Influence of extenders and cooling rates on epididymal sperm of Lewis rat strain

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Summary: The objective of this study was to determine the appropriate cooling rates and extender for improving the freezability of epididymal sperm of Lewis rat strain. The epididymal sperm from Lewis rats were suspended in different freezing extenders which were HEPES buffered Tyrode's lactate (TL-HEPES), modified Kreb's Ringer bicarbonate (mKRB), 3% dehydrated skim milk (SM), Salamon's Tris-citrate (TRIS), and TES/Tris (TES). The extenders contained 20% (v/v) egg yolk (EY), 0.75% equex paste (EP) and 0.1 M raffinose or 0.1 M sucrose. The sperm samples were frozen by automated freezer, using various cooling rates (10, 40, 70, and 100 °C/min). After thawing, sperm samples were examined for integrity of plasma and acrosomal integrity, and mitochondrial membrane potential (MMP). Type of extenders had significant effect on cryosurvival ($p<0.05$). The post-thaw highest motility (27.5%) was achieved for the sperm samples frozen in the presence of TES extender ($p<0.05$). As a result, although cooling rate did not significantly affect cryopreservation, the extenders affected post-thaw sperm parameters and TES considerably improved the freezability of epididymal Lewis rat sperm.

Key words: Cooling rate, epididymal sperm, extenders, freezing, Lewis rat strain.

Lewis ırkı rat sperması üzerine değişik sulandırıcı ve dondurma hızının etkisi

Özet: Çalışmanın amacı Lewis ırkı rat sperması için, uygun dondurma hızı ve sulandırıcılar tespit ederek spermanın dondurulabilirliğinin artırılmasıdır. Ratlardan alınan epididimal sperma HEPES buffered Tyrode's lactate (TL-HEPES), modified Kreb's Ringer bicarbonate (mKRB), %3 süt tozu (SM), Tris-citrate (TRIS) ve Tris/TES (TES) solüsyonları ile sulandırıldı. Sulandırıcılara 20% (v/v) yumurta sarısı, 0.75% Equex Paste ve 0.1 M rafinoz yada 0.1 M sükroz ilave edildi. Sperma örnekleri kontrollü dondurucu kullanılarak değişik soğutma hızında (10, 40, 70 ve 100 °C/dk) donduruldu. Dondurma sonrası spermalar plazma, akrozom ve mitokondri membran bütünlüğü yönünden değerlendirildi. Sulandırıcı tipinin dondurmada etkili olduğu saptandı ($p<0.05$). Dondurma-çözüm işlemi sonrası en yüksek motilite (%27.5) TES sulandırıcısından elde edildi ($p<0.05$). Sonuç olarak, dondurma hızının sperm dondurulmasına önemli bir etkisi tespit edilemese de, sulandırıcıların dondurma-çözüm sonrası sperm parametrelerini etkilediği ve TES'in epididimal Lewis rat sperması dondurulabilirliğini, önemli derecede geliştirdiği saptandı.

Anahtar sözcükler: Dondurma, dondurma hızı, epididimal sperma, sulandırıcı, Lewis rat ırkı.

Introduction

Rats have been used for biomedical and genomic research for long time and a useful model for human disease (26, 44). Currently, many inbred mutant and genetically modified rat strains are not available to researchers, and many mutant rat strains have been lost because of inadequate resources to maintain breeding population (1). Hence, cryopreservation and banking of embryos, spermatozoa, oocytes and reproductive tissues are important for the protection of rat strains (1, 33). Cryopreservation of spermatozoa is a simple and more economical alternative compared to cryopreservation of embryos, and recommended for the maintenance of transgenic and mutant rat strains (32). Rat sperm cryopreservation and in vitro fertilization (IVF) are still under development (17, 18, 31, 52). Up to now,

acceptable and repeatable sperm cryopreservation protocol in rat has not been achieved compared to other mammalian species (31, 52). The difficulty of freezing rat sperm is probably due to extreme sensitivity of membrane integrity and mitochondrial membrane to freezing process (46). Also an acceptable motility and fertility rate of rat sperm have not been reached (17, 18, 31, 46, 52, 54), compared with mouse sperm (29, 43).

