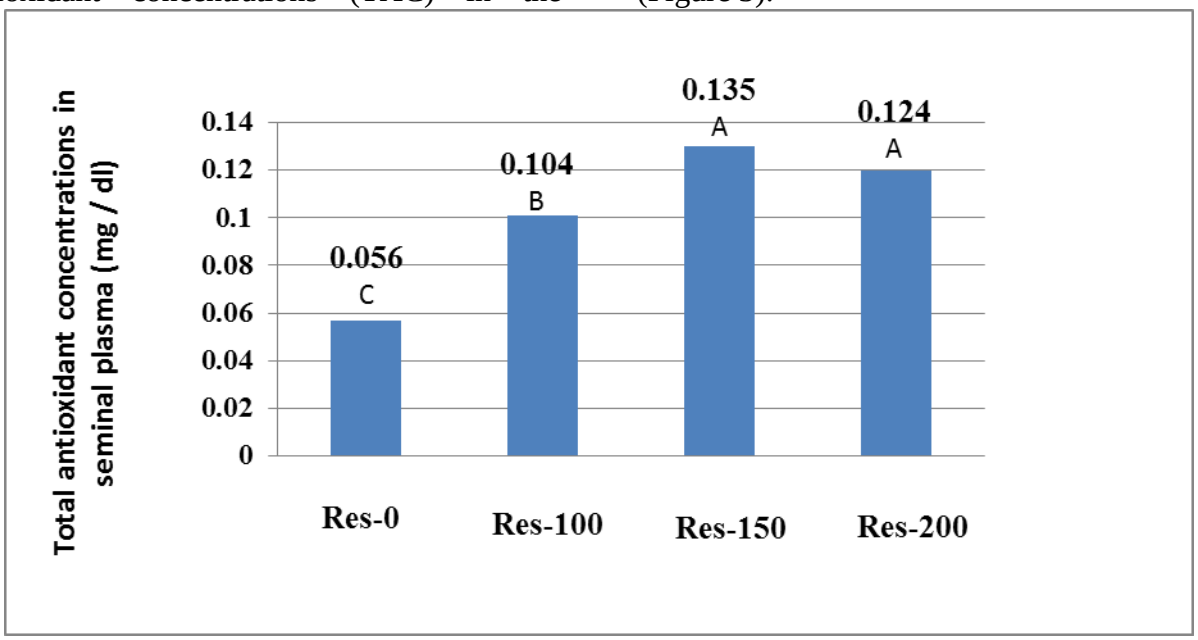

Figure 3. Effect of adding different resveratrol concentrations to milk-based extender on total antioxidant concentrations in the seminal plasma of Iraqi buffalo bulls two months post-cryopreservation

Different letters indicate significant differences (P≤0.01) among groups. Res-0= Milk-based extender group (control); 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 influence of milk-based extenders supplemented with the Res as the powerful antioxidant on the semen attributes of Iraqi buffalo bulls. However, the previous attempt was carried out by adding natural (*Melissa officinalis*) and synthetic (vitamin E, catalase, Trolox, and vitamin C) antioxidants to the milk-based extender and found similar improvement in semen characteristics of Holstein bulls in Iraq (1). A clear enhancement in the percentage of the integrity of the plasma membrane and the acrosome, as well as a significant decrease in the percent of total sperm abnormalities in the Res-150 group compared with the Res-0 in the different preservation periods, may be due to the crucial effect of resveratrol in maintaining cell viability of Bovidae sperms PC (11). Similar improvements in the percent of sperm cell individual motility and acrosome integrity of Iraqi buffalo bulls (*Bubalus bubalis*) were observed by adding 150 µM of resveratrol to soybean-lecithin extender (7). It is well-known that resveratrol has a high activity against free radicals and high polyphenol content. These are mainly due to its role as a reducing agent and a donor of hydrogen, in addition to containing flavonoids, which are capable of scavenging free radicals, and several hydroxyl groups through the phenol ring. This ring seems to enhance the antioxidant ability of the polyphenol molecule (36, 39, 40). The apparent decrease in the concentration of total antioxidants in the seminal plasma of the Res-0 group and its increase in the Res-supplemented groups to the milk-based extender (Figure 3) confirms the high oxidative stress suffered by the semen of buffalo bulls and the requirement of antioxidants supplementation to semen extenders, necessary to improve the quality of their semen. Moreover, the significant increase in the concentration of malondialdehyde in the seminal plasma of the Res-0 group and its decrease in the Res- supplementation groups (Figure 2) confirms what we mentioned above. Similarly, Nair et al. (31) and Kadirve et al. (28) found that the semen of buffalo bulls is more prone to oxidative damage than Holstein bulls. The reason behind that may be the return to the high contents of polyunsaturated fatty

