



RESEARCH ARTICLE

Basal medium eagle solution may improve the post-thaw parameters of Kangal dog semen

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Amino asit sıvısı Kangal köpeği spermasının çözümü sonu spermatolojik parametrelerini iyileştirebilir

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Öz

Amaç: Bu çalışmada dondurma-çözdürme sonrası Kangal köpeği spermatolojik parametreleri üzerine BME'nin etkisinin ortaya konulması amaçlandı.

Gereç ve Yöntem: Ejakülatlar hafta iki kez digital manipülasyon yardımıyla alındı. Alınan ejakülatlar 6 eşit hacme bölünerek temel Tris sulandırıcısı içeren (T), %5 gliserol (TG), %5 etilen glikol (TE), %5 gliserol (G) + %2.5 BME (TGB2.5), TG + %10 BME (TGB10), %5 etilen glikol (E) + %2.5 BME (TEB2.5) ve TE + %10 BME (TEB10) sulandırıcılar ile sulandırıldı. Sulandırılan spermalar 4°C'ta 1.5 saat ekilibrasyon sonrası sıvı azot buharında dondurularak sıvı azotta (~-196°C) saklandı.

Bulgular: Çalışmada progresif motilite oranı %10 BME içeren gliserollü sulandırıcı grubunda (%23.19), %5 etilen glikol içeren (%14.08) grubuna göre istatistiksel olarak yüksek bulundu. Akrozom bütünlüğü %2.5 BME içeren gliserollü sulandırıcı (%44.99) grubunda, %5 gliserol içeren (%33.63) grubuna göre istatistiksel olarak üstünlük gösterdi (P<0.05).

Öneri: Kangal köpeği spermasının dondurulmasında sulandırıcıya eklenen BME çözümü sonu spermatolojik parametreleri iyileştirebilir.

Anahtar kelimeler: BME, etilen glikol, gliserol, Kangal köpeği sperması

Abstract

Aim: The aim of this study was to investigate the effects of BME on Kangal dog sperm parameters following the freeze-thawing process.

Materials and Methods: Ejaculates were collected by digital manipulation twice a week. Ejaculates divided into six equal aliquots, and diluted with Tris-based extender (T) containing 5% glycerol (G) + BME 2.5% (TGB2.5), TG + BME 10% (TGB10), 5% ethylene glycol (E) + BME 2.5% (TEB2.5), TE + BME 10% (TEB10), TG and TE. Subsequently, the ejaculates were cooled to 4°C at 1.5 hours, and stored in liquid nitrogen (~-196°C).

Results: The extender supplemented with 10% BME + 5% G resulted in higher progressive motility (23.19%), in comparison to the extenders supplemented with 5% EG (14.08%). Better sperm acrosome integrity was achieved with the use of the extender containing 2.5% BME + 5% G (44.99%), when compared to the use of the extender supplemented with 5% G (33.63%) (P<0.05).

Conclusion: BME added to extender, may improve the post-thawed sperm parameters on freezing of Kangal dog semen.

Keywords: BME, ethylene glycol, glycerol, Kangal dog semen



Introduction

The Anatolian Shepherd Dog, otherwise known as the Kangal dog, has been bred in Anatolia for centuries, as a guardian of both households and livestock. The specific features of this breed are identified with Turkey, and the Kangal dog is the most popular dog breed in the country. Up to the present, Kangal dog owners have strived to protect this breed and its traits through natural selection. However the crossing of the Kangal dog with several different breeds in various regions has put the continuity of the breed-specific features of the Kangal dog under risk (Yilmaz 2005). Therefore, the cryopreservation of the semen of Kangal dogs has gained particular significance.

Functional changes occur in the organization of the lipid bilayer of the plasma membrane of spermatozoa during spermatogenesis and epididymal maturation. As a result of these changes, not only do plasmalogens in the mid-piece and tail become the predominating phospholipid structure of the plasma membrane, but also plasmalogens increase the cholesterol/phospholipid molarity two-fold during the migration of spermatozoa through the epididymis. These changes reduce the proportion of cholesterol in the plasma membrane of the spermatozoon, which in return contributes to the maturation of spermatozoa (Benoff 1993).