Spermatozoa of different species have marked differences in size, morphology, membrane phospholipids and metabolism. These differences reflect the high level of variation between species, and in some cases between individuals of the same species. Despite species variation, sperm freezing protocol has common stages (9). Rodent spermatozoa have unusual long tail, head shape and membrane composition characteristics

compared to domestic animals (11, 15, 19) and have extreme sensitivity to suboptimal conditions such as centrifugation, pipetting, chilling, and osmotic stress (22, 31, 40, 41, 47). Also it is known that the shape and the size of the sperm head could define its cryosensitivity (13). Therefore sperm preservation protocols are vary among animal species (5, 9).

During the cryopreservation process, spermatozoa exposed damaging factors such as extremely low temperatures, osmotic and toxic stresses factors and the formation and dissolution of ice in the extracellular environment (50). Successful cooling or freezing of sperm depend on storage temperature, cooling rate, thawing rate, chemical composition of the extender, reactive oxygen species (ROS) and seminal plasma composition (6, 10, 28, 51). Cooling rate has significant effect on post-thaw sperm viability (51) and should not be slower or faster than optimum rate in order to avoid an irreversible damage to sperm (12, 25, 27). The optimal cooling rate was reported to between 76 and 140°C/min in bull (51), 30°C /min in boar (12), 27°C-130 °C /min in mouse (25) and 10°C/min for human (16). An appropriate cooling rate for rat sperm has been reported to be 40 and 100°C/min in F344 and SD rats (46). Similarly one study (14) found optimum cooling rate between 53 and 70°C/min in SD rats. But there is no data on sperm of Lewis rat strain has been reported.

The optimal extender composition for each species should be determined in order to develop superior cryopreservation protocols (13, 35, 49). Rodent spermatozoa have different characteristics compared to domestic animals (11, 15, 19) and thus species-specific extender is needed for cryopreservation rat sperm. TES and mKRB containing 20% EY, 0.75% EP and either 0.1 M sucrose or raffinose was reported as useful extenders for rat sperm cryopreservation (23, 46, 53) although Tris–citrate, skim milk, egg yolk (EY), lactose, and N-tris (hydroxymethyl) methyl-2-aminoethanesulfonicd acid (TES) are most commonly used semen extenders (39). On the other hand TL-HEPES has also been reported to be a suitable extenders for sperm diluting in rats (47). TRIS is also a well-known diluent (39) and has a good potential for rat sperm handling and chilling (47). Mouse sperm is successfully cryopreserved with a basic freezing medium consisting of 18% raffinose and 3% skim milk (29). However skim milk has been demonstrated to be inefficient in rat strains (46). Another important chemicals for rat sperm cryopreservation is sodium dodecyl sulphate (SDS)-based products, which

play important role to improve the effectiveness of EY during sperm freezing in rat (31, 46, 53) mouse (37), cat (4), dog (36), and pig (8). Glycerol is the most common cryoprotectant used to freeze sperm from different species (38, 39). However, raffinose is an effective cryoprotectant for mouse and rat (24, 34, 52). Also sucrose has a cryoprotective capability similar to raffinose in rat (46).

In this study, we performed to determine appropriate nonpermeating CPA, extender and cooling rate to improve post thaw Lewis rat sperm viability. Also, this study designed for applying previous experience (46) and shows genetically differences of Lewis rat sperm in freezing.

Materials and Methods

All chemicals were purchased from Sigma (St Louis, MO) unless otherwise stated.

Animals and sperm collection: Six Lewis rats, 10 to 12 weeks of age were used as sperm donors. The rats were housed in accordance with the policies of the University of Missouri Animal Care and Use Committee and the Guide for the Care and Use of Laboratory Animals (20). The epididymal sperm was collected as described by Varisli et al. (46). The final concentrations of sperm samples were about $19-25 \times 10^6$ sperm/ml. Each experiment was performed by using a sample from a single donor.

Preparation of sperm extenders: Five extenders—TL-HEPES (7), Modified Kreb's Ringer bicarbonate (mKRB) (45, 52), Skim milk (SM), Tris–citrate (39) and TES (47)—were used. The osmolalities of the extenders were adjusted to 290-330 mOsm and determined by using a vapor-pressure osmometer (Vapro 5520, Wescor, Logan, UT). To obtain freezing extender, 0.1 M raffinose was added to extenders.

Centrifuged Egg yolk (20%; v/v) was prepared as described (46) and added to the each extender. Egg yolk phospholipids were then solubilized by adding 0.75% (v/v) Equex-Paste (Minitüb, Tiefenbach, Germany) to the extender.

