

SYNERGISTIC EFFECT OF SPERM FILTRATION TECHNIQUES AND ANTIOXIDANTS ADDED TO EXTENDER OF IRAQI BUFFALO SEMEN

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ABSTRACT

This study investigated the synergistic effect of glass wool and sephadex sperm filtration techniques on improving the low semen quality of buffalo bulls with or without antioxidants. Semen was collected for 14 weeks, evaluated weekly, and divided into 12 groups. Good and low semen have extended using a Tris extender. Good semen quality is divided into three groups [S1: Tris extender; S2: Tris + vitamins E (2 mM) and C (5 mM); S3: Tris + glutamine (20 mM) and arginine (1 mM)]. Low semen quality was divided into three main groups and subdivided into three sub-groups (S4; Tris extender; S5: Tris +vitamins E (2 mM) and C (5 mM); S6: Tris + glutamine (20 mM) and arginine (1 mM)). In the 2nd and 3rd main groups, glass wool and sephadex techniques are used with or without adding vitamins (E+C) and amino acids (Glutamine and arginine) and subdivided into three sub-groups with each technique, referred to as S7–S9 for glass wool and S10–S12 for sephadex techniques. Improving ($P<0.01$) motility, normal morphology, acrosome, and plasma membrane integrity and reducing total sperm abnormalities of low semen quality were noticed using sephadex with or without adding vitamins or amino acids post-cryopreservation. In conclusion, the sephadex technique removed immotile sperm, dead, abnormal, and good sperm harvested by this technique, which in turn may reflect positively on improving the pregnancy rate.

Keywords: glass wool, sephadex, vitamins E, C, glutamine, arginine, semen characteristics, buffalo bulls.

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التأثير التآزري لتقنيات ترشيح النطف ومضادات الاكسدة المضافة إلى السائل المنوي للجاموس العراقي

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المستخلص

أجريت هذه الدراسة بهدف معرفة التأثير التآزري لتقناتي الصوف الزجاجي والسيفادكس لترشيح النطف في تحسين السائل المنوي الرديء النوعية لثيران الجاموس مع او بدون استعمال مضادات اكسدة. تم جمع السائل المنوي لمدة 14 أسبوعاً، وتقييمه أسبوعياً، وقسم إلى 12 مجموعة. تم تخفيف السائل المنوي الجيد والرديء باستخدام مخفف Tris. قسم السائل المنوي الجيد النوعية إلى ثلاث مجموعات [S1؛ مخفف Tris، S2؛ Tris + فيتاميني E (2 مليمول) و C (5 مليمول)، S3؛ Tris + الكلوتامين (20 مليمول) والأرجينين (1 مليمول)]. قسم السائل المنوي الرديء النوعية إلى ثلاث مجموعات رئيسية والتي قسمت إلى ثلاث مجموعات فرعية (S4؛ Tris مخفف، S5؛ Tris + فيتاميني E (2 مليمول) و C (5مليمول)، S6؛ Tris + الكلوتامين (20 مليمول) والأرجينين (1 مليمول)]. المجموعتين الرئيسيتين الثانية والثالثة، تم استخدام تقناتي الصوف الزجاجي والسيفادكس مع أو بدون إضافة الفيتامينات (E + C) والأحماض الأمينية (الكلوتامين والأرجينين) وقسمت إلى ثلاث مجموعات فرعية لكل تقانة، وتعود S7– S9 لتقانة الصوف الزجاجي و S10–S12 للسيفادكس. تحسنت ($P < 0.01$) الحركة الفردية، والنطف الطبيعية، وسلامة الأكروسوم والغشاء البلازمي، وانخفضت التشوهات الكلية للنطف للسائل المنوي الرديء النوعية مع استخدام أو بدون إضافة الفيتامينات أو الأحماض الأمينية بعد الحفظ بالتجميد. نستنتج إن تقانة السيفادكس تتخلص من النطف غير المتحركة والميتة والمشوهة ونحصل على نطف جيدة بهذه التقانة، والتي بدورها قد تنعكس إيجاباً على تحسين معدل الحمل.

الكلمات المفتاحية: الصوف الزجاجي، سيفادكس، فيتامينات E و C، كلوتامين، أرجينين، صفات السائل المنوي، ثيران الجاموس.

