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

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ABSTRACT

This study was conducted to investigate the effect of swim-up and glass wool as sperm separation techniques with adding vitamin E and superoxide dismutase (SOD) to Tris extender on improving post-cryopreserved total sperm characteristics in straw and freezability percentage for low semen quality of Holstein bulls. Low and good of semen were extended using Tris extender. Good semen quality (GSQ) was divided into 3 groups (L1; Tris extender, L2; 2 mM vitamin E, L3; 200 IU SOD) Low semen quality (LSQ) was divided into two main groups, and subdivided into 3 sub-groups (L4; Tris extender, L5; 2 mM vitamin E, L6; 200 IU SOD). In the second main group, swim-up and glass wool techniques were used with adding vitamin E and SOD and subdivided into 3 sub-groups with each technique, and referred to L7, L8 and L9 for swim-up technique and L10, L11 and L12 for glass wool technique. Sperms with good motile intact acrosome and plasma membrane normal morphology of sperm were obtained using the glass wool separation technique with adding vitamin E.

Keywords: Vitamin E, SOD, Swim-up, Glass wool, Low semen quality, Holstein bulls.

تأثير تقانات Swim-up والصوف الزجاجي مع إضافة مضادات الأكسدة الى مخفف Tris في تحسين خصائص النطف الكلية في القصبة والنسبة المئوية للتجميد لدى السائل المنوي رديء النوعية لثيران الهولشتاين بعد الحفظ بالتجميد

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المستخلص

أجريت هذه الدراسة لبيان تأثير تقانات Swim-up والصوف الزجاجي لفصل النطف مع إضافة فيتامين E والسوبر أوكسيد دسميوتيز (SOD) الى مخفف Tris في تحسين العدد الكلي للنطف في القصبة وكذلك النسبة المئوية لقابلية التجميد بعد حفظها بالتجميد للسائل المنوي رديء النوعية لثيران الهولشتاين. ثم تخفيف كلا النوعين من السائل المنوي بإستخدام مخفف Tris. قسم السائل المنوي الجيد النوعية الى 3 مجاميع (L1: مخفف Tris، L2: 2 ملي مايكرون فيتامين E، L3: 200 وحدة دولية من SOD)، كما قسم السائل المنوي الرديء النوعية الى مجموعتين رئيستين، قسمت المجموعة الأولى الى ثلاث مجاميع فرعية (L4: مخفف Tris، L5: 2 ملي مايكرون فيتامين E، L6: 200 وحدة دولية من SOD). في المجموعة الرئيسية الثانية، تم استخدام تقانتي Swim-up والصوف الزجاجي لفصل النطف مع إضافة فيتامين E وSOD تم تقسيم كل مجموعة من مجموعتي التقانات الى ثلاث مجاميع فرعية تمت الإشارة اليها بالرموز L7، L8 و L9 لتقانة Swim-up وL10، L11 وL12 لتقانة الصوف الزجاجي. تحسن العدد الكلي للنطف ذات الحركة الجيدة والاكروسوم والغشاء البلازمي السليمين والشكل الطبيعي للنطف بإستخدام تقانة الصوف الزجاجي لفصل النطف مع إضافة فيتامين E الى مخفف السائل المنوي.

الكلمات المفتاحية: فيتامين E، السوبر أوكسيد دسميوتيز (SOD)، السباحة للأعلى، الصوف الزجاجي، السائل المنوي رديء النوعية، ثيران الهولشتاين