Sperm freezing and thawing: Sperm samples (100 µl) from Lewis rats were transferred into 1.5 ml centrifuge tubes containing 400 µl of each freezing extender and gently mixed. After dilution, motility analysis was performed. The sperm samples were then equilibrated at 4°C for 45 min and cryopreserved by Linkam cryostage (TMS- 94) at various cooling rates (10, 40, 70 and 100 °C/min) (46). After warming (46), motility analysis was

Table 1. Extender groups.
Tablo 1. Sulandırıcı grupları.

Extenders	TL-HEPES	mKRB	TRIS-R	TRIS-S	TES-R	TES-S
CPA	0.1 M sucrose	0.1 M raffinose	0.1 M raffinose	0.1 M sucrose	0.1 M raffinose	0.1 M sucrose

performed and the samples were transferred into 1.5 mL Eppendorf tubes containing 150 µL TL-HEPES base solution. All samples were underwent mitochondrial, acrosome and membrane integrity assessment.

Assessment of sperm motility: The percentage of motile spermatozoa was determined visually by direct observation and using a phase contrast microscope (Nikon Eclipse 600) equipped with an electrically heated (37 °C) stage.

Evaluation of sperm plasma membrane, acrosomal integrity and mitochondrial membrane potential (MMP): Sperm membrane and acrosomal integrity, and mitochondrial membrane potential (MMP) were assessed by SYBR-14/Propidium iodide (*Live/Dead sperm viability kit, catalog no: L-7011, FertiLight, Molecular Probes, Eugene, OR, USA*), Alexa Fluor-488-PNA conjugate (*catalog no: L-21409, Molecular Probes, Eugene, OR, USA*) and JC-1 (*M34152, Molecular Probes Inc*.), respectively. For fluorescent staining, Nikon Eclipse 600 using a dual fluorescence filter was used. To evaluate sperm by fluorescent staining, thawed sperm samples were washed to remove freezing extender. After thawing, 100 µl sperm sample centrifuged at 200g for 3 min than the supernatant was removed and 320 µl TL-HEPES was added to the tube containing the sperm pellet to resuspend the sperm pellet by gentle rotation of the tube. The detection of sperm membrane and acrosomal integrity and mitochondrial membrane potential (MMP) were established as described by Si et al. (41) and Varisli et al. (46).

Statistical analysis: Statistical analyses were performed using SPSS software (version 11.5 for Windows; SPSS Inc., Chicago, IL). Parametric data were analyzed by analysis of variance (Two-Way ANOVA) and if there were significant differences, Tukey test for multiple comparisons was used for post hoc analysis. The non-parametric data were analyzed by Kruskal-Wallis. Statistical significance was set at $p < 0.05$. Values were presented as the mean ± standard error of the mean (SEM).

Results

After dilution, the percentages of motile spermatozoa ranged from 58.3% to 75.0%. After equilibration, motility lost was seen very few and motility were from 48.3% to 70.0%. Post-thaw, the highest and the lowest motility were found in TES-S (27.5%) at 100 °C cooling rate and TL-HEPES (3.2%) at 10 °C cooling rate (Figure 1). While TES extender provided better motility compare to the others extenders, cooling rates did not affect sperm recovery ($p>0.05$).

