



The effect of different extenders on the sperm motility and viability of frozen Turkey semen[#]

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ABSTRACT

This study was conducted to develop long-term storage methods for turkey semen using different extenders. During the study, the massage method was used twice a week to collect the semen from five turkeys, a total of 44 times. The collected fresh semen's average ejaculate quantity, sperm concentration, motility and vitality values were determined as 0.22 ± 0.01 ml, $3.5 \pm 0.17 \times 10^9$ sp/ml, $77.0 \pm 1.44\%$ and $86.2 \pm 0.95\%$ respectively. In this study, glucose (G) including 5% Dimethyl sulphoxide (DMSO) cryoprotective, tris-glucose (TG), lactated Ringer's (LR), and lactated Ringer's glucose (LRG) extenders were used. The Turkey semen was combined and divided into four equal parts, and subsequently diluted at a ratio of 1:3 and equilibrated at $+4^\circ\text{C}$ for 90 minutes. Following equilibration, the samples were frozen in liquid nitrogen vapor to -80°C for five minutes, and stored at -196°C in liquid nitrogen. After freezing and thawing, the highest motility value was obtained from the G extender ($43.3\% \pm 1.62\%$) followed by LRG ($24.6 \pm 1.53\%$), LR ($12.6 \pm 0.92\%$) and TG ($12.2 \pm 0.66\%$). The vitality values were recorded as $55.8 \pm 1.89\%$, $23.8 \pm 1.58\%$, $21.5 \pm 1.10\%$ and $36.5 \pm 1.59\%$ respectively. The motility and vitality values were significantly ($p < 0.01$) more for the glucose extender than those for other extenders. Therefore, it was concluded that glucose extender is better option for the long-term storage of Turkey semen.

Key words: Cryopreservation, Motility, Sperm, Turkey, Vitality.

INTRODUCTION

Sperm cryopreservation is made for the purpose of protecting species, clinical studies and developing assisted reproduction techniques, such as artificial insemination (AI), embryo transfer and *in vitro* fertilization (Medeiros *et al.*, 2002). Turkey semen should be protected for a long time in order to effectively use in AI studies. The ratio for Turkey semen collected using the massage method is higher than during natural breeding. For a short period, by diluting the semen with physiological extenders, it can be used for the purpose of AI (Akçay *et al.*, 1997). Measurements, such as ejaculate ratio, motility, vitality, abnormal defects, pH, and semen concentration should be made to determine semen quality (Alkan *et al.*, 2002).

Poultry semen has a very low resistance to the inner development of ice crystals. One of the most sensitive points of a good freezing curve is the transfer between temperatures, and these transfers are applied by freezing semen at a slow speed. Faster freezing is mostly applied to fish and mammals because this technique have little cellular damaging affects (Seigneurin and Blesbois, 1995). Different procedures may be required for certain species of poultry and these procedures can be differentiated in terms of certain factors, such as suitable timing, cooling, equalizing temperature,

diluter and cryoprotectant suitability (Lukaszewicz, 2001; Blesbois, 2007; Graham, 2007).

It is recommended that cryoprotectants with impermeable characteristics are used (Massip, 2001; Tarvis, 2013) to decrease crystallization in cellular water. Although there are studies in which various available cryoprotectant combinations have been tried (Long *et al.*, 2014). However, to our knowledge no researcher has yet established which cryoprotectant should be used and at which ratio.

Glucose based cryoprotectants are used to protect against intracellular crystal formation, which can occur at the conclusion of cell freezing, cold shocking, decrystallization following freezing/thawing, and membrane destabilization. The cell is exposed to cryoprotectants before being frozen, and the cell achieves intracellular equilibrium. Cryoprotectants are significant as they have low molecular weight and although their effect is toxic, they may be used at specific ratios (Palasz and Mapletopt, 1996; Bucak and Tekin, 2007; Tarvis, 2013; Tunali, 2014). The objective of this study is to evaluate the pre (in fresh semen) and post thaw (frozen semen) motility and viability of turkey semen using various extenders based on glucose.

