

The Time-Dependent Motility and Longevity of Stallion Spermatozoa Diluted in Different Spermatozoal Concentrations and Extenders during Cool-Storage

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SUMMARY

The aim of this study was to investigate the time-dependent changes in motility and longevity of stallion spermatozoa diluted in different spermatozoal concentrations and extenders during cool-storage at 5 °C. Ten purebred Arabian stallions, ranging from 10-24 years of age, were used. Three semen samples were collected from each stallion by artificial vagina. Each ejaculate of 10 stallions was diluted in 3 spermatozoal concentrations (25, 50, and 75×10^6 in 1 ml of extender) in 3 different extenders (milk-, TRIS-based, and glucose-lactose). Sperm motility was determined at 0, 2, 4, 24, 48, 72, 96 and 120th h after the semen was diluted. All ejaculates of each Arabian stallion had a mean initial motility $\geq 80\%$. When it was compared the time-dependent effects of different spermatozoal concentrations in the same extender; the diluted semen with low (25×10^6) spermatozoal concentration had greater motility and longevity than medium (50×10^6) and high (75×10^6) spermatozoal concentration for each extender at all times during examination period. Similarly, when it was compared the time-dependent effects of different extenders in the same spermatozoal concentration; spermatozoa diluted with milk-based extender had higher motility and longevity than TRIS-based and glucose-lactose extenders for each spermatozoal concentration at all times. In conclusion, the stallion semen must be mixed with an appropriate extender that helps to the protection of spermatozoa, and spermatozoal concentration in the extender should be low level in order to gain maximum benefit from cooled stallion semen. Among the tested extenders the milk based extender preserved motility for a longer time.

Keywords : spermatozoal concentration, extender, sperm motility, longevity, stallion.

RESUME

Effet du milieu de dilution et du niveau de dilution du sperme sur la motilité et la longévité des spermatozoïdes d'étalon au cours de leur conservation réfrigérée

L'objectif de cette étude était d'évaluer les changements de la motilité et de la longévité des spermatozoïdes d'étalon en fonction du milieu de dilution et de la concentration en spermatozoïdes au cours de leur conservation à 5 °C. Dix étalons pur sang arabe, âgés de 10 à 24 ans ont été utilisés. Trois échantillons de sperme ont été collectés par étalon à l'aide d'un vagin artificiel. Chaque éjaculat a été dilué à 3 niveaux de concentrations, 25, 50, et 75×10^6 spermatozoïdes (sp) par ml dans 3 milieux de dilution à base de lait, de tris, ou de glucose-lactose. La motilité des spermatozoïdes a été évaluée aux temps 0, 2, 4, 24, 48, 72, 96 et 120 h post-dilution. Le pourcentage de spermatozoïdes motiles de tous les éjaculats était initialement supérieur ou égal à 80%. La comparaison de l'effet de différentes concentrations en spermatozoïdes pour un milieu de dilution donnée a montré que le faible niveau de dilution (25×10^6 sp/ml) était associé à une motilité et longévité supérieure aux niveaux de dilution intermédiaire (50×10^6 sp/ml) et élevé (75×10^6 sp/ml) quelle que soit le milieu de dilution quelle que soit la période de mesure. De la même façon, l'effet des différents milieux de dilution a été comparée pour une concentration donnée en spermatozoïdes ; les spermatozoïdes dilués dans un milieu à base de lait ont présenté une motilité et une longévité supérieures à ceux dilués dans les milieux à base de tris ou de glucose-lactose quelle que soit la période de mesure. En conclusion, le sperme d'étalon doit être dilué dans un milieu approprié qui assure la protection des spermatozoïdes et la concentration en spermatozoïdes doit être faible pour permettre un bénéfice maximal du sperme réfrigéré d'étalon. Parmi les milieux de dilution, le milieu à base de lait apparaît comme celui qui préserve le plus longtemps la motilité des spermatozoïdes.

Mots-clés : spermatozoïdes, diluant, motilité, longévité, étalon

Introduction

The transportation of cooled stallion semen has continued to gain popularity among breeders due to the fact that its acceptance increases among breed registries. In the many horse industries of different countries, this popularity results in an increase of artificial inseminations (AI) of mares. There is also a considerable variation in success rates in terms of fertility together with this widespread use. Some of this varia-

bility can be attributed to differences in the inherent fertility of the mares or stallions or in the ability of the semen from certain stallions to survive rigors of cooling, storage and transport [23].