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INTRODUCTION

The enhancement in quality of semen is crucial aspect for maximum utilization of genetically-superior sub-fertile sire, for the reason of age and/or non-specific factors like thermal stress, transport and vaccination stress during their dynamic life show a low-grade semen quality than previously (25). In Iraq, increasing quantities of poor-quality semen from genetically-superior bulls used in artificial insemination (AI) center, at moderate season (October and November) in particular (2), is a big burden and of economic loss, namely, $ 3-4 per bull per day (personal communication). These will negatively reflect in low conception rates of cattle herds as well as to decrease in their breeder income (4). Using of semen separation techniques for *invitro* fertilization (IVF) processes became an important routine work for yielding a higher number of motile and morpho- logically normal spermatozoa, that would be successfully used for long-term cryopreservation processes (34). The basic presupposition of any sperm preparation technique is to (a) eliminate any factors detrimental to fertilization, (b) to block factors such as prostaglandins that would other- wise cause uterine contractions, (c) to increase sperm concentration, motility and (d) to form sperm capacitation. This is to be achieved by separating the seminal plasma from spermatozoa in a rapid and efficient fashion (13). Swim-up technique is based on migration of motile sperm from the bottom to the top fraction of the media, which separates motile sperms from the non-motile sperm and debris, as it is based on the ability of sperm to swim into the culture medium. This method may be performed by layering the culture medium directly over the semen, or layering the culture medium over the pellet, which is obtained after the centrifugation of the sample (33). This technique is an easy reliable and effective sperm processing method for insemination purposes, and an increased sperm count, motility and pregnancy rate were obtained after the swim up procedure in human (14). Moreover, Risopatron (29) found 63.20% of bull's live sperm with intact plasma membrane using swim up technique. Jeyendran (18) confirmed motility-based separation of the swim-up technique is based on metabolic differences. Furthermore, the upper fraction of bull sperm has improved motility compared to the cells in the lower fraction and shows higher metabolic rates. Glass wool filtration is a low-cost, simple, and highly effective procedure to select functionally competent sperm for reproductive technologies in the bull, which may be useful for other domestic and farm animals, as well as for endangered species (5).Glass wool filtration to remove debris and round cells from human ejaculates is four decades old (24). The method was first proposed for artificial insemination (16), and later for *invitro* fertilization (IVF; 32). Greater sperm's cell individual motility and plasma membrane integrity percentages of post-thawed bull's semen samples noticed as compared with the control group using glass wool technique (17). The continuous releasing of reactive oxygen species (ROS) from abnormal and immature sperms, as well as those produced from freezing-thawing processes is often accompanied by low concentrations of antioxidants in seminal plasma and semen extenders. That would consequently lead to deleterious effects of oxidative stress on spermatozoa (30). Vitamin E is a fat-soluble compound and also known as α-tocopherol (31). It acts as most effective non-enzymatic antioxidants due to its ability to inhibit free radicals (21). Overwhelming results of improved post-cryopreserved (PC) semen quality of Holstein bulls were obtained by adding vitamin E to Tris extender (1, 2,3, 12). On the other hand, superoxide dismutase (SOD) is an antioxidant that catalyzes dismutation of superoxide into oxygen and hydrogen peroxide. It scavenges both extracellular and intracellular superoxide anion and prevents lipid peroxidation of the plasma membrane (7). A good result of cooled (6) and cryopreserved (20) semen characteristics of bulls were got with adding SOD to semen extenders. The comparison among the above-mentioned semen separation techniques with adding vitamin E or SOD and their effects on low-quality semen characteristics of Holstein bulls were not previously investigated either in Iraq or in the world. Therefore, this study was undertaken to explore the effect of using swim-up and glass wool techniques with

adding vitamin E or SOD to separated, low and good-quality semen on improving post-cryopreserved totals sperm normal morphology counts, total acrosome integrity count ,total plasma membrane integrity, total function of sperm fraction and freezability percentage pertaining to low semen quality of Holstein bulls. This study was aimed the effect of using swim-up and glass wool techniques with addition of vitamin E or SOD to separated, low and good-quality semen on PC sperm characteristics of Holstein bulls.

MATERIALS AND METHODS

The present study was performed on Holstein bulls at the Department of Artificial Insemination pertaining to the Directorate of Animal Resource, Ministry of Agriculture, Abu-Ghraib, Baghdad, during the period from February, 2018 to May 2019. Both low fresh semen (sperm's cell individual motility less than 40%) from nine bulls and good fresh semen quality from three bulls were collected via artificial vagina at two ejaculates/bull/week. Measuring of ejaculate volume, next concentration of sperm was calculated by a Neubauer Haemocytometer counting chamber was used for sperm concentration (28). Low and good semen quality was weekly collected from 12 Holstein bulls (9 for low and 3 for good semen quality) for 16 weeks experimental periods. Both types of semen were extended using Tris extender and divided as follows: Good semen quality (GSQ) were divided into 3 groups, first group was regarded as positive control group containing Tris extender only (L1), whereas, vitamin E (2 mM) and superoxide In the second main group, swim-up and glass wool techniques were used for semen separation. Each of the technique groups was subdivided into 3 sub-groups. First was extended with Tris extender only, whereas, vitamin E and SOD were added with similar concentration to the second and third groups respectively. The sub-groups were referred to as L7, L8 and L9 for swim-up technique and L10, L11 and L12 for glass wool technique. Acrosome integrity percentage was performed according to the staining procedure followed the protocol of (12). The hypo-osmotic swelling test was performed conforming to the methods explained by (15) method. Swim-up technique as described by (23). Glass wool filtration described by (32). Parameters were assessed at four preservation periods (cooling, 96 hrs., 3- and 6-months post-cryopreservation; PC). semen quality (LSQ) were divided into two main groups, the first main groups was regarded as negative group subdivided into 3 sub-groups; first group was extended with Tris extender only (L4); while, vitamin E (2 mM) dismutase (SOD; 200 IU) were added to the Tris per the second (L2) and third (L3) groups respectively. Low and SOD (200 IU) were added to the second (L5) and third group (L6) respectively.