The generation of heat and the occurrence of osmotic changes during cooling, freezing and thawing affect lipid composition and organisation, calcium flow, permeability and integrity in the plasma membrane of spermatozoa, and create mechanical stress (Noiles et al 1995). Differences observed between animal species for the lipid composition of spermatozoa result in differences in the susceptibility of spermatozoa to freezing and thawing. It has been reported that bull, boar and ram spermatozoa, all characterized by a plasma membrane rich in unsaturated fatty acids, are more susceptible to freezing than human, dog and rabbit spermatozoa (Noiles et al 1995). The exposure of sperm to heat changes and aerobic oxygen during freezing and thawing results in the production of reactive oxygen species (ROS) and non-reactive oxygen species (Agarwal et al 2005).

The antioxidant capacity of spermatozoa is limited due to radical-scavenging antioxidants being contained only by a small cytoplasmic component (Bilodeau et al 2000).

For this reason, mammalian sperm cells may fail in preventing lipid peroxidation (LPO) when they were cooled (Storey 1997). Substances with antioxidant properties have been reported to decrease the effect of ROS-based and heat stress detriments (Alvarez et al 1983), and thereby, to improve ram (Aisen et al 2000), goat (Bucak et al 2009a), boar (Pena et al 2003), dog (Micheal et al 2007), hamster (Alvarez et al 1998) and human (Storey 1997) sperm quality.

The freezing of dog sperm has evolved over a long period, from the discovery of the cryoprotective property of glycerol by Polge et al (1949) to the subsequent development of various freezing protocols. Glycerol is a highly hydrophilic polyol compound and prevents intracellular crystallization by means of its distribution in between intracellular water and the cytoplasm (Vidament et al 1997). The equal number of carbon atoms and hydroxyl groups contained by glycerol and the lyophilic/hydrophilic properties of this compound increase its cryoprotective effect during the freezing of sperm (Storey et al 1998). The use of different glycerol concentrations for the freezing of sperm has proven lower concentrations to show better cryoprotective effect (Cristanelli et al 1985).

Ethylene glycol is a cryoprotectant, which contains an equal number of carbon atoms and hydroxyl groups, similar to glycerol. The molecular weight of ethylene glycol (62.07 g/mol) is lower than that of glycerol (92.10 g/mol). Therefore, it has been indicated that, when compared to glycerol, ethylene glycol has a higher level of intracellular permeability and a lower level of toxic effect (Storey et al 1998).

In some organisms, amino acids play a protective role against low temperatures. Some researchers have reported that several amino acids protect spermatozoa against cold shock by forming a layer on the plasma membrane of the spermatozoon (Ateşşahin et al 2008, Cristanelli et al 1985). The present study was aimed at the investigation of the efficiency of amino acid solution (basal medium eagle, BME) and the cryoprotectants glycerol and ethylene glycol in the cryopreservation of Kangal dog sperm. The post-thaw effects of BME and the cryoprotectants, which were added to the extenders, were assessed on the basis of subjective and CASA motilities, sperm kinetic parameters (VCL $\mu\text{m/s}$, VSL $\mu\text{m/s}$, VAP $\mu\text{m/s}$, ALH μm and LIN %), fluorescent staining (viability, acrosome integrity, mitochondrial activity) and DNA integrity (COMET assay).

Materials and Methods

Animals and semen collection

Semen samples from 5 Kangal dogs (1 and 3 years of age), of superior genetic merit and fertility capacity, were used in this study. This study ethical committee approval was taken SUSDAMEK (2013/016) and the animals were housed at the Education, Research and Practice Farm of Selcuk University, Faculty of Veterinary Medicine, and were maintained under uniform nutritional conditions. Ejaculates were collected twice a week from the Kangal dogs by digital manipulation.