In vitro survival and maintenance of fertilizing capacity of spermatozoa are affected by semen handling procedures and storage or transport time (12 to 24 h), and successful storage is reported for up to 80 h [7]. The capability of spermatozoa to sustain their fertilizing capacity during storage varies

among stallions, therefore, the capability of each stallion sperm to tolerate cooling and storage should be tested before semen is used for commercial purposes [11].

In general, the use of an extender enhances the spermatozoon survival rate outside the stallion and, with the addition of antibiotics, reduces bacterial contamination and associated risk of pathogen transfer. An extender may also protect spermatozoa from unfavourable external environmental conditions [5, 9].

The aim of this study was to investigate the time-dependent changes in motility and longevity of stallion spermatozoa diluted in different spermatozoal concentrations and extenders during cool-storage at 5 °C.

Material and Methods

ANIMALS AND LOCATION

In this investigation, 10 purebred Arabian stallions, ranging from 10-24 years of age, were used. The stallions are raised at the Farm of General Directorship of Sultansuyu Agriculture Business Enterprises in Turkey, and have routinely been used in an AI program. The horses were kept in boxes and fed with oats and hay three times a day. Drinking water was provided *ad libitum*.

SEMEN COLLECTION, FILTRATION AND EVALUATION

Semen was collected by an artificial vagina (Hannover model), using a teaser mare during breeding season (April). Three semen samples were collected from each stallion twice a week during investigation. As soon as semen samples were collected, the first process was to filter the sample to remove the gel fraction by using clean dry sterile muslin. After filtration, the semen-rich fraction of the ejaculate poured a warm graduated cylinder for determination of gel-free semen volume. Semen volume was determined by direct reading the graduations on collection tubes. To determine the percentage of initial sperm motility, light microscope with heated stage was used. A slide was placed on microscope stage and allowed to warm a temperature of 37 °C, several droplets of 3% sodium citrate were then dropped on the slide, and a small droplet of gel-free semen at 37 °C was placed on the sodium citrate droplets and mixed by coverslip. The percentage of motility was evaluated visually at 400 x magnification. Motility estimations were performed on 3 droplets and 5 different fields per drop in each sample. The mean value of fifteen successive estimations was used as the final motility score. Sperm concentration was determined with a hemocytometer [2]. Semen samples were not centrifuged.

DILUTION AND COOLING OF SEMEN

All ejaculates had $\geq 50\%$ progressively motile spermatozoa were immediately diluted in 3 different extenders (milk-, TRIS-based and glucose-lactose) and 3 spermatozoal concen-

trations (25, 50 and 75×10^6 spermatozoa in 1 ml of each extender) at 37 °C at anaerobic conditions. The components of these 3 extenders [4] are given in Table I. For this purpose, each ejaculate from each stallion was diluted in the 9 treatments based on spermatozoal concentration and extender type ;

Milk-Based Extender

Component	Quantity
Non-fat dry milk	2.4 g
Glucose monohydrate	4.9 g
Sodium bicarbonate (7.5% sol.)	2.0 ml
Polymixin B sulphate (50 mg/ml)	2.0 ml
Distilled water	92.0 ml

First the liquids was mixed and then the powders was added

TRIS-Based Extender

Component	Quantity
TRIS (hydroxymethyl) aminomethane	2.44 g
Citric acid monohydrate	1.36 g
Glucose	0.82 g
Egg yolk	20.0 ml
Polymixin B sulphate	100.000 IU
Distilled water	100.0 ml

Glucose-Lactose Extender

Component	Quantity
Glucose	30.0 g
Sodium citrate	1.85 g
Sodium EDTA	1.85 g
Polymixin B sulphate	100.000 IU
Sodium bicarbonate	0.6 g
Distilled water	100.0 ml

50 ml of this solution was added to 50 ml of 11% lactose solution, and then 20% egg yolk was supplemented.

TABLE I : The components of Milk-, TRIS-Based and Glucose-Lactose extender used in this study.