Statistical analyses

The statistical computation was performed using SAS program (29) based on study on completely randomized deign (CRD) to study the effect of different factors on the studies characteristics. Means with significant differences were compared using Duncan multiple range test (9).

RESULTS AND DISCUSSION

Total normal morphology count ($\times 10^6$)

morphology counts ($\times 10^6$) within the L1-L2, L4-L5, and L7 and L10-L11 groups at different cryopreservation periods (Table 1). A greater ($p \leq 0.0001$) total normal morphology counts ($\times 10^6$) were noticed for groups L1-L3, and L7-L12 in comparison with the L4-L6 groups at 96 hrs. PC period (Table 1). The L2 (14.29±0.12), L8 (14.26±0.06), L11 (14.36±0.05), and L12 (14.20±0.33) groups exhibited greatest ($P \leq 0.0001$) total normal morphology count as compared with the L3 (13.46±0.21) group at 96 hrs. (Table 1). The total normal morphology counts ($\times 10^6$) were a higher ($P \leq 0.0001$) for groups L1-L3 (12.97±0.18-13.86±0.19) and L7-L12 (13.23±0.23-14.66±0.08) in comparison with L4-L6 (11.68±0.43-12.39±0.17) groups at 3rd months PC period (Table 1). No differences were observed among L1-L2, L7-L8, and L10-L12 groups (Table 1). Moreover, non-significant differences were observed among L4-L6 groups (Table 1). A higher ($P \leq 0.0001$) total normal morphology counts ($\times 10^6$) were demonstrated for groups L1-L2, L7-L8, and L10-L12 in comparison with L4-L6 groups at 6th months PC period (Table 1). The differences in total normal morphology counts ($\times 10^6$) among groups L1-L3, L7-L10, and L12

at 6th months PC period lacked significance (Table 1). Moreover, non-significant differences were demonstrated among L4-L6 groups for similar trait and period (Table 1). Non-significant differences were Moreover, non-significant differences were demonstrated among L4-L6 groups for similar trait and period (Table 1). Non-significant differences were observed in the total normal morphology counts ($\times10^6$) within the L1-L2, L4-L5, and L7 and L10-L11 groups at different cryo-preservation periods (Table 1). The ninety-six hrs. PC exhibited greater (P≤0.03) total normal morphology counts ($\times10^6$) as compared with 3rd and 6th months PC period in L3 and L6 groups (Table 1). Non- significant differences were noticed in the total normal morphology counts ($\times10^6$) at 3rd and 6th months PC period in L3 and L6 groups (Table 1). As mentioned above, the ninety-six hrs. PC revealed a greater (P≤0.03) total normal morphology counts ($\times10^6$) as compared with the 3rd and 6th months PC period within L8 group (Table 1). Furthermore, the third months PC revealed a higher (P≤0.03) total normal morphology counts ($\times10^6$) in comparison with the 6th months PC period within L8 group (Table 1). A higher (P≤0.03) total normal morphology counts ($\times10^6$) at 96 hrs. PC was recorded as compared with the 6th months PC period in L9 and L12 groups (Table 1). The differences in the total normal morphology counts ($\times10^6$) at 3rd as compared with 96 hrs. and 6th months PC period within L3 and L6 groups lacked significance (Table 1). As mentioned above, the ninety-six hrs. PC revealed greater (P≤0.03) total normal morphology counts ($\times10^6$) as compared with the 3rd and 6th months PC period in L8 group (Table 1). Also, the third months PC exhibited a higher (P≤0.03) total normal morphology counts ($\times10^6$) as compared with the 6th months PC period in L8 group (Table 1). A higher (P≤0.03) total normal morphology counts ($\times10^6$) at 96 hrs. recorded as compared with 6th months PC period within L9 and L12 groups (Table 1). Non-significant differences were demonstrated in the total normal morphology counts ($\times10^6$) at 3rd as compared with 96 hrs. and 6th months PC period in L3 and L6 groups (Table 1).