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[#]This study has produced the first author of the master thesis prepared in consultation with the second author.

MATERIALS AND METHODS

Animal materials: This study was conducted with the permission of Ahi Evran University Animal Experiments Local Ethics Committee (AEU-HADYEK). In this study, five one-year-old American bronze turkeys (*Meleagris gallopavo*), raised at Ahi Evran University Agriculture Faculty Poultry Unit, were used. The turkeys were sheltered in closed pens 2m x 6m and 2m x 18m open divisions enclosed with poultry netting, and could go in and out whenever they wanted. The birds were fed with turkey breeder ration.

Collecting the semen: Abdominal massage was performed in turkeys three to four weeks before the study commenced with the purpose of adapting the turkeys to giving semen using the dorso-abdominal massage method. The semen was stripped from turkeys twice a week using the massage method. To avoid the stripped semen becoming mixed with feces another researcher collected it. The collected semen was preserved at an ambient temperature of 37°C until dilution.

Preparation of extenders

Four different extenders were prepared as follows:

1. Glucose extender (G): 0.37M D(+)-Glucose-monohydrate into 5%(v/v) Dimethyl sulfoxide (DMSO) (370 mOsm/L).
2. Tris+glucose extender (TG): 30mM/L Tris (hydroxymethyl)-aminomethane + 300mM/L D(+)-Glucose-monohydrate into 5% (v/v) DMSO.
3. Lactated ringer extender glucose free (LR): 0.31 g sodium lactate, 0.03 g potassium chloride, 0.02 g calcium chloride, 0.60 g sodium chloride, for 100 ml distilled water into 5%(v/v) DMSO.
4. Lactated Ringer+Glucose extender (LRG): 0.31 g sodium lactate, 0.03 g potassium chloride, 0.02 g calcium chloride, 0.60 g sodium chloride, for 100ml distilled water + 1 g D(+)-Glucose-monohydrate) into 5%(v/v) DMSO.

Determination of spermatological characteristics: Semen volume (ml): Ejaculate was collected with 1 ml plastic syringes. Volume of ejaculate was recorded in ml.

Sperm Motility (%): It was recorded in fresh semen by diluting with 1:2 serum physiological (SF), for better observations. No extra dilution was required for the post-thaw motility. Motility was examined under three different microscope by means of counting at least 200 progressively sperm for each one, considering the sperm's mobility with 400x magnification in a heating staged microscope, and stated as percentages.

Sperm concentration: A hemocytometer was used to identify sperm concentrations of the semen samples. Some of the collected semen was diluted with Hayem solution (Tekin, 1990) to become 1:250. The numbers of spermatozoa in five large squares at diagonal of the chamber were counted in 400x magnification using. Sperm concentration was $\times 10^9$ sp/ml and was calculated using the hemocytometrical counting equation. The hemocytometrical counting equation is following: Concentration (μ l) = counted spermatozoa / great square area \times great frame height \times dilution rate.

Sperm Vitality (viability): Examination of live/dead sperm ratio for ejaculate was carried out via Eosin-Y staining method (Kadioglu, 2011). A 5 μ l semen sample mixed at the rate of 1:1 Eosin-Y stain was placed on the slide to and covered with lamella. After preparation and waiting for 30 seconds, the live/dead ratio was calculated using 100x magnification and the vitality ratio was calculated at least 200 spermatozoa under phase contrast microscope at the third field. There was no color change in the living sperm but it determined that for dead sperm Eosin-Y stained sperm head (Fig. 1). Vitality values evaluated in three different categories, as fresh, extended and post-thaw semen.