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INTRODUCTION

Semen is affected by many factors, including genetics, nutrition, diseases, age, management, vaccination, stress, transportation, and others (9, 13, 16, 21, 27,36). There is a strong relationship between fertility and semen characteristics fresh of buffalo bulls (7, 8, 37,38) and bovine bulls (6,16, 17). Buffalo is a low-grade reproductive efficiency animal (4). The females' artificial insemination using low-fertility bull semen leads to a significant loss for the dairy buffalo industry. Therefore, it is necessary to find reliable tests or tools to improve the semen quality of buffalo bulls or to determine the latent fertility of buffalo bulls. The bulls used in artificial centers do not always produce good-quality semen (35). The artificial insemination centers are a huge burden or an economic loss that is not insignificant, estimated at millions of \$ due to the use of sperm from low-fertility bulls. These losses are estimated at 3-4 \$/day/bull in Iraq (24) and had negatively reflected in the low financial return of breeders and artificial insemination centers. Thus, improving semen quality is necessary to get the most benefit from genetically-superior bulls or/and have poor quality semen. The sperm filtration techniques significantly improved semen quality in vitro fertilization. Filtration techniques have become very important in the processes of in vitro fertilization and artificial insemination. In semen filtration techniques, motile sperms filtered from debris, non-motile, and abnormal. Sperm filtration techniques include dilution and washing, selective filters such as glass wool, Sephadex (5,10, 24, 26), density gradient centrifugation such as Bovipure (43) Sill Select Plus (11,32,33), and self-migration techniques such as the swim-down or swim-up technique (25, 26, 27,33, 34). Some techniques of semen filtration from fresh semen or post-thawing semen gave good results for bull semen characteristics (24, 25,26). The plasma membrane of buffalo sperm has a higher percentage of polyunsaturated fatty acids than that of bovine bulls (42), which makes it more susceptible to oxidative stress during cryopreservation. Thus, there is a decrease in motility and the integrity of the plasma membrane, acrosome, and chromatin in buffalo sperm. This weakness is

mainly due to the low concentrations of antioxidants in buffalo semen. Antioxidants have been reduced during the cryopreservation and thawing processes. The continuous production of free radicals in the seminal plasma from abnormal, immature, dead sperm, and cryopreservation processes had accompanied by a decrease in antioxidants concentrations in the seminal plasma, which has a significant effect on bull semen quality (3, 8, 19, 20, 22, 39), which leads to oxidative stress on the sperm. The addition of some antioxidants to semen bulls gave good results in improving the semen quality post-cryopreservation (7, 8,15, 19, 22). Vitamins E and C are antioxidants and act as inhibitors of the oxidation of unsaturated fatty acids in the sperm plasma membrane through their ability to inhibit free radicals very efficiently and can act synergistically (17). Numerous studies have indicated an improvement in the characteristics of bull semen after cryopreservation when vitamin E or C had added to the semen diluents (18, 29). The glutamine plays an antioxidant and protects sperm from freezing damage in bulls (2). It found that glutamine, egg yolk, and glycerol act synergistically in bull semen diluents to prevent freezing damage. Abdel-Razek and El-Shamaa (1) showed that the addition of glutamine to semen diluents of buffalo bulls improved individual motility, viability, integrity of the sperm plasma membrane, and fertility rate. It also reduced the formation of reactive oxygen species inside the sperm compared with the sperm of the control group. Arginine is an amino acid that plays a crucial role in sperm motility. A decrease in arginine concentration causes disturbance of sperm metabolism, lack of sperm motility, spermatogenesis, and oxidation of sperm membranes (12), as it is associated with free radicals. Badr (12) found that the addition of 0.5 mmol of arginine to the semen diluent of Egyptian buffalo bulls improved the individual motility and viability of sperm and the pregnancy rate with a decrease in the concentration of malondialdehyde compared with the control group. The amino acids arginine and glutamine act synergistically as antioxidants in cells. The semen filtration techniques for low-grade-quality semen from

buffalo bulls have not been addressed previously in Iraq. This study has been conducted to investigate the effect of using glass wool and sephadex techniques to filtrate low-quality semen from Iraqi buffalo bulls. The use of antioxidants (vitamin C with E or glutamine with arginine) may give good results in improving or activating the filtered or unfiltered semen through one of the filtration technologies for buffalo bulls by taking advantage of both and the possibility of preserving it by freezing and using it for commercial purposes (increasing the number of straw produced by the artificial insemination center).

MATERIALS AND METHODS

Experimental design

The study carried out at the Artificial Insemination Department/ Directorate of Animal Resource, Ministry of Agriculture. Ten Iraqi buffalo bulls (500–750 kg weighting and 6–8 years old) were trained for semen collection using an artificial vagina. The experimental buffalo bulls have good health. Bulls with low-quality fresh semen (n=6; individual motility $\leq 40\%$) and good-quality (n=3; individual motility $\geq 50\%$) were collected via artificial vagina at one ejaculate/buffalo bull/week for 14 weeks. Both low and good-quality semen divided into 12 groups. Good and low semen was extended using a Tris extender. Good semen quality was divided into three groups [S1; Tris extender, S2; Tris+ vitamins E (2 mM) and C (5 mM), S3; Tris +glutamine (20 mM) and arginine (1 mM)]. Low semen quality was divided into three main groups and subdivided into three sub-groups (S4; Tris extender, S5; Tris +vitamins E (2 Mm) and C (5mM), S6; Tris + glutamine (20 mM) and arginine (1 mM)]. In the 2nd and 3rd main groups, glass wool and sephadex techniques used with or without adding vitamins (E+C) and amino acids (Glutamine and arginine) and subdivided into three sub-groups with each technique and referred to as S7- S9 for glass wool and S10-S12 for sephadex technique. All buffalo bulls were allocated on a standardized diet, as a concentrate ration (protein 18% and 2146 kcal) was provided daily at a rate of 4-6 kg/bull. Roughage consisted of alfalfa hay (7-9 kg/bull/day) besides green forage (50-60

kg/bull/day). Fresh water and salt blocks were available ad libitum to the bulls.