- diluted in 25×10^6 spermatozoa in 1 ml of milk-based extender
- diluted in 50×10^6 spermatozoa in 1 ml of milk-based extender
- diluted in 75×10^6 spermatozoa in 1 ml of milk-based extender
- diluted in 25×10^6 spermatozoa in 1 ml of TRIS-based extender
- diluted in 50×10^6 spermatozoa in 1 ml of TRIS-based extender

Number of Stallion	Age (year)	Semen volume [gel free, (ml)]	Gel volume (ml)	Sperm concentration ($\times 10^6$ sperm / ml)	Initial Sperm motility (%)
1	18	50.0 $\pm$ 5.7 ^{abc}	114.3 $\pm$ 2.3 ^d	109.3 $\pm$ 2.3 ^a	81.6 $\pm$ 1.6 ^{ab}
2	10	17.0 $\pm$ 2.0 ^a	28.3 $\pm$ 2.0 ^a	186.6 $\pm$ 3.3 ^{bc}	90.3 $\pm$ 1.6 ^a
3	10	29.6 $\pm$ 3.1 ^{ab}	29.3 $\pm$ 3.4 ^a	106.6 $\pm$ 4.4 ^a	83.3 $\pm$ 3.3 ^{ab}
4	16	35.3 $\pm$ 2.0 ^{ab}	68.0 $\pm$ 5.2 ^{bc}	208.3 $\pm$ 3.1 ^c	83.3 $\pm$ 1.6 ^{ab}
5	20	73.0 $\pm$ 1.5 ^{bc}	93.6 $\pm$ 2.6 ^{cd}	188.0 $\pm$ 4.1 ^{bc}	80.0 $\pm$ 0.0 ^b
6	11	56.6 $\pm$ 5.2 ^{abc}	62.6 $\pm$ 5.6 ^{abc}	309.3 $\pm$ 8.0 ^d	85.3 $\pm$ 1.6 ^{ab}
7	20	101.6 $\pm$ 5.1 ^c	92.0 $\pm$ 9.5 ^{cd}	99.6 $\pm$ 7.2 ^a	86.6 $\pm$ 1.6 ^{ab}
8	24	36.3 $\pm$ 2.8 ^{ab}	50.6 $\pm$ 1.4 ^{ab}	200.0 $\pm$ 2.8 ^c	80.0 $\pm$ 0.0 ^b
9	13	80.6 $\pm$ 0.6 ^{bc}	206.0 $\pm$ 12.2 ^e	170.0 $\pm$ 6.0 ^{bc}	86.6 $\pm$ 1.6 ^{ab}
10	14	30.3 $\pm$ 0.3 ^{ab}	48.3 $\pm$ 1.6 ^{ab}	287.3 $\pm$ 9.0 ^d	80.0 $\pm$ 0.0 ^b
Average	15.6 $\pm$ 1.5	51.1 $\pm$ 6.4	79.3 $\pm$ 9.6	185.7 $\pm$ 14.3	83.7 $\pm$ 3.5

- Different superscript letters (a, b, c, d and e) within same column indicate significant ($P < 0.05$) differences among stallions.

TABLE II : The mean values of age and spermatological characteristics of purebred Arabian stallions used in the present study.

f.-diluted in 75×10^6 spermatozoa in 1 ml of TRIS-based extender

g.-diluted in 25×10^6 spermatozoa in 1 ml of glucose-lactose extender

h.-diluted in 50×10^6 spermatozoa in 1 ml of glucose-lactose extender

i.-diluted in 75×10^6 spermatozoa in 1 ml of glucose-lactose extender

The semen:diluent ratios were 1:4 for 25×10^6 spermatozoa/ml sample, 1:2 for 50×10^6 spermatozoa/ml sample and 1:1.5 for 75×10^6 spermatozoa/ml sample. Diluted semen samples were removed from the incubator for storage and were rapidly cooled from 37 °C to 20 °C. The heat of diluted semen was reduced from 20 °C to 5 °C by dropping one degree at a minute, and then all the samples were stored in a refrigerator at 5 °C for 144 h [12, 25]. This process was made within a chamber containing the samples. Then the samples warmed up before evaluation after cooled preservation and percentage of motility was evaluated as described in the determination of initial sperm motility. The suspensions of sperm were re-sampled at 0, 2, 4, 24, 48, 72, 96 and 120th to determine the motility of the spermatozoa. All of these processes were conducted for each semen sample collected from same stallion.