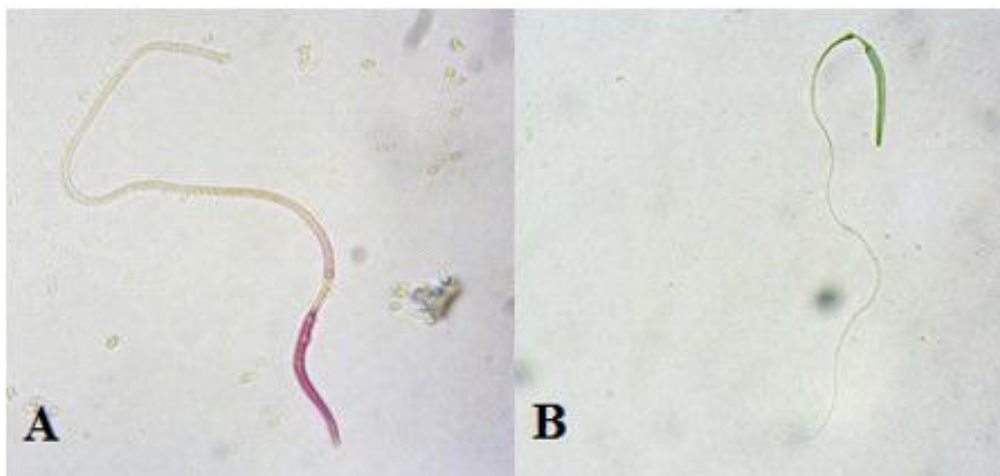


Fig 1: Stained dead (A) and non-stained living (B) Microscopic view of sperm

Dilution of semen and equilibration: After the general spermatological characteristics of fresh semen belonging to each turkey, all the semen samples were pooled. The pooled semen was placed in four different tubes, so their volumes were equal. Previously prepared G including 5% DMSO TG, LR, and LRG extenders were added every tube at a ratio of 1:3 (Akçay *et al.*, 2006) (1 part semen: 3 part extender). Following the dilution of the semen, the motility and vitality values were recorded and they were transferred to the paillette, where they were equilibrated at +4°C for 90 minutes.

Freezing and thawing of semen: After being diluted, the semen remained in the paillettes for 90 minutes at +4°C, then they were frozen for five minutes in nitrogen vapor to -80°C. The paillettes were taken from the nitrogen vapor and stored at -196°C in liquid nitrogen. After freezing, paillettes were taken out from the liquid nitrogen and held at 37°C for five seconds for thawing.

Statistical analysis: The data was analyzed using the statistical software program SPSS 15.0 for Windows. A one-way ANOVA test was used to compare the groups. All results were given as means \pm standard deviation. Differences of $p < 0.05$ were considered to be significant, $p < 0.01$ highly significant and $p > 0.05$ not significant.

RESULTS AND DISCUSSION

Fresh semen characteristics: Mean \pm SEM values of various semen parameters in different fresh ejaculates of turkey is presented Table 1. The fresh semen characteristics of different ejaculates collected from individual turkey were non-significantly different. The mean \pm SEM values for semen volume, sperm concentration, motility and vitality were 0.22 ± 0.01 ml, $3.5 \pm 0.17 \times 10^9$ sp/ml, $77.0\% \pm 1.44$ and $86.2\% \pm 0.95$, respectively.

The volume of the semen collected from turkey varied from 0.16 to 0.4 ml in previous studies (Akçay *et al.*, 2006). The average semen volume obtained in current study was 0.22 ± 0.04 ml which was within range as obtained by

other researchers. The reason for the differences between the fresh semen values can be determined by factors such as the individual's age, breed, stripping frequency, nutrition, light, temperature, and genetics, all of which have an effect on turkeys (Turkoglu *et al.*, 2005; Bakst and Dymond, 2015). Akçay *et al.*, (2006) found sperm concentrations of $5.1 \pm 1.12 \times 10^9$ sp/ml in their study, Ilgaz and Tekin (1990) found it to be $5.03 \pm 0.34 \times 10^9$ sp/ml. The results in current study established the average sperm concentration to be $3.5 \pm 1.13 \times 10^9$ sp/ml, which was lower than the results obtained from other researchers.

Akçay *et al.*, (2006) reported motility of fresh sperm to be $84.8 \pm 0.34\%$ and Ilgaz and Tekin (1990), reported it to be $67.69 \pm 2.36\%$. Average motility was found to be $77.0 \pm 9.56\%$ in this current study, higher than findings obtained from Ilgaz and Tekin (1990). The result was lower than those obtained from Akçay *et al.*, (2006).