Table 1. Effect of swim-up and glass wool separation techniques and adding superoxide dismutase or vitamin E to semen extender on post-cryopreserved (PC) total normal morphology ($\times10^6$) of Holstein bulls (Mean±SE).

Period / Groups	96 hrs. PC	3 months PC	6 months PC	Level of Significance
L1	13.92±0.13 ab A	13.86±0.19 ab A	13.69±0.18 ab A	N.S.
L2	14.29±0.12 a A	13.85±0.40 ab A	13.70±0.61 ab A	N.S.
L3	13.46±0.21 b A	12.97±0.18bc B	12.67±0.27 bc B	P≤0.03
L4	11.73±0.37 e A	11.68±0.43 e A	11.31±0.36 e A	N.S.
L5	12.65±0.25 cd A	12.39±0.17 de A	12.42±0.34 c A	N.S.
L6	12.57±0.15 d A	12.06±0.14 de B	11.85±0.16 ce B	P≤0.01
L7	13.92±0.26 ab A	13.74±0.11 ab A	13.56±0.12 ab A	N.S.
L8	14.26±0.06 a A	14.66±0.08 a B	13.83±0.05 ab C	P≤0.0006
L9	13.78±0.14 ab A	13.23±0.23 bc AB	12.96±0.26 bc B	P≤0.04
L10	14.09±0.10 ab A	13.87±0.13 ab A	13.71±0.12 ab A	N.S.
L11	14.36±0.05 a A	14.20±0.28 a A	14.06±0.10 a A	N.S.
L12	14.20±0.33 a A	13.58±0.10 ab AB	13.41±0.13 ab B	P≤0.04
Level of Significance	P≤0.0001	P≤0.0001	P≤0.0001	---

Means with small superscripts within each column indicated significant differences among groups and large superscripts within each row indicated significant differences among periods; N.S.= no significant; L1= Good Semen Quality (GSQ) + Tris extender; L2= GSQ + Tris extender + 2 mM vitamin E; L3= GSQ + Tris extender + 200 IU SOD; L4= Low Semen Quality (LSQ) + Tris extender; L5= LSQ + Tris extender + 2 mM vit. E; L6= LSQ + Tris extender + 200 IU SOD; L7= SU Semen tech.+Tris extender; L8= SU Semen tech.+ Tris extender + 2 mM vit. E; L9= SU Semen tech.+Tris extender + 200 IU SOD; L10= GW Semen tech.+ Tris extender; L11= GW Semen tech.+ Tris extender + 2mMvit. E, L12= GW Semen tech.+Tris extender + 200 IU SOD.

Total acrosome integrity count (×10^6)

The results of total acrosome integrity count were greater (P≤0.003) in L1-L2, L5, and L11 groups as compared with the L4, L7, and L9 groups at 96 hrs. PC period (Table 2). The total acrosome integrity count did not significantly different among L1-L3, L5-L6, L8, and L10-L12 groups at 96 hrs. PC period. Concomitantly, differences among L3-L4, L6, L8-L9, and L12 groups lacked significance (Table 2). The L2 and L5 exhibited greater (P≤0.0006) total acrosome integrity count than those of L3-L4, L7, and L9 at 3rd months PC period (Table 2). Non- significant differences were noticed t among L1-L2, L5-L6, L8, and L10-L12 groups as well as, among L3-L4 and L7-L8 groups at 3rd months PC period in total acrosome integrity count (Table 2). The total acrosome integrity count was overwhelmingly greater (P≤0.0001) in L1-L2, L5, and L10-L12 groups (11.23±0.49-12.27±0.42×10^6) than L4, L7 and L9 groups (9.38±0.61-9.78±0.33×10^6) at 6th month PC period (Table 2). At similar time, non-significant differences were mentioned among L1-L2, L5-L6, L8, and L10-L12 groups, and also among L3-L4, L6, and L8 groups (Table 2). All cryopreservation periods did not significantly affect total acrosome integrity count within all groups, except for L5 group (Table 2). The 96 hrs. PC period was higher (P≤0.1) than 6th months PC in total acrosome integrity count in L5 group (Table 2). The acrosome integrity considered a good indicator of bull's fertility (19). Acrosomal integrity and enzyme maintenance are essential for acrosomal reaction (11) and membrane fusion is required for sperm-oocyte fusion, even though, sperm membranes are fixed to oocytes in the free zone and the occurrence of fertilization (26). The lesser count of total acrosome integrity obtained currently in each straw (9.38±0.61×10^6; Table 2) will provide an enough number of sperm for successful fertilization.