DATA ANALYSES

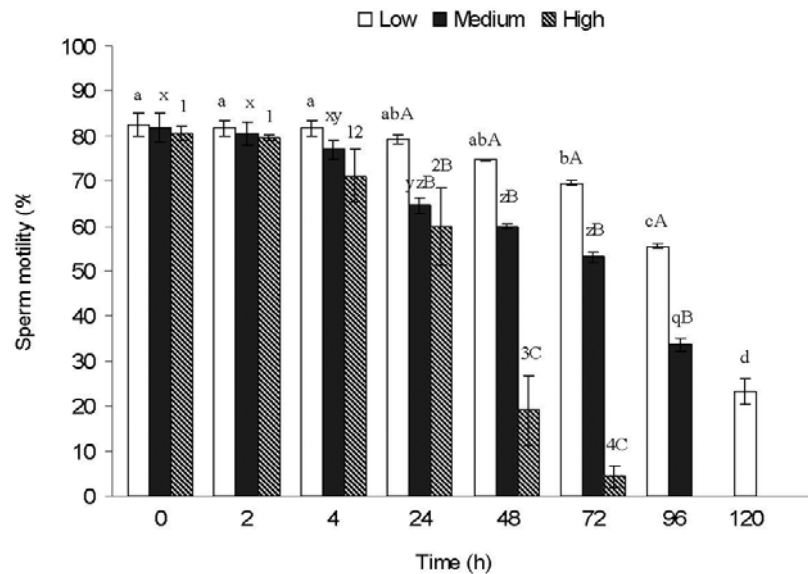
The data are presented as the mean $\pm$ Standard Error of Means (SEM). The degree of significance was set at $P < 0.05$. All initial spermatological characteristics and motility of semen diluted in different extenders and spermatozoal concentrations at every time were analyzed by General Linear Model (GLM) procedures to determine the effect of stallion, ejaculate, extender, spermatozoal concentration and time. Two-way analysis of variance for repeated measures and post-hoc Tukey-HSD test for multiple comparisons was

used to determine the differences in percentage of motility of semen diluted in different extenders and spermatozoal concentrations. The differences observed in the percentage of sperm motility among different time segment were evaluated by Paired sample t test. The relationship between spermatozoal concentration and sperm motility of diluted semen for each extender during examination period was evaluated with Pearson correlation test. All analyses were carried out using the SPSS/PC (Version 10.0) software package program [1].

Results

The initial semen quality of purebred Arabian stallions used in the present study is given in Table II. Although statistically significant individual differences ($P < 0.05$) were found in spermatological characteristics among stallions, no significant differences ($P > 0.05$) were observed in terms of these parameters among non-diluted semen samples collected from the same stallion. However, all ejaculates of each Arabian stallion had a mean initial motility $\geq 80\%$.

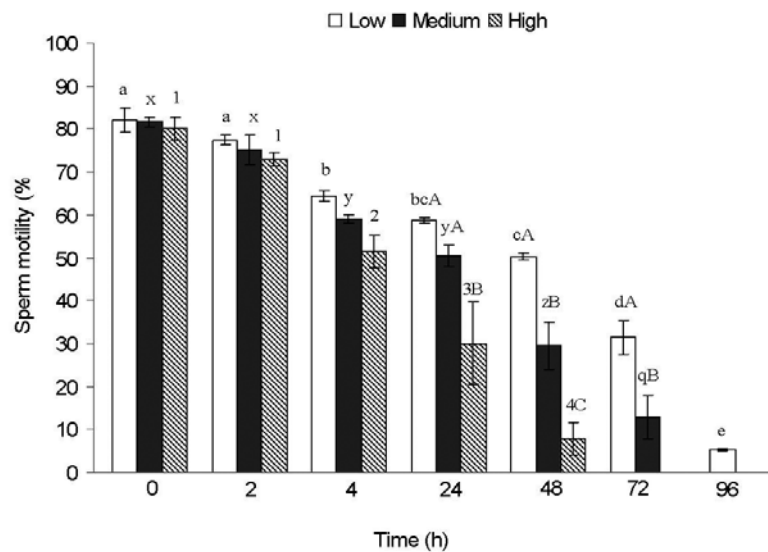
The time-dependent effects of milk-based, TRIS-based and glucose-lactose extenders on motility of stallion semen, which diluted with different spermatozoal concentrations, were illustrated in Fig 1, Fig 2 and Fig 3, respectively. Time-dependent significant differences were found in the motility in terms of spermatozoal concentration and extenders ($P < 0.05$, $P < 0.01$). The diluted stallion semen with low (25×10^6) spermatozoal concentration had greater motility and longevity than diluted semen with medium (50×10^6) and high (75×10^6) spermatozoal concentration for each extender during examination period at 5 °C. There were significant ($P < 0.01$) negative correlations among all spermatozoal concentrations and sperm motility of diluted semen belonging to each extender during all examination period.



The bars bearing different uppercases (A, B and C) show significant ($P < 0.01$) differences among different spermatozoal concentrations within the same time segment.