Rosato *et al.*, (2012) obtained an average vitality of 84.48 ± 0.45 from turkey fresh sperm. According to the results of the current study, the general average vitality value was $86.2 \pm 6.28\%$, while it was found to be compatible with and higher than results obtained from Rosato *et al.*, (2012). These difference may be explained by breed, location, nutrition, age and climate differences because the sperm parameters can vary depending on these factors.

Diluted semen characteristics: Average motility values after dilution is presented Figure 2. The fresh semen collected during the study was diluted in four different extenders and the Ringer-Glucose extender had the highest average motility value ($83.5 \pm 1.65\%$) following dilution.

In their study, Akçay *et al.* (2006) obtained the highest motility value after dilution with $84 \pm 2.2\%$ for Ringer extender using 10% DMSO. In their study about local, white and hybrid turkeys, Pandian and Prabakaran (2012) reported average motility values as $72.18 \pm 0.20\%$ with 9% glycerol, as $68.71 \pm 0.12\%$ with 5% ethylene glycol, as $65.08 \pm 0.07\%$ with 7% ethylene glycol. Kowalczyk and Lukaszewicz

Table 1: Mean \pm SEM values of various semen parameters in different fresh ejaculates of turkey

Stripping Number	Semen Volume	Sperm Concentration	Sperm Motility	Sperm Vitality
1.	0.21 ± 0.02	2.70 ± 0.44	83.6 ± 3.23	89.0 ± 2.51
2.	0.23 ± 0.02	2.92 ± 0.10	82.8 ± 1.16	89.2 ± 0.80
3.	0.22 ± 0.02	3.72 ± 0.27	71.0 ± 4.43	80.8 ± 2.69
4.	0.24 ± 0.02	4.56 ± 0.43	77.8 ± 3.50	86.8 ± 3.44
5.	0.21 ± 0.02	3.01 ± 0.42	67.2 ± 5.74	85.0 ± 3.51
6.	0.21 ± 0.01	3.95 ± 0.70	75.8 ± 6.01	87.2 ± 3.41
7.	0.22 ± 0.02	3.40 ± 0.74	74.2 ± 3.81	84.2 ± 3.47
8.	0.21 ± 0.00	4.30 ± 0.75	79.0 ± 2.08	85.7 ± 2.96
9.	0.23 ± 0.03	2.97 ± 0.22	82.7 ± 2.60	89.7 ± 0.88
10.	0.21 ± 0.01	3.57 ± 0.48	80.7 ± 3.48	85.7 ± 4.06
General average	0.22 ± 0.01	3.5 ± 0.17	77.0 ± 1.44	86.2 ± 0.95
Time	F 0.488	1.143	1.178	0.554
	p 0.864	0.384	0.365	0.816

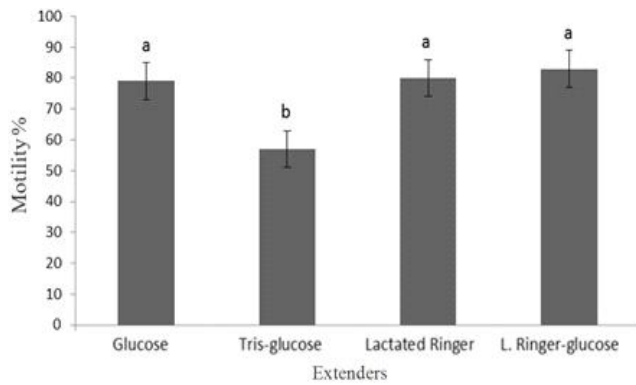


Fig 2: Average motility values after dilution. (Time: $F= 2.032$; $p=0.057$ Time x Extender: $F= 1.186$; $p=0.300$ Extender: $F= 41.133$; $p=0.001^{**}$)

(2012) determined values of $50.0\pm 7.0\%$ and $55.0\pm 4.0\%$ in two different experiments in their study about cocks using 6% DMSO. Roushdy *et al.*, (2014) reported the best result was $59.23\pm 0.54\%$ with 3% DMA in their study about two different breeds of chicken. Akcay *et al.*, (2006) used sugar-based extenders at their study, and reported the highest average motility value was $91.3\pm 2.6\%$ after dilution. In current study, the highest motility value was found to be LRG as $83.5\pm 9.04\%$ after dilution. According to results the highest average motility value after dilution was similar with findings obtained from other researchers.