Table 2. Effect of swim-up and glass wool separation techniques and adding of superoxide dismutase or vitamin E to semen extender on post-cryopreserved (PC) total acrosome integrity count (×10^6) of Holstein bulls (Mean±SE).

Period / Groups	96 hrs. PC	3 months PC	6 months PC	Level of Significance
L1	11.88±0.20 ab A	11.59±0.26 ab A	11.55±0.35 abc A	N.S.
L2	12.08±0.38 ab A	12.25±0.35 a A	12.27±0.42 a A	N.S.
L3	10.73±0.12 abcd A	10.20±0.23 bc A	10.65±0.24 cdef A	N.S.
L4	10.32±0.40 cde A	9.93±0.44 bc A	9.78±0.33 def A	N.S.
L5	12.40±0.13 a A	12.09±0.10 a AB	11.76±0.21 ab B	P≤0.1
L6	11.44±0.37 abcde A	11.19±0.34 ab A	10.91±0.39 abcd A	N.S.
L7	10.01±0.62 e 13.05A	9.60±0.71 c A	9.38±0.61 f A	N.S.
L8	11.45±0.59 abcde A	10.92±0.43 abc A	10.50±0.37 abcd A	N.S.
L9	10.26±0.58 de A	9.93±0.45 c A	9.74±0.42 ef A	N.S.
L10	11.81±0.36 abc A	11.44±0.48 ab A	11.23±0.49 abc A	N.S.
L11	11.93±0.38 ab A	11.36±0.37 ab A	11.31±0.33 abc A	N.S.
L12	11.74±0.57 abcd A	11.33±0.46 ab A	11.27±0.41 abc Aq.	N.S.
Level of Significance	P≤0.003	P≤0.0006	P≤0.0001	---

Means with small superscripts within each column indicated significant differences among groups and large superscripts within each row indicated significant differences among periods; N.S.= no significant; L1= Good Semen Quality (GSQ) + Tris extender; L2= GSQ + Tris extender + 2 mM vitamin E; L3= GSQ + Tris extender + 200 IU SOD; L4= Low Semen Quality (LSQ) + Tris extender; L5= LSQ + Tris extender + 2 mM vit. E; L6= LSQ + Tris extender + 200 IU SOD; L7= SU Semen tech.+Tris extender; L8= SU Semen tech.+ Tris extender + 2 mM vit. E; L9= SU Semen tech.+Tris extender + 200 IU SOD; L10= GW Semen tech.+ Tris extender; L11= GW Semen tech.+ Tris extender + 2mMvit. E, L12= GW Semen tech.+Tris extender + 200 IU SOD.

Total plasma membrane integrity count (×10^6)

The L2 group exhibited a greater total plasma membrane integrity as compared with the all groups at whole cryopreservation periods (Table 3). Non-significant differences were demonstrated among L1, L3-L6, and L8-L12 groups, as well as among L4-L9 and L12 groups for 96 hrs. and 3rd months PC periods (Table 3). The higher (P≤0.003) count of total plasma membrane integrity at 6th months PC period was in the L2 group (13.60±0.13×10^6), while lesser in the L7 group (10.05±0.98×10^6). Non-significant differences were noticed among L1, L3-L4 and L8-L12 groups, as well as among L3, L5-L6, L9-L10, and L12 groups (Table 3). The 96 hrs. PC period was significantly greater than 3rd and 6th months PC period within L1, L9, and L11-L12 groups (Table 3). On the other hand, no differences were observed between 3rd and 6th months PC periods within similar groups (Table 3). Non-significant differe- nces were illustrated in total plasma membrane integrity within L1 and L4-L8 groups at all cryopreservation periods (Table 3). The maintenance of the sperm fertilization potential depends on the integrity and functionality of the different cellular structures. The plasma membrane integrity is crucial for sperm survival inside the female reproductive tract to maintain the fertilization capability and the cell's osmotic equilibrium, and acting as a barrier between the extracellular and intracellular media (26). As in Table (3), lesser count of total plasma membrane integrity was 10.05±0.98 ×10^6 in each straw will provide an enough number of good sperm for successful fertilization. The adding of vitamin E to Tris extender of good semen quality (L2) was significantly improved of plasma membrane integrity at all preservation periods. These results were inconsistent with (3) findings, who found non-significant differences in plasma membrane integrity between vitamin E (0.2 mM) and control (Tris extender) groups at all preservation periods (cooling, 48 hrs., 1st, 2nd, and 3rd months PC periods).