Semen characteristics

The semen characteristics had evaluated (45) post-cooling and cryopreservation (sperm's cell individual motility, live sperm, normal morphology, sperm's acrosome, and plasma membrane integrity, total antioxidants and malondialdehyde concentrations, DNA damage, and freezability).

Statistical analyses

The statistical computation had performed using the SAS program based on a study on completely randomized design (CRD) to study the effect of different factors in the study characteristics. Means with significant differences had compared using Duncan multiple range test. The statistical models for comparison among groups $Y_{ij} = \mu + T_i + e_{ij}$
 Y_{ij} = dependent variable pertaining to the j observation of the i treatment

μ = overall mean

T_i = effect of i treatment (i- positive, negative controls, glass wool, sephadex techniques)

e_{ij} = error term.

RESULTS AND DISCUSSION

Post-cooling: The S1-S3 and S10- S12 groups were superior ($P \leq 0.01$) in sperm cell individual motility than S4, S7, and S8 groups (Table 1). Non-significant differences among S1-S3 groups, also among S12 with S3, and S10-S11 groups in the sperms cell motility during the cooling period. The higher ($P \leq 0.01$) live sperm (%) were in S1-S3, S9, and S12 as compared with S4 group. The S11 and S12 groups exhibited greater ($P \leq 0.01$) normal morphology (%) as compared with the S1-S10 groups. The S1-S3 and S11-S12 revealed higher ($P \leq 0.01$) acrosome and plasma membrane (except S11 group) integrity percentages than the S4-S6 groups (Table 1).

Post- cryopreservation

The results of sperm cell individual motility exhibited significant differences ($P \leq 0.01$) among groups post- cryopreservation period of buffalo bulls' semen (Table 2). Greater individual motility was recorded in the S1-S3 groups (36.25-38.33%) while lesser in the S4 group (15%). The S1-S3 groups revealed higher ($P \leq 0.01$) live sperm than the rest groups. The S10-S12 groups recorded higher ($P \leq 0.01$) normal morphology (%) than the

S1-S8 groups. The results demonstrated significant differences among groups in acrosome and plasma membrane integrity (Table 2). A high ($P \leq 0.01$) amount of total antioxidant activity has found in the S3 group as compared with the S1–S2, S5–S9, and S11 groups (Figure 1A). Less ($P \leq 0.01$) DNA damage percentages were found in the glass

wool (S7) and sephadex (S10) filtration groups than in the S12 group (Figure 1B). The S6-S12 groups revealed lower ($P \leq 0.01$) malondialdehyde concentrations than the S1-S5 groups (Figure 1C). The S9 group exhibited higher freezability, while the S4 group was less so (Figure 1D).

Table1. Effect of glass wool and sephadex selection techniques with or without adding antioxidants on some of semen characteristics in Iraqi buffalo bull's post cooling (mean \pm standard error)

Groups	Individual motility (%)	Live sperm (%)	Normal morphology (%)	acrosome membrane integrity(%)	plasma membrane integrity(%)
S1	48.3 \pm 1.42 a	87.91 \pm 1.68 a	96.54 \pm 0.42 bc	78.5 \pm 1.49 a	81.6 \pm 1.76 a
S2	47.50 \pm 0.8a	83.45 \pm 2.32 ab	96.04 \pm 0.32 bcd	78.66 \pm 2.39 a	79.6 \pm 2.60 a
S3	46.3 \pm 1.6 ab	81.8 \pm 2.89 abc	96.06 \pm 0.53 bcd	78.2 \pm 3.92 a	75.8 \pm 2.97 a
S4	25.71 \pm 2.39 f	61.07 \pm 4.51 e	92.57 \pm 0.5 f	56.32 \pm 3.94 c	55.1 \pm 3.17 c
S5	27.9 \pm 2.32 ef	64.28 \pm 4.34 de	93.14 \pm 0.70 f	59.6 \pm 3.97 bc	60.4 \pm 3.3 bc
S6	30 \pm 1.96 def	66.82 \pm 4.50 cde	93.78 \pm 0.63 ef	59.32 \pm 4.1 c	61.3 \pm 4.2 bc
S7	23.8 \pm 2.79 f	72.9 \pm 5.5 abcde	94.81 \pm 0.82 de	66.75 \pm 5.11 abc	67.9 \pm 6.4 abc
S8	25.0 \pm 2.31 f	71.9 \pm 6.3 bcde	96.31 \pm 0.68 bcd	70.0 \pm 4.82 abc	71.31 \pm 5.8 ab
S9	30 \pm 1.33 def	77.2 \pm 5.2 abcd	95.37 \pm 0.47 cde	69.8 \pm 5.54 abc	70.93 \pm 6.7 ab
S10	36 \pm 3.23 cd	74.4 \pm 5.1 abcde	97.55 \pm 0.17 ab	73.1 \pm 4.55 ab	73.8 \pm 4.0 ab
S11	35 \pm 3.49 cde	73.3 \pm 6.7 abcde	98.75 \pm 0.08 a	73.7 \pm 5.48 a	74.1 \pm 6.01 ab
S12	40 \pm 3.49 bc	79.2 \pm 6.0 abcd	98.95 \pm 0.05 a	77.9 \pm 5.18 a	79.15 \pm 5.0 a
Level of significance	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01