Average vitality values after dilution is presented Figure 3. The time x extender and the differences between the time were found to be statistically significant ($p<0.01$)

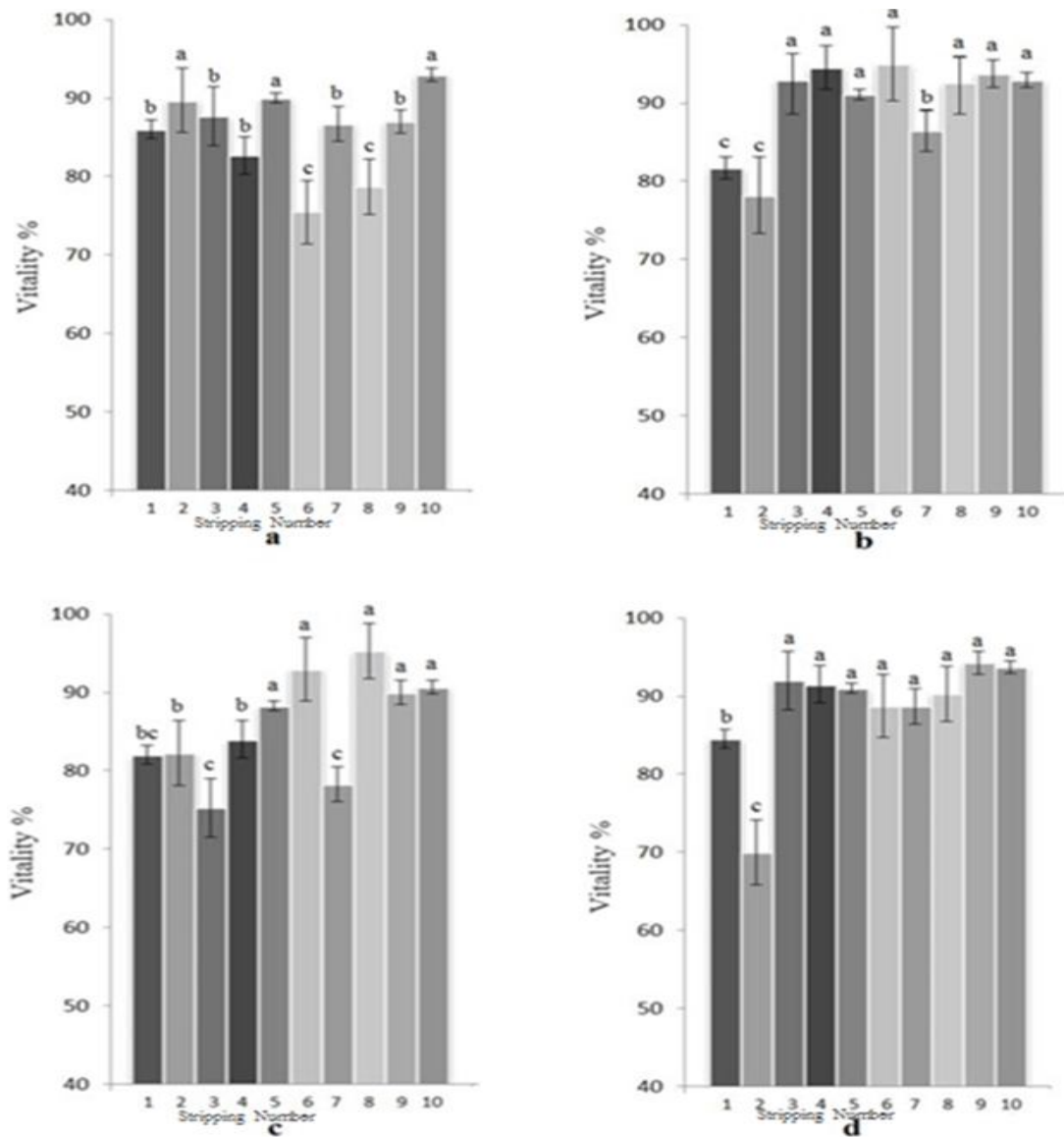


Fig 3: Average vitality values after dilution. a. G b. TG c. LR d. LRG vitality values after dilution (Time: $F= 5.161$; $p=0.000^{**}$, Time x Extender: $F= 2.844$; $p<0.001^{**}$ Extender: $F= 0.855$; $p=0.521$)

in terms of vitality values belonging to different extenders following dilution there was no statistical difference between the extenders. The differences in terms of motility and vitality values were found to be significant (as $p < 0.01$) amongst various extenders used.

Kowalczyk and Lukaszewicz (2012) determined $83.2 \pm 7.0\%$ and $80.7 \pm 7.4\%$ values at two different experiment in their study about cocks that used EK extender (developed by Łukaszewicz) added 6% DMSO. Roushdy *et al.*, (2014) reported the best result was $59.43 \pm 0.57\%$ with 3% DMA. Lukaszewicz (2001) found $92.56 \pm 1.96\%$ for filtrated semen, and $93.83 \pm 2.62\%$ for unfiltered semen with DMA cryoprotectant in their study about cocks. According to obtained data the highest average vitality value was $89.1 \pm 6.66\%$ for LRG, and it was found to be similar or higher than values obtained from researchers.

After freezing-thawing: Average motility and vitality values after freezing-thawing is presented Figure 4. In terms of belonging to different extenders after freezing-thawing, time x extender and the differences between the extenders were not found to be statistically significant. The highest motility value was obtained from the glucose extender ($43.3\% \pm 1.62\%$) followed by LRG ($24.6 \pm 1.53\%$), LR ($12.6 \pm 0.92\%$) and