The bars bearing different lowercases (a, b, c and d; low spermatozoal concentration), (x, y, z and q; medium spermatozoal concentration) and numbers (1, 2, 3 and 4; high spermatozoal concentration) show the significant ($P < 0.05$) differences between different time points.

Figure 1. The time-dependent motility rates of stallion semen diluted with milk-based extender in low (25×10^6), medium (50×10^6) and high (75×10^6) spermatozoal concentration during examination period.



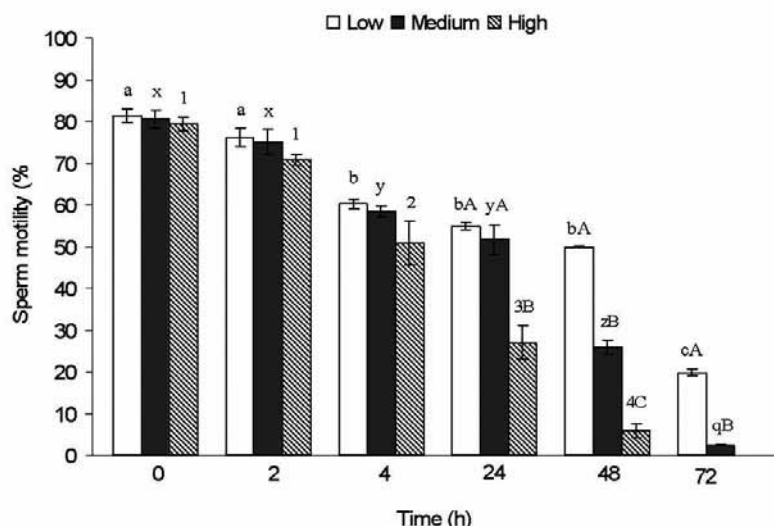
The bars bearing different uppercases (A, B and C) show significant ($P < 0.01$) differences among different spermatozoal concentrations within the same time segment.

The bars bearing different lowercases (a, b, c, d and e; low spermatozoal concentration), (x, y, z and q; medium spermatozoal concentration) and numbers (1, 2, 3 and 4; high spermatozoal concentration) show the significant ($P < 0.05$) differences between different time points.

Figure 2. The time-dependent motility rates of stallion semen diluted with TRIS-based extender in low (25×10^6), medium (50×10^6) and high (75×10^6) spermatozoal concentration during examination period.

The stallion spermatozoa diluted in milk-based extender had greater motility and longevity than diluted in TRIS-based and glucose-lactose extenders for each spermatozoal concentration at all times. Similarly, the motility of spermatozoa diluted in TRIS-based extender was also higher than diluted in glucose-lactose extender belonging to each spermatozoal concentration

at all times. However, differences between these 2 extenders were statistically insignificant. The semen diluted in low, medium and high spermatozoal concentrations protected its available motility (50%) up to 96, 72 and 24 hours within milk-based extender, and up to 48, 24 and 4 hours in both TRIS-based and glucose-lactose extenders, respectively.



The bars bearing different uppercases (A, B and C) show significant ($P < 0.01$) differences among different spermatozoal concentrations within the same time segment.

The bars bearing different lowercases (a, b and c; low spermatozoal concentration), (x, y, z and q; medium spermatozoal concentration) and numbers (1, 2, 3 and 4; high spermatozoal concentration) show the significant ($P < 0.05$) differences between different time points.

Figure 3. The time-dependent motility rates of stallion semen diluted with glucose-lactose extender in low (25×10^6), medium (50×10^6) and high (75×10^6) spermatozoal concentration during examination period.

Discussion

Currently, storage and cryopreservation protocols are not still standardized for stallion semen as bulls, and the success of preservation varies substantially. In general, the rates of success in fertility are lower when compared with other farm animals. One of the prerequisites for successful preservation of stallion semen is high initial quality [26]. The present study also showed that all ejaculates of each Arabian stallion had good initial semen quality.