Means with different superscripts within similar column indicated significant differences among groups. S1 = good quality semen + Tris extender; S2 = S1 with vitamin E (2 mmol) + vitamin C (5 mmol); S3 = S1 + glutamine (20 mmol) + arginine (1 mmol); S4= low-quality semen +Tris extender; S5= S4 with vitamin E + vitamin C; S6= S4 with glutamine + arginine; S7= Glass wool technique; S8= S7 + Vitamin E + Vitamin C; S9= S7 with glutamine + Arginine;S10= Sephadex technique;S11 = S10 + vitamin E + vitamin C;S12 = S10 + glutamine + arginine

Table 2. Effect of glass wool and sephadex selection techniques with or without adding antioxidants on some of semen characteristics in Iraqi buffalo bull's post- cryopreservation (mean \pm standard error)

Groups	Individual motility (%)	Live sperm (%)	Normal morphology (%)	acrosome membrane integrity(%)	plasma membrane integrity(%)
S1	38.3 \pm 1.42 a	74.2 \pm 2.9 a	83.08 \pm 0.35 cd	70.6 \pm 3.8 a	72.20 \pm 3.47 a
S2	37.5 \pm 1.44 a	75.9 \pm 3.5 a	83.04 \pm 0.25 cd	70.08 \pm 3.7 a	75.04 \pm 4.12 a
S3	36.6 \pm 2.8 a	74.25 \pm 4.8 a	82.7 \pm 0.6 cde	62 \pm 3.67 ab	66.62 \pm 5.17 ab
S4	15 \pm 1.81 c	43.6 \pm 5.5 b	80.10 \pm 0.53 h	40.8 \pm 4.60 c	39.35 \pm 5.29 c
S5	19.3 \pm 2.5 bc	40.28 \pm 4.3 b	80.7 \pm 0.50 gh	40.2 \pm 3.73 c	40 \pm 4.53 c
S6	19.3 \pm 2.3 bc	42.9 \pm 4.4 b	81.14 \pm 0.6 fgh	39.42 \pm 3.7 c	39.67 \pm 3.44 c
S7	18.8 \pm 2.8 bc	42.8 \pm 6.2 b	82.2 \pm 0.5 def	53.3 \pm 5.1 bc	52.87 \pm 5.40 bc
S8	17.5 \pm 1.6 bc	44.9 \pm 1.8 b	81.62 \pm 0.4 efg	51.6 \pm 5.4 bc	51.12 \pm 5.22 c
S9	25 \pm 1.33 b	55.8 \pm 2.6 b	83.75 \pm 0.29 bc	52.1 \pm 4.2 bc	53.68 \pm 4.42 bc
S10	24 \pm 2.66 b	51.1 \pm 5.5 b	84.6 \pm 0.19 ab	50.4 \pm 4.8 bc	49.9 \pm 4.72 c
S11	23 \pm 3.09 b	52 \pm 5.68 b	85.7 \pm 0.11 a	51.1 \pm 5.3 bc	50.1 \pm 6.05 c
S12	23.6 \pm 2.86 b	51.6 \pm 5.2 b	85.4 \pm 0.20 a	50.0 \pm 4.6 bc	52.9 \pm 4.15 bc
Level of Significance	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01

Means with different superscripts within similar column indicated significant differences among groups. S1 = good quality semen + Tris extender; S2 = S1 with vitamin E (2 mmol) + vitamin C (5 mmol); S3 = S1 + glutamine (20 mmol) + arginine (1 mmol); S4= low-quality semen +Tris extender; S5= S4 with vitamin E + vitamin C; S6= S4 with glutamine + arginine; S7= Glass wool technique; S8= S7 + Vitamin E + Vitamin C; S9= S7 with glutamine + Arginine;S10= Sephadex technique;S11 = S10 + vitamin E + vitamin C;S12 = S10 + glutamine + arginine.