Table 3. Effect of swim-up and glass wool separation techniques and adding of superoxide dismutase or vitamin E to semen extender on post-cryopreserved (PC) total plasma membrane integrity count (×10^6) of Holstein bulls (Mean±SE).

Period / Groups	96 hrs. PC	3 months PC	6 months PC	Level of Significance
L1	12.90±0.31 b A	12.15±0.01 b B	12.05±0.06 b B	P≤0.01
L2	14.03±0.11 a A	13.65±0.24 a A	13.60±0.13 a A	N.S.
L3	12.85±0.07 b A	12.30±0.12 b B	11.95±0.14 bc C	P≤0.0008
L4	12.45±0.19 bc A	12.20±0.33 b A	12.10±0.12 b A	N.S.
L5	11.18±0.39 bc A	10.55±0.44 bc A	10.43±0.33 cd A	N.S.
L6	12.10±0.73 bc A	10.92±0.27 bc A	10.70±0.53 cd A	N.S.
L7	10.55±1.01 c A	10.20±0.958 c A	10.05±0.98 d A	N.S.
L8	12.45±0.62 bc A	12.19±0.624 b A	12.13±0.60 b A	N.S.
L9	12.15±0.09 bc A	11.40±0.10 bc B	11.20±0.05 bcd B	P≤0.0003
L10	12.90±0.07 b A	12.35±0.25 b AB	12.00±0.27 bc B	P≤0.06
L11	12.95±0.05 b A	12.60±0.08 b B	12.40±0.21 b B	P≤0.01
L12	12.30±0.09 bc A	11.90±0.06 b B	11.78±0.05 bc B	P≤0.001
Level of Significance	P≤0.02	P≤0.003	P≤0.003	---

Means with small superscripts within each column indicated significant differences among groups and large superscripts within each row indicated significant differences among periods; N.S.= no significant; L1= Good Semen Quality (GSQ) + Tris extender; L2= GSQ + Tris extender + 2 mM vitamin E; L3= GSQ + Tris extender + 200 IU SOD; L4= Low Semen Quality (LSQ) + Tris extender; L5= LSQ + Tris extender + 2 mM vit. E; L6= LSQ + Tris extender + 200 IU SOD; L7= SU Semen tech.+Tris extender; L8= SU Semen tech.+ Tris extender + 2 mM vit. E; L9= SU Semen tech.+Tris extender + 200 IU SOD; L10= GW Semen tech.+ Tris extender; L11= GW Semen tech.+ Tris extender + 2mMvit. E, L12= GW Semen tech.+Tris extender + 200 IU SOD.

Total function sperm fraction ($\times10^6$)