acids (PUFA), leading to damage associated with freezing-thawing processes and a low conception rate. Alhelal and Abdulkareem (7) found similar results in Iraqi buffalo bulls (*Bubalus bubalis*) when adding Res to the soybean-lecithin extender. The good results of the semen characteristics of the buffalo bulls proved the success of the milk-based extender as a future extender that can be relied upon and used successfully in artificial insemination centers in Iraq and the world. It is more successful as an extender in preserving the sperms of buffalo bulls (14), rams (9), and horses (38) compared to the Tris extender. In conclusion, adding 150 µM Res to the milk extender can be recommended as a suitable supplementation for the enhancement of PC buffalo sperm. However, in vivo fertility trials are required to confirm these recommendations.

REFERENCES

1. Abdulkareem, T. A. and O. H. Al-Zaidi. 2018. Effect of adding an aqueous extract of Melissa officinalis leaves and some other antioxidants to milk–based extender on post-cooling and post-cryopreservative sperm's individual motility and live sperm percentage of Holstein bulls. Al-Anbar J. Vet. Sci., 11 (1), 37-53

2. Abdulkareem, T. A., S. M. Eidan, S. A. Al-Sharifi, S. M. Abd and M. N. Dhaydan. 2021. Effect of hCG hormone administration on estrus induction and reproductive performance of anestrus Iraqi buffaloes (*Bubalus bubalis*). Buffalo Bulletin, 40 (3): 499-502

3. Abdulkareem, T. A. S. M. Eidan, F. K. Al-Saidy, and N. K. Al-Hassani. 2023. Effect of pre-and post-mating vitamins AD3E treatment on reproductive performance of Awassi ewes. Iraqi Journal of Agricultural Sciences 54(2),431-437. https://doi.org/10.36103/ijas.v54i2.1717

4. Abdulkareem, T. A., R. I., Khalil, and M. A. O., Al-Ezzi, 2024. Effect of sperm freezability on some semen attributes and amino acid concentrations in seminal plasma of Holstein bulls. Iraqi Journal of Agricultural Sciences, 54 (6), [In Press].

5. Abdulkareem, T. A., F. F. Ibrahim, M. S. Hassan, and O. A. Mohamed. 2020. Effect of adding amino acid combinations to Tris extender for improving post-cryopreserved semen quality of Holstein bulls. Biochem. Cell. Arch., 20 (1), 697-701. https://doi.org/10.35124/bca.2020.20.1.697

6. Abdulkareem, T.A., M. S. Noon, and K. H. Sultan. 2017. The synergistic Influence of some antioxidants added to Tris extender on sperm cell's individual motility of Holstein bulls following different cooling and cryopreservation periods. Al-Anbar J. Vet. Sci., 10 (1): 10-20

7. Alhelal, A. M. and T. A. Abdulkareem. 2023. Effect of adding resveratrol to soybean-lecithin extender on some semen attributes of buffalo bulls. Iraqi Journal of Agricultural Sciences, 54 (4), 1074-1083. https://doi.org/10.36103/ijas.v54i4.1797

8. Al-Saedi, A. J. A. and T. A. Abdulkareem. 2022. Comparison of semen quality for three lines of Holstein bulls :1. Some immediate and microscopic characteristics. Iraqi Journal of Agricultural Sciences, 53 (4), 752-759. https://doi.org/10.36103/ijas.v53i4.1585

9. Ari, U., C. Kulaksiz, and R. Y. Öztürkler. 2011. Freezability of Tushin ram semen extended with goat or cow milk-based extenders. Reprod. Domest. Anim., 46(6), 975-979. https://doi.org/10.1111/j.1439-0531.2011.01769.x.

10. Andraszek, K., D. Banaszewska, M. Czubaszek, E. Wójcik and M. Szostek, 2014. Comparison of different chromatin staining techniques for bull sperm. Arch. Tierz. 57(1), 1-15. https://doi.org/10.7482/0003-9438-57-013

11. Assuncao, C. M., V. R. A. Mendes, F. Z. Brandao, R. I. T. P. Batista, E. D. Souza, B. C. de Carvalho and L. S. A. Camargo. 2021. Effects of resveratrol in bull semen extender on post-thaw sperm quality and capacity for fertilization and embryo development. Anim. Reprod. Sci., 226, Article #106697. https://doi.org/10.1016/j.anireprosci.2021.106697

12. Branco, C.S., ME. Garcez, FF. Pasqualotto, 2010. Resveratrol and ascorbic acid prevent DNA damage induced by cryopreservation in human semen. Cryobiology, 60(2), 235-237. https://doi.org/10.1016/j.cryobiol.2009.10.012