Also, the S9 group exhibited higher ($P \leq 0.01$) freezability than the S1–S8 groups (Figure 1D). The superiority of S1-S3 treatments in individual motility, live sperm, acrosome, and plasma membrane integrity (Tables 1 and 2) over most experimental treatments is because the quality of the semen used for cryopreservation was good. There are several antioxidants in semen, some of which are found in the nucleus (vitamin E), lysosomes (vitamin C and E, and carotenoids), mitochondria (such as vitamin E, C, manganese, catalase, and glutathione peroxidase), cytoplasm (vitamin C, E, and glutathione peroxidase), and cell membranes. However, these antioxidants are present in small amounts (40), and their sufficient presence in mammalian sperm prevents and reduces oxidative damage to the lipid membranes of sperm during cryopreservation and thawing processes. Non-significant differences in most semen characteristics post cooling and cryopreservation (Tables 1 and 2) were found in the current study among the S1-S3 groups. Contrary to our results of the cryopreservation of bull semen, Eidan and Al-Zaidi (18) reported that adding a combination of vitamin C and E to the semen extender improved acrosome integrity and reduced mid-piece abnormalities as compared with control group. We suppose the lack of significant differences in most semen characteristics in the current study among S1-S3 groups may be a return of several reasons: First, the amount of vitamin E with C (S2) was insufficient, or the semen of buffalo bulls didn't suffer from a deficiency of glutamine with arginine acids (S3) as mentioned in Figure 1A. The second reason is that buffalo semen may need treatment or the addition of other antioxidants like enzymatic, non-enzymatic, and natural antioxidants (7, 8, 15, 16, 17, 19, 24, 25, 28, 34, 39). A third reason may be a need for genetic improvement by selecting genes related to semen characteristics (9, 21, 44, 46, 47). Sperm individual motility percentage had frequently been determined in artificial insemination laboratories, an essential requirement in oocyte fertilization (14). The cervical mucus selectively allows only progressively motile sperm of normal morphology to penetrate and migrate through

the cervix (45). The sephadex filtration with or without antioxidants (S10-S12) improved most of the semen traits in this study than the negative control group (S4; Table 1, 2 and Figure 1). Semen filtration technology through Sephadex columns (G10-G200) which is a dextran gel, was used in bulls (31) and buffaloes (25). This technology relies on the passage of normal morphology of sperm and motile through the pores between the sephadex particles and their collection at the bottom of the collecting tube with the accumulation of dead and non-motile sperm on the surface of the sephadex gel (6). Any improvement in the percentages of sperm motility has regarded as a normal response for sperm activity post the removal of seminal plasma since it contains dead and abnormal sperms, leukocytes, epithelial cells, debris, and microbial contamination that produce many oxygen radicals that negatively influence the sperm functions. Mammalian fertilization and subsequent embryonic development depend on the integrity of the sperm's DNA (41). Understanding the complex packaging of mammalian sperm chromatin and the assessment of DNA integrity could potentially provide a benchmark in clinical infertility (30, 41). The DNA damage percentage in this study was still within the permissible (Figure 1B) limit value (7–20%) for a bull's fertility (23, 40), indicating good bull fertility. The S7 (Glass wool group) and S10 (Sephadex group) recorded the lowest DNA damage sperm percentages than other groups (Figure 1C), which may be because glass wool filtration has mainly based on the fact that due to the sticky nature of dead, non-motile, and abnormal sperm, they easy get attached to fiber (26, 45). Also, during the sephadex filtration process, dead and non-motile sperms accumulate on the surface of the sephadex gel (26, 45). A higher ($P \leq 0.01$) freezability recorded in the S9 group than the S1–S8 groups (Figure 1D) may be due to the fact that arginine and glutamine act synergistically as antioxidants and reduce the production of free radicals, which form due to oxidative stress during the cryopreservation process. Although most of the sperm filtration treatments recorded significantly or mathematically higher characteristics of the semen than a negative control, they were not

within the level required for artificial insemination, perhaps due to the long period of sperm filtration within each technology, which causes the sperm to suffer from high acidity in the filtering tubes as a result of the metabolic processes that it performs before cooling and cryopreservation, which affects the motility of the sperm and membrane integrity. Therefore, we need to reduce the filtration time of the sperm or add substances that modify