A greater ($P\leq0.001$) total function of sperm fraction ($\times10^6$) was recorded in all positive groups (L1-L3), swim-up separation with vitamin E (L8) and glass wool separation with (L11) or without vitamin E (L10), while, lesser count was observed in all negative control groups (L4-L6) at 96 hrs. period (Table 4). Furthermore, non- significant differences were noticed among all positive groups (L1-L3) and all sperm separation techniques groups (L7-L12) as well as among L5-L7, L9, and L12 at 96 hrs. period (Table 4). All positive groups (L1-L3) and glass wool separation with (L11) or without vitamin E (L10) groups exhibited higher ($P\leq0.0005$) total function of sperm fraction ($\times10^6$) than the L4-L5and L7 groups at 3rd months PC period (Table 4). Moreover, non- significant differences were showed among all positive groups (L1-L3), L6, and L8-L12 as well as among L5-L9 and L12 3rd months PC period (Table 4). It was illustrated from Table (4); that significant differences were noticed in total function sperm fractions ($\times10^6$) among groups at the 6th month PC period. The L1-L3 and L11 groups (8.11 ± 0.02-$9.65\pm0.18\times10^6$) recorded higher total function sperm fractions, while, the L6 ($3.28\pm0.46\times10^6$) and L4 ($3.96\pm0.24\times10^6$) were lesser. Non-significant differences in total function sperm fractions ($\times10^6$) were observed among all positive groups (L1-L3), all glass wool sperm separation technique groups (L10-L12) and among L4-L7 and L9 groups at similar period (Table 4). Non- significant differences in the total functions sperm fractions ($\times10^6$) among all cryopreservation periods within all groups (Table 4). Non-significant differences between L1 and vitamin E (L2) groups at all cryopreservation periods. These results were disagreed with (3), who revealed higher total functions of sperm fractions in vitamin E (0.2 mM) group as compared with the control group (Tris extender) at all preservation periods in Holstein bull semen. The total function sperm fractions ($\times10^6$) were higher (significantly or numerically) in the SOD groups (L3, L9, and L12) than L4 group at most preserved periods. The SOD is an important antioxidant enzyme which rapidly catalyzes the dismutation of superoxide anion ($O^{-\bullet}$) and thus acts as a first line antioxidant defense. In the case of SOD deficiency or increased superoxide production, it reacts with nitric oxide to produce peroxynitrite ($ONOO^-$), which is a potent oxidant and nitrosating agent that can cause direct damage to proteins, lipids, and DNA (8, 22). All groups of glass wool sperm separation technique (L10-L12) were superior in total function sperm fractions ($\times10^6$) in comparison with the negative control (L4) at all cryopreservation periods. Mean- while, non-significant differences in total function sperm fractions among L10-L12 groups and the L1 group. The lacked significance with the positive control group will provides good tools for improvement of low semen quality in the nearest future.

Table 4. Effect of swim-up and glass wool separation techniques and adding of superoxide dismutase or vitamin E to semen extender on post-cryopreserved (PC) total function sperm fraction ($\times 10^6$) of Holstein bulls (Mean±SE).

Period / Groups	96 hrs. PC	3 months PC	6 months PC	Level of Significance
L1	9.22±0.27 a A	8.69±0.38 a A	8.58±0.37 a A	N.S.
L2	11.72±0.20 a A	9.94±0.23 a A	9.65±0.18 a A	N.S.
L3	10.44±0.01 a A	8.56±0.11 a A	8.11±0.02 a A	N.S.
L4	4.11±0.07 c A	4.01±0.20 c A	3.96±0.24 d A	N.S.
L5	6.66±0.61 bc A	5.92±0.55 bc A	5.22±0.55 cd A	N.S.
L6	6.24±0.86 bc A	6.18±0.56 abc A	3.28±0.46 d A	N.S.
L7	8.40±0.87 ab A	5.30±0.74 bc A	5.36±0.72 cd A	N.S.
L8	9.06±1.00 a A	7.31±0.99 ab A	6.71±0.96 bc A	N.S.
L9	8.21±0.89 ab A	6.67±0.19 ab A	5.73±0.31 cd A	N.S.
L10	9.03±0.12 a A	7.52 ±0.31 a A	7.18±0.31 ab A	N.S.
L11	9.93±0.62 a A	8.82±0.53 a A	8.61±0.68 a A	N.S.
L12	8.41±0.03 ab A	7. 42±0.11 ab A	7.10±0.11 ab A	N.S.
Level of Significance	P≤0.001	P≤0.0005	P≤0.001	---

Means with small superscripts within each column indicated significant differences among groups and large superscripts within each row indicated significant differences among periods; N.S.= no significant; L1= Good Semen Quality (GSQ) + Tris extender; L2= GSQ + Tris extender + 2 mM vitamin E; L3= GSQ + Tris extender + 200 IU SOD; L4= Low Semen Quality (LSQ) + Tris extender; L5= LSQ + Tris extender + 2 mM vit. E; L6= LSQ + Tris extender + 200 IU SOD; L7= SU Semen tech.+Tris extender; L8= SU Semen tech.+ Tris extender + 2 mM vit. E; L9= SU Semen tech.+Tris extender + 200 IU SOD; L10= GW Semen tech.+ Tris extender; L11= GW Semen tech.+ Tris extender + 2mMvit. E, L12= GW Semen tech.+Tris extender + 200 IU SOD.