13. Bucak, M. N., M. B. Ataman, N. Başpınar, O. Uysal, M. Taşpınar, A. Bilgili, and E. Akal. 2015. Lycopene and resveratrol improve post-

thaw bull sperm parameters: sperm motility, mitochondrial activity, and DNA integrity. Andrologia., 47(5), 545-552. https://doi.org/10.1111/and.12301
14. Chaudhari, D. V., A. J. Dhami, K. K. Hadiya and J. A. Patel. 2015. Relative efficacy of egg yolk and soya milk-based extenders for cryopreservation (− 196 C) of buffalo semen. Vet. World., 8(2), 239-244. https://doi.org/10.14202/vetworld.2015.239-244
15. Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics 11,1–42
16. Eidan, S. M. 2016. Effect on post-cryopreserved semen characteristics of Holstein bulls of adding combinations of vitamin C and either catalase or reduced glutathione to Tris extender. Anim. Reprod. Sci., 167, 1-7. https://doi.org/10.1016/j.anireprosci.2016.01.014
17. Eidan, S. M., O. H. Al-Zaidi, F. F. Ibrahim, B. A. R. Al-Timimi and W. E. Lateef. 2015. Effect of adding catalase and glutathione reduce to Tris extender on freezing ability of Holstein bulls following different cryopreservation periods. The Iraqi J. Vet. Med., 39 (2), 19-24
18. Eidan, S.M., A.J. Al-Nuaimi, O.A. Abd Sultan, F.F. Ibrahim, T.A. Abdulkareem, and W.E. Lateef. 2020. Effect of adding α-lipoic acid on some post-cryopreserved semen characteristics of Holstein bulls. Plant Archives, 20(2), 11-16.
19. Eidan, S. M. and S. A. Khudhir. 2023. Association between ATP1A1 gene polymorphisms with semen characteristics in Holstein bulls. Iraqi Journal of Agricultural Sciences, 54 (2): 330-337. https://doi.org/10.36103/ijas.v54i2.1706
20. Gambini, J., M., G. Inglés, Olaso, R. Lopez- Grueso, V. Bonet-Costa, L. Gimeno-Mallench, and C. Borras, 2015. Properties of resveratrol: in vitro and in vivo studies about metabolism, bioavailability, and biological effects in animal models and humans. Oxid. Med. Cell. Longev., 2015, Article # 837042. https://doi.org/10.1155/2015/837042
21. Garcez, M. E., C. dos Santos Branco, L. V. Lara, F. F. Pasqualotto, and M. Salvador, 2010. Effects of resveratrol supplementation on cryopreservation medium of human semen. Fertil. Steril., 94(6), 2118-2121. https://doi.org/10.1016/j.fertnstert.2010.01.058
22. Hancock, J. L. 1951. A staining technique for the study of temperature shock in semen. Nature, 167, 323-324
23. Hassan, M.S. and S.M. Eidan. 2021. Effect of swim-up and glass wool techniques, with adding antioxidants to Tris extender on improving post-cryopreserved some semen attributes of low semen quality for Holstein bulls. Iraqi Journal of Agricultural Sciences, 52(3), 552-563. https://doi.org/10.36103/ijas.v52i3.1342
24. Hassan, M.S., S.M. Eidan, F.F. Ibrahim, and K.J. Yahya. 2021. Effect of swim-up and glass wool techniques, with adding antioxidants to Tris extender on improving post cryopreserved total sperm characteristics in straw and freezability percentage for low semen quality of Holstein bulls. Iraqi Journal of Agricultural Sciences, 52(4), 885-895. https://doi.org/10.36103/ijas.v52i4.1396
25. Hasanain, M. H., K. G. M., Mahmoud, Y. F., Ahmed, M. F., Nawito, A. A., EL-Menoufy, and S. T. Ismail, 2022. Polymorphism Investigation of Sex Determining Factor gene (SRY) and the association of Semen Criteria with Field fertility in Egyptian Buffalo Bulls. Egypt. J. Chem., 65(4), 1-2. https://doi.org/10.21608/EJCHEM.2021.96085.4509
26. He, W. H., X. H. Zhai, X. J. Duan, and H. S. Di. 2020. Effect of resveratrol treatment on apoptosis and apoptotic pathways during boar semen freezing. J. Zhejiang Univ. Sci., 21(6), 485-494. https://doi.org/10.1631/jzus.B1900520
27. Jeyendran, R. S., H. H. Van der Ven, M. Perez-Pelaez, B. G. Crabo and L. J. D. Zaneveld. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertile., 70(1), 219-228. https://doi.org/10.1530/jrf.0.0700219
28. Kadirve, G. S. K. G., S. Kumar, S. K. Ghosh and P. Perumal. 2014. Activity of antioxidative enzymes in fresh and frozen-thawed buffalo (*Bubalus bubalis*) spermatozoa in relation to lipid peroxidation and semen quality. Asian Pac. J. Reprod., 3(3), 210-217.