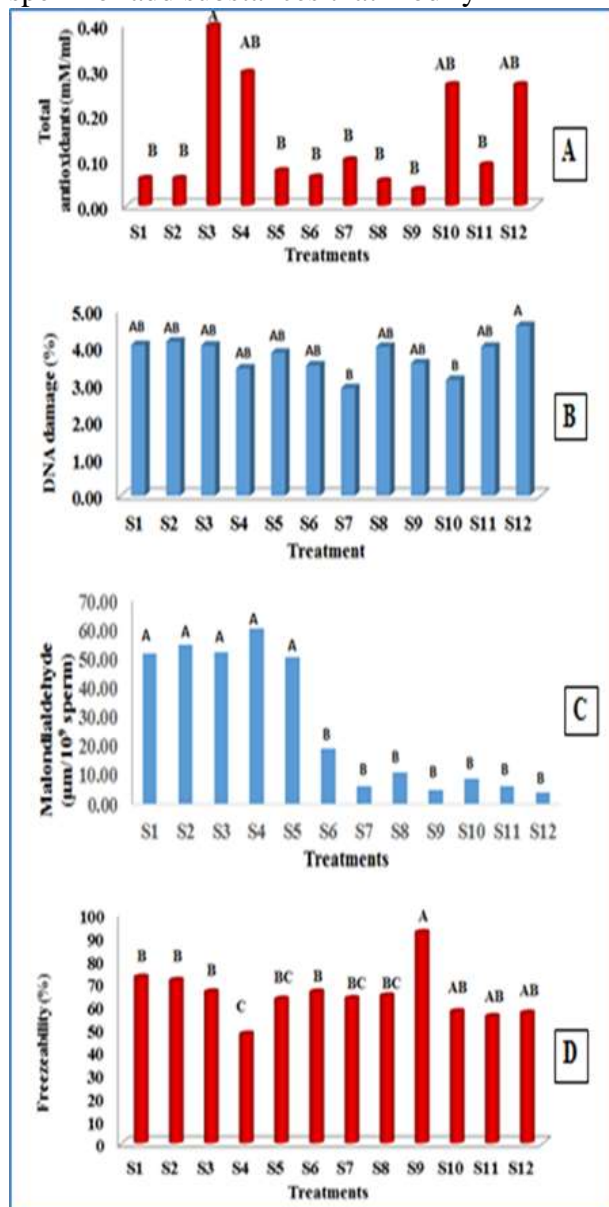


Figure1. Effect of glass wool and sephadex selection techniques with or without adding antioxidants on freezability, total antioxidants, malondialdehyde and DNA damage in Iraqi buffalo bull’s semen post-cryopreservation

Means with different superscripts indicated significant differences (P<0.01) among groups. S1 = good quality semen + Tris extender; S2 = S1 with vitamin E (2 mmol) + vitamin C (5 mmol); S3 = S1 + glutamine (20 mmol) + arginine (1 mmol); S4= low-quality semen +Tris extender;

S5= S4 with vitamin E + vitamin C; S6= S4 with glutamine + arginine; S7= Glass wool technique; S8= S7 + Vitamin E + Vitamin C; S9= S7 with glutamine + Arginine;S10= Sephadex technique;S11 = S10 + vitamin E + vitamin C;S12 = S10 + glutamine + arginine. the pH or increase the sperm's activation in the future

REFERENCES

1. Abdel-Razek, I. and I. El-Shamaa. 2016. Effect of glutamine addition to freezing extender on cryopreserved semen parameters and subsequent fertility of Egyptian buffalo bull Asian. *J. Anim. Vet. Adv.*, 11, 296-302. <https://doi.org/10.3923/ajava.2016.296.302>
2. Abdulkareem, T.A., F.F. Ibrahim, M.S. Hassan, O.A. Mohamed, and W.E. Lateef. 2020. Effect of adding amino acids combinations to Tris extender for improving post cryopreserved semen characteristics of Holstein bulls. *Biochem. Cell. Arch.*, 20(1), 697-701. <https://doi.org/10.35124/bca.2020.20.1.697>
3. Abdulkareem, T.A. and O.H. Alzaidi. 2018. Effect of adding aqueous extract of *Melissa officinalis* leaves and some other antioxidants to Tris extender on post-cooling and post-cryopreservative plasma membrane and acrosome integrity percentages of Holstein bulls. *Al-Anbar JVS*, 11(1), 54-69
4. Abdulkareem, T.A, S.A.Al-Sharifi, M.A., Ishak, S.M. Eidan, M.A. Alnimr, C.W. Passavant, J.R. Branen, and R.G. Sasser.2011. Early pregnancy detection of Iraqi riverine buffalo (*Bubalus bubalis*) using the BioPRYN enzyme-linked immunosorbent assay for PSPB and the progesterone assay. *Reprod. Domest. Anim.*, 46(3),455-462. <https://www.doi:10.1111/j.1439-0531.2010.01689.x>
5. Ahmad, Z., M. Anzar, M. Shahab, N. Ahmad, and S. Andrabi. 2003. Sephadex and sephadex ion-exchange filtration improves the quality and freezability of low-grade buffalo semen ejaculates. *Theriogenology*, 59(5-6), 1189-1202. [https://www.doi.org/10.1016/S0093-691X\(02\)01159-7](https://www.doi.org/10.1016/S0093-691X(02)01159-7)
6. AL-Gebouri, F.G. and S.M. Eidan. 2023. Metabolites and semen characteristics in different bulls fertility. *Iraqi J. Agric. Sci.*, 55(Special Issue), 206-216
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 J. Agric. Sci., 54(4),1074-1083. <https://doi.org/10.36103/ijas.v54i4.17978>
- 8 Alhelal, A.M. and T.A. Abdulkareem. 2023. Ameliorating post-thawed semen of buffalo bulls using a milk-based extender supplemented with resveratrol Iraqi J. Agric. Sci., 55(Special Issue),186-194
9. 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>
- 10 . Alwaeli, S.N. and S.M. Eidan. 2023. Effect of Glass wool and Sephadex sperm separation techniques on improving the poor quality semen of Iraqi buffalo bulls. IOP Conf. Ser.: Earth Environ. Sci. 1262, 072003. doi:10.1088/1755-1315/1262/7/072003
11. Alwaeli, S.N. and S.M. Eidan. 2023. The Effect of Sil-Select and Swim-Down Techniques with Antioxidant Added to Diluent on Semen Quality of Iraqi Buffalo Bulls. IOP Conf. Ser.: Earth Environ. Sci.(In press).
12. Badr, M.R., A.-K. Abdel-Khalek, A.M. Sakr, M.M. Hegazy, and Z.M. Rawash. 2020. Effect of level and time of l-arginine addition to semen extender on the freezability and fertilizing potentials of buffalo spermatozoa. Assiut Veterinary Medical Journal, 66(166), 19-30. <https://www.doi.org/10.21608/avmj.2020.167299>
13. Bhakat, M., T. Mohanty, A. Gupta, A. Chakravarty, P. Singh, and M. Abdullah. 2015. Effect of HS and BQ vaccination on semen quality parameters of Murrah buffalo bulls. J. Inf. Mol. Biol., 3(1), 24-27. <http://dx.doi.org/10.14737/journal.jimb/2015/3.24.27>
14. Dcunha, R., R.S. Hussein, H. Ananda, S. Kumari, S.K. Adiga, N. Kannan, Y. Zhao, and G. Kalthur. 2020. Current insights and latest updates in sperm motility and associated applications in assisted reproduction. *Reprod. Sci.*, 29, 7–25. <https://www.doi.org/10.1007/s43032-020-00408-y>
15. 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://www.doi.org/10.1016/j.anireprosci.2016.01.014>
16. 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.
17. Eidan, S.M., O.A.A. Sultan, F.F. Ibrahim, K.F. Hurish, and H.H. Naseer. 2017. Effect of adding Manganese chloride and Co-enzymes (α -lipoic acid and Q10) on post-cryopreservation semen quality characteristics of Holstein bulls. *Iraqi J. Vet. Med.*, 41(2), 86-93. <https://doi.org/10.30539/iraqijvm.v41i2.53>
18. Eidan, S.M. and O.H.A. Al-Zaidi. 2017. Effect of adding some vitamins, omega-3 and their combinations to tris extender on sperm acrosome deformation percentage of Holstein bulls after different preservation periods. *IJAR*, 22(4), 117-128.
19. Eidan, S.M., O.H. Al-Zaidi, F. F Ibrahim, B.A.R. Timimi, and W.Y. Lateef. 2015. Effect of adding catalase and glutathione reduce to Tris extender on freezing ability of Holstein bulls following different cryopreservation periods. *Iraqi J. Vet. Med.*, 39(2), 19-24. <https://doi.org/10.30539/iraqijvm.v39i2.161>
20. Eidan, S.M., R.I. Khalil, and Z.H. Ali 2017. Effect of melatonin implantation on Semen quality Holstein bulls: 1-Ejaculate volume and Total normal morphology sperm. *Al-Anbar JVS*, 10(1), 145-153
21. 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>
22. Eidan, S.M., T.A. Abdulkareem, and O.A.A. Sultan. 2015. Influence of adding manganese to Tris extender on some postcryopreservation semen attributes of Holstein bulls. *IJAAS*, 1, 26-30. <http://dx.doi.org/10.11648/j.ijaas.20150102.12>
23. Evenson, D.P. 2016. The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. *Anim. Reprod. Sci.*, 169,