Freezability (%)

They were significant differences (P≤0.007) in freezability among all groups at 96 hrs. PC period (Table 5). The higher freezability recorded in L5, L2, and L3 groups (92.26±0.60-95.36±3.10%) while lesser was noticed in L7 (65.69±6.02%) at 96 hrs. PC period. In addition, non-significant difference was showed in freezability at similar period among L1-L3, L5-L6 groups, L9-L12 as well as among L4 and L7-L9 groups (Table 5). The highest (P≤0.0001) freezability percentage was observed in L5 group (94.06±3.05%) and lowest in L7 (64.50±5.83%) at the 3rd month PC period (Table 5). The freezability did not differ among the L1-L6 and L9-L11 groups as well as among L7-L8 and L12 groups for similar period (Table 5). At the 6th month PC period, the L5 group (92.76±2.91 %) had greater (P≤0.003) freezability percentage and the L7 (63.31±4.96%) was the lowest (Table 5). The freezability percentage did not differ among all the cryopreservation periods in the L1-L2, L4-L5, and L7-L11 groups (Table 5). The freezability was higher at the 96 hrs. than the 3rd and 6th month PC period within the L3 and L12 groups (Table 5). Moreover, non-significant differences were npticed between 3rd and 6th month PC period for within similar groups. Within the L6 group, freezability was greater at 96 hrs. than 6th month PC period (Table 5). The freezability of the negative control group did not differ at all PC periods. Thus, these results prompted us to perform a good sperm separation for low-semen quality. This separation was performed for enhancing an artificial insemination, maximizing the productive and economic benefits from elite

bulls. According to the current results, sperms with good motile, intact acrosome and plasma membrane, normal morphology of sperm as well as lower were obtained using the glass wool separation technique with adding vitamin E to semen extender.

Table 5. Effect of swim-up and glass wool separation techniques and adding of superoxide dismutase or vitamin E to semen extender on post-cryopreserved (PC) freezability (%) of Holstein bulls semen (Mean±SE).

Period / Groups	96 hrs. PC	3 months PC	6 months PC	Level of Significance
L1	87.20±2.50 ab A	86.51±2.65 abc A	84.65±1.64 abc A	N.S.
L2	92.26±0.60 a A	89.28±2.99 ab A	89.10±2.85 ab A	N.S.
L3	92.71±0.14 a A	85.44±0.28 abc B	80.30±3.39 abc B	P≤0.005
L4	78.28±9.58 abc A	88.48±5.18 ab A	78.28±9.58 abcd A	N.S.
L5	95.36±3.10 a A	94.06±3.05 a A	92.76±2.91 a A	N.S.
L6	87.86±1.51 ab A	84.29±2.50 abcd AB	78.12±2.85 abcd B	P≤0.02
L7	65.69±6.02 c A	64.50±5.83 e A	63.31±4.96 d A	N.S.
L8	73.36±6.44 bc A	73.36±6.44 cde A	73.36±6.44 bcd A	N.S.
L9	82.48±5.97 abc A	74.74±5.45 abcd A	73.19±5.10 bcd A	N.S.
L10	89.83±2.27 ab A	86.43±3.37 abc A	82.95±2.10 abc A	N.S.
L11	84.47±5.40 ab A	82.35±4.99 abcd A	81.25±4.74 abc A	N.S.
L12	85.29±2.69 ab A	72.68±1.83 de B	71.56±1.64 cd B	P≤0.0001
Level of Significance	P≤0.007	P≤0.0001	P≤0.003	---

Means with small superscripts within each column indicated significant differences among groups and large superscripts within each row indicated significant differences among periods; N.S.= no significant; L1= Good Semen Quality (GSQ) + Tris extender; L2= GSQ + Tris extender + 2 mM vitamin E; L3= GSQ + Tris extender + 200 IU SOD; L4= Low Semen Quality (LSQ) + Tris extender; L5= LSQ + Tris extender + 2 mM vit. E; L6= LSQ + Tris extender + 200 IU SOD; L7= SU Semen tech.+Tris extender; L8= SU Semen tech.+ Tris extender + 2 mM vit. E; L9= SU Semen tech.+Tris extender + 200 IU SOD; L10= GW Semen tech.+ Tris extender; L11= GW Semen tech.+ Tris extender + 2mMvit. E, L12= GW Semen tech.+Tris extender + 200 IU SOD.

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