Figures 2 and 3 show the mean percentages of acrosome integrity and mitochondrial membrane potential of frozen-thawed Lewis rat sperm were subjected to different cooling rate. Post- thaw membrane integrity ranged between 5.7% and 13.8%. After freezing and thawing, there was an overall decrease in acrosome integrity and mitochondrial membrane potential. But severe decline was detected in MMP. The cooling rate did not affect of sperm parameters except acrosome integrity in TRIS-S group and acrosome integrity ranged between 17.3% and 39.3%, also the extender affected sperm parameters significantly ($p<0.05$). Post-thaw sperm membrane integrity was seen lower than motility except TL-HEPES.
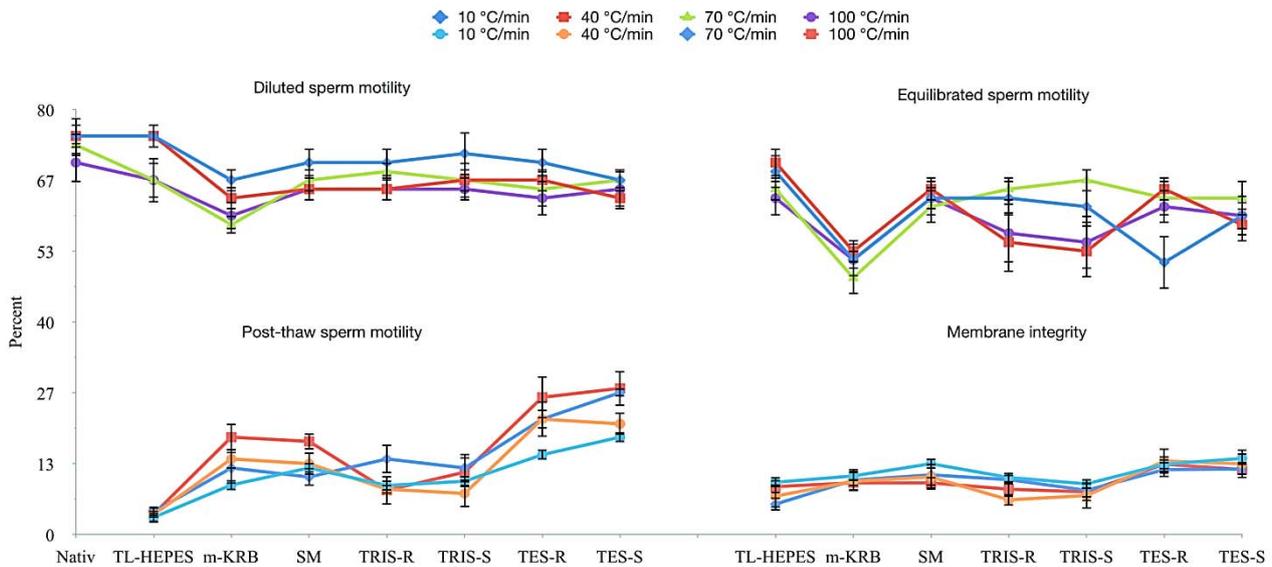

Figure 1. Motility (diluted, equilibrated and frozen-thawed) and membrane integrity of Lewis rat sperm in various extenders and at cooling rates.
Şekil 1. Lewis rat spermasının değişik sulandırıcı ve dondurma hızında, motilite (sulandırma, alışım ve dondurma sonrası) ve membran bütünlüğü.
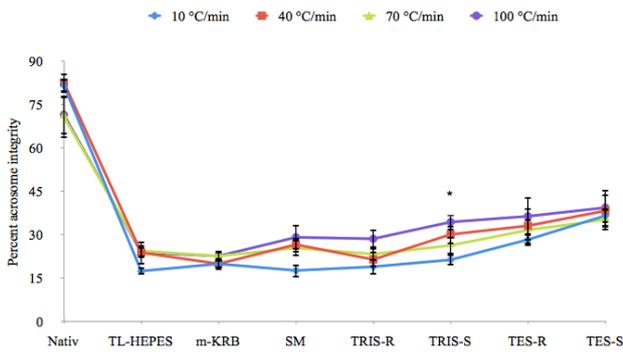
Figure 2. Percent post-thaw intact acrosome integrity of Lewis rat sperm using various cooling rates and freezing extenders (values are mean percentages ± SEM, n = 6; * Differences between the same lines are significant, p<0.05)
Şekil 2. Lewis rat spermasının değişik sulandırıcı ve dondurma hızında, dondurma sonrası sağlam acrozom bütünlüğü (değerler, ortalama yüzde± SEM, n=6; *Aynı sütündaki faklılıklar önemlidir)
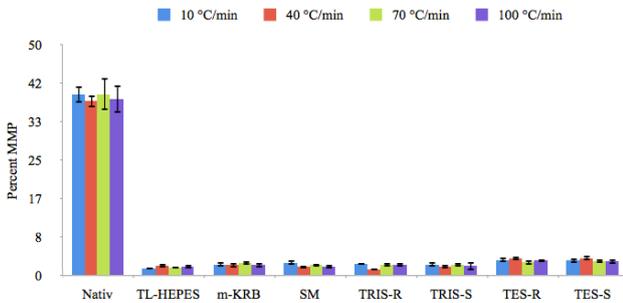

Figure 3. Percent post-thaw mitochondrial membrane integrity (MMP) of Lewis rat sperm using various cooling rates and freezing extenders (values are mean percentages ± SEM, n = 6).
Şekil 3. Lewis rat spermasının değişik sulandırıcı ve dondurma hızında, dondurma sonrası mitokondrial membrane potansiyeli (değerler, ortalama yüzde± SEM, n=6)

The greatest disruption was shown in mitochondrial membrane potential compare to motility, membrane and acrosome integrity. The cooling rates between 10 °C and 100 °C did not affect post-thaw sperm parameter (p>0.05). The sperm samples that were frozen in TES exhibited higher sperm motility when compared to those frozen in others extenders (p<0.05).