- 56-75.
<https://www.doi.org/10.1016/j.anireprosci.2016.01.017>
24. 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>
25. 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>
26. Husna, A.U., R. Ejaz, S. Qadeer, A. Azam, B.A. Rakha, M.S. Ansari, Q. Shahzad, M.T. Javed, M.H. Vazquez-Levin, and S. Akhter. 2016. A comparative analysis of sperm selection procedures prior to cryopreservation for Nili-Ravi buffalo bull (*Bubalus bubalis*) semen-: Assessment of its impact on post-thaw sperm functional quality. *Anim. Reprod. Sci.*, 174, 29-36.
<https://www.doi.org/10.1016/j.anireprosci.2016.08.015>
27. Javed, M.T., M. Ahmad, G. Saleem, H. Sadia, F. Manzoor, and G. H. Dilbar. 2018. Effect of age and season on semen characteristics of buffalo bull: *Areview. J. INNOV. SCI.*, 4(1), 65-82.
<http://dx.doi.org/10.13140/RG.2.2.13510.09287>
28. Kowalczyk, A. 2022. The role of the natural antioxidant mechanism in sperm cells. *Reprod. Sci.*, 29(5), 1387-1394.
<https://www.doi.org/10.1007/s43032-021-00795-w>
29. Kumar, N., A. Singh, R. Cheema, A. Kumar, H. Kaur, and P. Brar. 2018. Effect of vitamin E supplementation during buffalo Semen cryopreservation on sperm characteristics and oxidative stress. *Journal of animal research*, 8(5), 797-805.
<https://www.doi.org/10.30954/2277-940X.10.2018.9>
30. Kumaresan, A., M. Das Gupta, T.K. Datta, and J.M. Morrell. 2020. Sperm DNA integrity and male fertility in farm animals: Review. *Front. vet. sci.*, 7, 1-15.
<https://www.doi.org/10.3389/fvets.2020.00321>
31. Lee, H., S. Kim, D. Ji, and Y. Kim. 2009. A comparative study of Sephadex, glass wool and Percoll separation techniques on sperm quality and IVF results for cryopreserved bovine semen. *J. Vet. Sci.*, 10(3), 249-255.
<https://www.doi.org/10.4142/jvs.2009.10.3.249>
32. Li, K.-p., X.-s. Yang, and T. Wu. 2022. The effect of antioxidants on sperm quality parameters and pregnancy rates for idiopathic male infertility: a network meta-analysis of randomized controlled trials. *Front. Endocrinol.*, 13, 1-14.
<https://www.doi.org/10.3389/fendo.2022.810242>
33. Meitei, H.Y., S. Uppangala, K. Sharan, S.G. Chandraguthi, A. Radhakrishnan, G. Kalthur, S. Schlatt, and S.K. Adiga. 2021. A simple, centrifugation-free, sperm-sorting device eliminates the risks of centrifugation in the swim-up method while maintaining functional competence and DNA integrity of selected spermatozoa. *Reprod. Sci.*, 28, 134-143. <https://doi.org/10.1007/s43032-020-00269-5>
34. Mohammed, O.A., T. A. Abdulkareem, F. Ibrahim, O. Al-Zaidi, W. Latif, and S. Alwan. 2020. Effect of adding pentoxifylline and nitric oxide to Tris extender on some post-cryopreserved semen attributes of Holstein bulls. *Iraqi J. Agri. Sci.*, 51(2): 619-628.
<https://doi.org/10.36103/ijas.v51i2.989>
35. Mohsin, A.S. and S.M. Eidan 2019. Ffect of adding antioxidants to Tris extender on cooling and post-cryopreservative semen characteristics of Holstein bulls 1. melatonin hormone. *Biochem. Cell. Arch*, 19(1): 1843-1848
36. Musa, K. S. and T. A. Abdulkareem. 2023. Protein profiles in seminal plasma of Iraqi buffalo bulls (*Bubalus bubalis*) associated with fresh and cryopreserved semen quality. *IOP Conf. Ser.: Earth Environ. Sci.* 1262, 072095.
[doi:10.1088/1755-1315/1262/7/072095](https://doi.org/10.1088/1755-1315/1262/7/072095)
37. Musa, K.S. and T. A. Abdulkareem. 2023. Regression coefficient of semen characteristics