TG ($12.2 \pm 0.66\%$). Statistically, the difference between times was not significant. The highest and lowest mean \pm SEM motility was $26.1 \pm 4.81\%$ and $21.0 \pm 3.34\%$ respectively at the first and sixth strip, respectively. The differences between times x extender were not statistically significant.

Akçay *et al.*, (2006) used 10% DMSO cryoprotectant in addition to G extender (300 mOsm/L), and obtained the highest motility value as $40.5 \pm 2.8\%$ in their study. Current study, 5% DMSO was used in addition to G extender (370 mOsm/L), and the highest value obtained was $43.3 \pm 1.62\%$ motility. Makhafola *et al.*, (2009) determined motility value after freezing-thawing according to 0, 30, 60 and 90 min holding time after thawing in their study

conducted using 5% DMSO about three different breeds of cock. Their results are nearly similar with current study' results. Pandian and Prabakaran (2012) reported average motility values to be $28.88 \pm 0.09\%$ with 9% Glycerol, as $16.47 \pm 0.10\%$ with 5% Ethylene Glycol, and $19.77 \pm 0.09\%$ with 7% Ethylene Glycol. Keskin *et al.*, (1995) determined $36.7 \pm 2.6\%$ value for BPSV-1, $41.7 \pm 2.1\%$ for BPSV-2, $46.7 \pm 1.4\%$ for Ringer-1, $44.2 \pm 1.9\%$ for Ringer-2 respectively in their study carried out using Glycerol (5% and 8%) and DMSO (5% and 4%) cryoprotectants. Kowalczyk and Lukaszewicz (2012) determined $30.0 \pm 10.0\%$ motility in the experiment EK extender was used adding 6% DMF in their study about cocks. Roushdy *et al.*, (2014) stated the best result to be $36.07 \pm 1.16\%$ with 7% Glycerol in their study about two different chicken breeds. The results obtained in the current study were found to be similar or higher than those obtained by researchers.