Discussion and Conclusion

Sperm freezing procedures need to be optimized for species (2, 3, 38, 39, 51) and also for strains. Studies demonstrated that post-thaw sperm quality was highly variable between mouse strains (30, 43). Effective freezing medium consisting of 18% raffinose and 3% skim milk without any permeating CPA has been found successful for sperm from many inbred and outbred mouse strains (25, 30, 38, 43). In rat strains (31, 52), cryopreservation of sperm has not been sufficiently optimized compared to mouse (30, 43).

Skim milk with nonpermeating CPA has been successfully used to cryopreserve sperm from many inbred and outbred mouse strains (30, 43). However in this and one study (46), it has been demonstrated that skim milk is not as effective as TES in protecting rat sperm from freezing injury. A study (52) revealed success of cryopreservation of epididymal rat sperm in modified Krebs-Ringer bicarbonate containing 0.1 M raffinose, 0.75% of an SDS-based product, and 20% EY provided 39.3% motility index. This study also demonstrated efficiency of the extender solution containing 0.1 M raffinose or 0.1 M sucrose dissolved in TES medium with 0.75% Equex-Paste and 20% centrifuged egg yolk and TES extender improved post-thaw motility compered to TL-HEPES and m-KRB. Also it was reported (46) that cooling rate significantly affected post-thaw motility depended on extenders. Cooling rate did not statistically affect any sperm motility when m-KRB and TRIS was used in F344 rats as well as SM, TRIS and TES-S extenders in SD rat sperm.

Sperm cryo-survival is depended significantly on the cooling rate, and less strongly associated with the warming rates (25, 42). The optimum-cooling rate may change among species. For mouse sperm optimum-cooling rate was reported to vary from 40°C/min to 300°C/min (42) and from 27°C/min to 130°C/min (25). However, optimum cooling rate was found 10°C/min for human sperm (16). Also, it was suggested two stage cooling rate, which is 3-5 °C/min up to -5°C, 20-50 °C/min up to -196°C for boar sperm (12). Previous studies has indicated that maximum motility for rat sperm was obtained when cooling rates between 50-80 °C/min for SD (14) and 40-100°C/min for SD and F344 (46) rat strains. But in this study, statically differences between cooling rates were not found. It has been reported that the composition of medium and cooling rates have significant effects on sperm (51). However in this study, the cooling rate did not affect motility, plasma, acrosome and mitochondrial membrane integrity in extenders except for acrosome membrane integrity in TRIS-S extender. In agreement with our study, reported that any relations between motility, plasma membrane integrity, mitochondrial function and cooling rate was found (16).

In this study, motility was better than membrane and mitochondrial membrane integrity. Acrosome integrity after freezing was ranging from 17.3% to 39.3% and was not statically different in cooling rate and extenders groups. These values were lower than reported data (52), in which acrosome integrity was observed 89.3%. The differences in both studies may be due to classification of intact and damaged spermatozoa. In this study, post-thaw acrosome integrity was closely in consistency with others sperm parameters compare to cited study (52). Also in consistence with cited study (46), post-thaw acrosome integrity of Lewis rat was

almost same with sperm acrosome integrity of SD and F344 Rats. The lower sperm parameters compared to motility can help us to explain the too weak progressive motility. Motility and mitochondrial membrane potential of sperm affect fertility and low sperm mitochondrial membrane potential may explain infertility (21). The highest motility and acrosome integrity were 27.5% and 39.3% in TES respectively. But, highest plasma and mitochondrial membrane integrity was only 13.8% and 3.7%. In addition, although progressive motility was nearly absent in this study, in cited study (23), addition of 2 g/L adenosine 50-triphosphate (ATP) to TES-sucrose-EY extender, managed to obtain little higher motility (36.5%) and progressive motility (6.0%) from this study but plasma membrane integrity and mitochondrial membrane potential was nearly same.

In summary, freezing decreased the sperm parameters but effect of cooling rate was not detected in cryopreservation of Lewis rat sperm. The results revealed weak interaction between extenders and the cooling rate on the rat sperm characteristics. Our finding suggests that TES extender with raffinose or sucrose works well and in rat sperm freezing study but differences of post-thaw sperm parameters in rat strains should be considered in future sperm freezing study.

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