https://doi.org/10.1016/S2305-0500(14)60028-2

29. Mohammed O.A., T.A. Abdulkareem, F. F. Ibrahim, O.H. Al-Zaidi, W.E. Latif, and S.H. Alwan. 2020. Effect of adding pentoxifylline and nitric oxide to Tris extender on some post-cryopreserved semen attributes of Holstein bulls. Iraqi Journal of Agricultural Sciences, 51(2),619-628. https://doi.org/10.36103/ijas.v51i2.989

30. Mohsin, A. S. and S. M. Eidan. 2019. Effect of adding antioxidants to Tris extender on cooling and post-cryopreservative semen characteristics of Holstein bulls. 2. *Salvia officinalis* aqueous extract. Biochem. Cell Arch., 19 (1), 1157-1164. https://doi.org/10.35124/bca.2019.19.1.1157 .

31. Nair, S. J., A. S. Brar, C. S. Ahuja, S. P. S. Sangha and K. C. Chaudhary. 2006. A comparative study on lipid peroxidation, activities of antioxidant enzymes, and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. Anim. Reprod. Sci., 96(1-2), 21-29. https://doi.org/10.1016/j.anireprosci.2005.11.002

32. Paulenz, H., L. Söderquist, T. Ådnøy, O. H. Fossen and K. A. Berg. 2003. Effect of milk-and Tris-based extenders on the fertility of sheep inseminated vaginally once or twice with liquid semen. Theriogenology, 60(4), 759-766. https://doi.org/10.1016/s0093-691x(03)00048-7

33. SAS/STAT 2012. User's Guide for Personal Computers. Release 9.1 SAS Institute Inc., Cary, N. C., USA

34. Shah, S. A. H., S. M. H. Andrabi, and I. Z. Qureshi, 2017. Freezability of water buffalo bull (*Bubalus bubalis*) spermatozoa is improved with the addition of curcumin (diferuloylmethane) in the semen extender. Andrologia, 49(8), Article # e12713. https://doi.org/10.1111/and.12713

35. Silva, E. C. B., J. F. P. Cajueiro, S. V. Silva, P. C. Soares, and M. M. P. Guerra. 2012. Effect of antioxidants resveratrol and quercetin on *in vitro* evaluation of frozen ram sperm. Theriogenology, 77(8), 1722-1726. https://doi.org/10.1016/j.theriogenology.2011.11.023

36. Silvestre, M. A., J. I. Yániz, F. J. Peña, P. Santolaria and M. Castelló-Ruiz. 2021. Role of antioxidants in cooled liquid storage of mammal spermatozoa. Antioxidants (Basel), 10(7), Article # 1096. https://doi.org/10.3390/antiox10071096

37. Tejada, R. I., J. C. Mitchell, A. Norman, J. J. Marik and S. Friedman. 1984. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil. Steril. 42(1), 87-91. https://doi.org/10.1016/s0015-0282(16)47963-x

38. Tsunoda, R. H., R. P. Arruda, E. C. Serrano-Recalde, B. M. M. Oliveira, S. A. Florez-Rodriguez, M. B. R. Alves, R. Lanconi, M. Nichi, E. C. C. Celeghini and C. C. Celeghini. 2015. Addition of pentoxifylline to skim milk–based extender on frozen-thawed equine sperm. J. Equine Vet. Sci., 35, 823-829. https://doi.org/10.1016/j.anireprosci.2019.106128

39. Wittayarat, M., S. Panyaboriban, N. Kupthammasan, T. Otoi and K. Chatdar-Ong. 2022. Effects of green tea polyphenols and α-tocopherol on the quality of chilled cat spermatozoa and sperm IZUMO1 protein expression during long-term preservation. Anim. Reprod. Sci., 237, Article # 106926. https://doi.org/10.1016/j.anireprosci.2022.106926

40. Zhang, R., H. Dong, P. Zhao, C. Shang, H. Qi, Y. Ma and P. Lin. 2022. Resveratrol and lycium barbarum polysaccharide improve Qinling giant panda (*Ailuropoda melanoleuca Qinlingensis*) sperm quality during cryopreservation. Vet. Res., 18(1), 1-11. https://doi.org/10.1186/s12917-021-03122-2