Although the dilution process preserves sperm motility, unbalanced ratio of semen:extender may be harmful to fertility. The use of 1:3 or 1:4 dilution ratios (semen:extender) are widely accepted and employed. However, LOVE *et al.* [14] reported that the greatest percentage of motile sperm was 74%, which was found in the non-centrifuged stallion semen after diluted in Kenney extender at ratio 1:9. BOZKURT *et al.* [3] suggested that the motility of ram semen which diluted in egg yolk, sodium citrate, and glucose at the rate of 1:1 and refrigerated at 4 °C maintained for 96 h (49% motility). The motility of equine semen is influenced by the spermatozoal concentration within an extender and, the longevity of sperm during cool-storage may vary in different fractions of the ejaculate. High semen:extender ratios (low spermatozoal concentration) are known to reduce the deleterious effects of seminal plasma on cooled, stored spermatozoa [10]. It has been documented [24, 27] that to store the cooled semen, optimal concentration should be 25×10^6 sperm in 1 mL of extender. VARNER *et al.* [24] also reported that better motility rates were achieved with spermatozoa concentrations of 25×10^6 in 1 mL of diluents compared to both 50×10^6 and 100×10^6 in 1 mL of diluents. WEBB *et al.* [27] have also reported that different sperm concentrations in the skimmed milk and glucose extender

affect progressive motility. It has been suggested [11] that the motility rates of cooled semen diluted in 25×10^6 , 50×10^6 or 100×10^6 sperm in 1 mL of non-fat skimmed milk extender are equal for 24 hours. RIGBY *et al.* [20] reported that the motility of non-centrifuged semen which diluted at a ratio of 1:4 (approximately 60×10^6 sperm/mL) and 1:9 (approximately 25×10^6 sperm/mL) in KMT extender and stored for 48 h in an Equitainer-I, were 43% and 64%, respectively. For successful AI, 1 mL of diluted and cooled semen should contain 20–400 $\times 10^6$ sperm [6, 8, 9, 21] in each insemination dose. SHORE *et al.* [22] reported that the motility of diluted stallion semen including 500 and 250 million spermatozoa after 24 h storage was 59.5% and 50.5%, respectively. It has been reported [13] that the motility percentages of semen diluted in low and high spermatozoal concentrations are similar after cooled storage and/or after thawing.

There is no definitive view amongst workers about whether the spermatozoal concentration in the diluted semen for cool-storage should be low or high. In the present study, the diluted stallion semen with low spermatozoal concentration had greater motility and longevity than those of medium and high for each extender at all times during cool-storage at 5 °C. There were significant ($P < 0.01$) negative correlations between all spermatozoal concentrations and sperm motility of diluted semen belonging to each extender during all examination period. The extenders including low spermatozoal concentration have greater amounts of energy substrates, lipoproteins and phospholipids, which are necessary for per sperm and help to the protection of motility and life span of semen, than extenders containing high spermatozoal concentration. On the basis of this status and non optimal semen:diluent ratio, the time-dependent motility and longevity of semen diluted in low spermatozoal concentration was found higher than diluted in high spermatozoal concentration.

It is not possible to preserve semen for more than several hours without using the semen extender. Semen extenders prolong the longevity of spermatozoa by stabilizing enzyme systems and maintaining membrane integrity, protect spermatozoa from unfavourable environmental conditions such as cold shock, toxic by-products produced by spermatozoa, and prevent growth of micro-organisms [17]. Numerous studies have been carried out to compare extenders of varying compositions. In a study by PICKETT *et al.* [18] was reported that cream-gel extender was superior contained 2.4% Tris extender with 5% glycerol. HOUSEHOLDER *et al.* [8] reported that conception rates for cream-gel were lower than for both of the skim milk-extenderns and heated skim milk that preserved sperm motility better than unheated fresh skim milk. However, Kenney's extender, a non-fat dried milk solid - glucose extender, is superior to heated skim milk [17]. Egg yolk-TRIS and egg-yolk-bicarbonate are less satisfactory than heated skim milk in maintaining sperm motility at 5 °C [19]. Semen kept in glycine egg yolk extender at 5 °C survives less than semen in a skim-milk extender, when evaluation is based on motility and acrosome morphology [15]. Supplementing of a skim-milk glucose extender with a modified Tyrode's medium, which also contains BSA, to the semen maintains motility better than a skim-milk extender [16].

The results of the present study showed that the stallion spermatozoa diluted in milk-based extender had greater motility and longevity than diluted in TRIS-based and glucose-lactose (both extenders include egg yolk) extenders for each spermatozoal concentration at all times. There is an adverse reaction between seminal plasma and egg yolk leading to reduced spermatozoa survival in stallion semen that had been diluted with an extender including egg yolk [9]. This situation may be explained with the absence of egg yolk in the milk-based extender.

In conclusion, the stallion semen must be mixed with an appropriate extender that helps to the protection of spermatozoa, and spermatozoal concentration in the extender should be low level in order to gain maximum benefit from cooled stallion semen. Among the tested extenders the milk based extender preserved motility for a longer time.

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