The highest average vitality value was $55.8 \pm 1.89\%$ for the G extender, followed by LRG ($36.5 \pm 1.59\%$) for, and TG extender ($23.8 \pm 1.58\%$). The vitality was obtained minimum for LR extender ($21.5 \pm 1.10\%$) in current study. The research by Lukaszewicz (2002) on frozen semen, using EK extender added 6% DMF, reported a vitality value after freezing-thawing of 34.7%, and a normal sperm ratio as 14.1%. Kowalczyk and Lukaszewicz (2012) determined $53.5 \pm 7.5\%$ vitality value in their experiment using EK extender added 6% DMF in their study about geese.. Roushdy *et al.*, (2014) reported $37.02 \pm 1.13\%$ as their best result with 7% Glycerol in their study about two different breeds of chicken. Tai *et al.*, (2001) reported vitality value after freezing-thawing as $27.0 \pm 3.2\%$, $7.3 \pm 4.7\%$ for 9% DMA and 4% DMSO, respectively. Rosato *et al.* (2012) stated vitality values after freezing-thawing as $36.2 \pm 2.92\%$ in their study using DMSO and lycopene. According to the results in current study it was found similar or higher than the other researchers' findings.

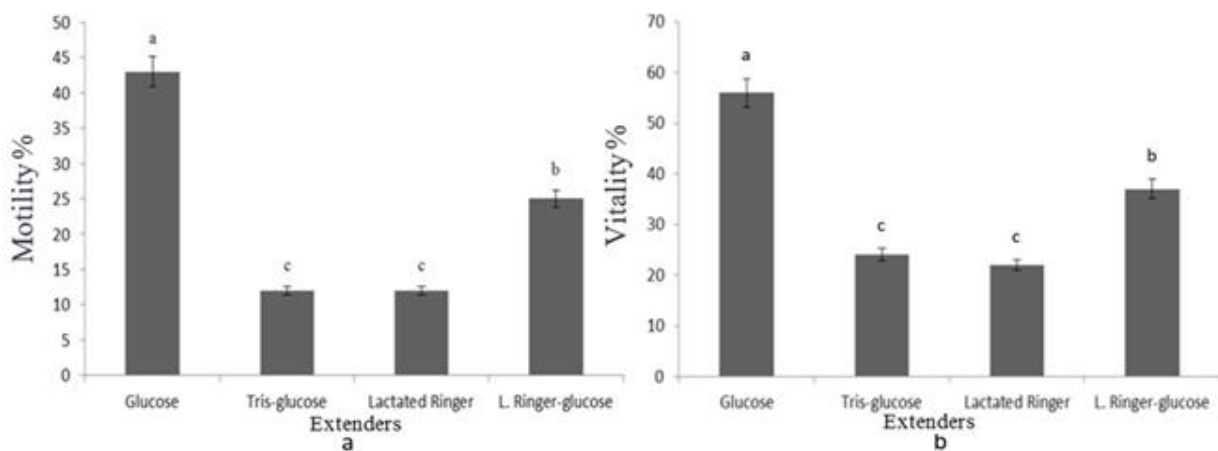


Fig 4: After freezing-thawing, a Motility, b Vitality (Time: $F = 1.202$; $p = 0.317$, Time x Extender $F = 1.316$; $p = 0.204$, Extender $F = 146.172$; $p = 0.000^{**}$)

CONCLUSION

It was determined that LRG extender had the highest average motility value after dilution, followed by LR as the second extender with the highest motility value. However G was the extender with the highest average motility value after freezing-thawing. It was concluded that further experiments should be conducted with modified extenders, either alone or using other chemicals, for the purpose of determining these extenders' effectiveness and the results should be examined to see whether or not they are at a sufficient level for the turkey meat sector. Moreover, it was determined that these extenders were not only high for motility values, but also high enough for vitality values following dilution. The high ratios obtained show that the extenders could meet the semen's requirements. In conclusion, it was determined

that the Glucose (370 mOsm/L) extender including 5% DMSO, was better in terms of motility and vitality than the other extenders used in this study. However, fertility trials are required to confirm the effectiveness of glucose extender at. Besides this factors, which can affect motility directly, such as extender variety, cryoprotectant variety, equilibration time and freezing-thawing time should also be considered.

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