- of buffalo bulls on seminal plasma proteins. Ser.: Earth Environ. Sci.,(In press)
38. Musa, K. S. and T. A. Abdulkareem. 2024. Some biochemical attributes in seminal plasma of Iraqi buffalo bulls and their relation to the semen quality. Iraqi J. Agri. Sci., 55(1): In press.
39. Nsiab, Z.M. and S.M. Eidan. 2023. Effect of Melatonin Implantation on Sexual Behavior and some of Semen Quality of Iraqi Buffalo Bulls. 4th International. IOP Conf. Ser.: Earth Environ. Sci. 1262, 072013. doi:10.1088/1755-1315/1262/7/072013
40. Qamar, A.Y., M.I. Naveed, S. Raza, X. Fang, P.K. Roy, S. Bang, B.M. Tanga, I.M. Saadeldin, S. Lee, and J. Cho. 2023. Role of antioxidants in fertility preservation of sperm—A narrative review. Anim. Biosci., 36(3), 385-403. <https://www.doi.org/10.5713/ab.22.0325>
41. Shahverdi, A., A. Rastegarnia, and T.R. Topraggaleh. 2014. Effect of extender and equilibration time on post thaw motility and chromatin structure of buffalo bull (*Bubalus bubalis*) spermatozoa. Cell J., 16(3), 279–288.
42. Silva, L.K.X., J.d.B. Lourenço Júnior, A.O.A.d. Silva, J.S.d. Sousa, A.G.M. Silva, A.N.d. Reis, M.d.S. Miranda, S.d.S.D. Santos, O.M. Ohashi, and L.G. Martorano. 2020. Increased quality of in natura and cryopreserved semen of water buffaloes supplemented with saturated and unsaturated fatty acids from the palm oil industry. Anim. Reprod., 17(4), 1-17. <https://www.doi.org/10.1590/1984-3143-AR2020-0522>
43. Samardzija, M., M. Karadjole, M. Matkovic, M. Cergolj, I. Getz, T. Dobranic, A. Tomaskovic, J. Petric, J. Surina, and J. Grizelj. 2006. A comparison of BoviPure® and Percoll® on bull sperm separation protocols for IVF. Anim. Reprod. Sci., 91(3-4), 237-247. <https://doi.org/10.1016/j.anireprosci.2005.04.005>
44. Shao, B., H. Sun, M. J. Ahmad, N. Ghanem, H. Abdel-Shafy, C. Du, T. Deng, S. Mansoor, Y. Zhou, and Y. Yang. 2021. Genetic features of reproductive traits in bovine and buffalo: Lessons from bovine to buffalo. Front. genet., 12, 1-16. <https://www.doi.org/10.3389/fgene.2021.617128>
45. Srivastava, N. and M. Pande. 2017. Semen Analysis: An Overview. Protocols in Semen Biology (Comparing Assays): 1-6. https://www.doi.org/10.1007/978-981-10-5200-2_1
46. Sultan, O.A.A. and S.M. Eidan. 2020. Association of CD9 gene with semen quality of Holstein bulls: 1. Fresh semen. Biochem. Cell. Arch., 20(1), 2721-2725.
47. Sultan, O.A.A. and S.M. Eidan. 2020. Association of CD9 gene with semen quality of Holstein bulls: 2. Post cryopreservative semen. Biochem. Cell. Arch., 20(1), 2697-2701.