Semen extending, freezing and thawing

A Tris-based extender (Tris 297.58 mM, citric acid 96.32 mM, fructose 82.66 mM, egg yolk 20% (v/v), pH 6.8) was used as





the base extender (T). Only ejaculates including spermatozoa with >80% progressive motility and a concentration higher than 400×10^6 spermatozoa/mL were pooled, and the sperm contribution of each male was balanced to eliminate individual differences. The obtained ejaculates were used for each pooling process. Five pooled ejaculates were included in the study. Each pooled ejaculate was divided into six equal aliquots and diluted at 37°C with Tris-based extenders containing 5% glycerol (G) + 2.5% basal medium eagle solution (BME, SIGMA B6766) (TGB2.5), TG + 10% BME (TGB10), T + 5% ethylene glycol (E) + 2.5% BME (TEB2.5), TE + 10% BME (TEB10), TG and TE, at a final concentration of approximately 100×10^6 spermatozoa/straw in a 15 mL-plastic centrifuge tube. Diluted semen samples were aspirated into 0.25 mL French straws, sealed with polyvinyl alcohol powder, and equilibrated at 5°C for 1.5 h. After equilibration, the straws were frozen in liquid nitrogen vapour, 5 cm above the liquid nitrogen, for 12 min and plunged into liquid nitrogen for storage (Baspınar et al 2011). After stored, the frozen straws were thawed individually at 37°C for 30 s in a water bath for microscopic evaluation.

Evaluation of microscopic sperm parameters

Analysis of subjective and CASA motilities

Subjective motility was assessed using a phase-contrast microscope (200x magnification), with a warm stage maintained at 37°C. A wet mount was made using a 5 µL drop of semen placed directly on a microscope slide and covered by a coverslip. Sperm motility estimations were performed in three different microscopic fields for each semen sample. The mean of the three successive estimations was recorded as the final motility score. Besides estimating subjective sperm motility, computer-assisted sperm motility analysis (CASA), Sperm Class Analyzer (SCA® v.4.2, Barcelona, Spain) was also used to analyse sperm motion characteristics.

The CASA was set up as follows: phase contrast; frame rate – 20 Hz; minimum contrast – 50; low and high static size gates – 0.6 to 4.32; low and high intensity gates – 0.20 to 1.92; low and high elongation gates 7 to 91; default cell size – 10 pixels; default cell intensity – 80. Thawed semen was diluted (5 µL semen + 95 µL extender) in a Tris-based extender (without egg yolk and cryoprotectant) and evaluated immediately after dilution. A 4-µL sample of diluted semen was placed on a pre-warmed chamber slide (Leja 4, Leja Products, Luzernestraat B.V., Holland) and sperm motility characteristics were determined at 37°C. The following motility values were recorded: motility (%), progressive motility (%), VSL (straight linear velocity, µm/s), VCL (curvilinear velocity, µm/s), VAP (average path velocity, µm/s), ALH (amplitude of lateral head displacement, µm) and LIN [linearity of the curvilinear path (LIN = (VSL/VCL) x 100)]. For each evaluation, 10 microscopic fields were analysed to include at least 300 cells.

Assessment of sperm plasma membrane integrity

This assessment was performed by staining with the Sperm Viability Kit (SYBR-14/PI Molecular Probe: L 7011 Invitrogen). The staining protocol was modified from Garner and Johnson (1995). A working solution of SYBR-14 was diluted 1:10 with DMSO (Applichem A3006), then divided into equal aliquots (30 µl) after being filtered through a 0.22 µm Millipore Millex GV filter, and was stored at -20°C. Propidium iodide (PI) was dissolved in distilled water at 2 mg/mL, divided into equal aliquots (30 µL) after being filtered through a 0.22 µm Millipore Millex GV filter, and was stored at -20°C. The thawed straws were diluted 1:3 with Tris stock solution without cryoprotectants and egg yolk, and then 30 µL of diluted semen was mixed with 6 µL of SYBR-14 and 2.5 µL of PI. The sample was gently mixed, incubated at 37°C in the dark for 20 min, then added 10 µL of Hancock's solution (Schafer and Holzmann 2000) to stop sperm movement. A wet mount was made using a 2.5 µL drop of sample placed directly on a microscope slide and covered by a coverslip.

At least 200 spermatozoa were examined at 400x magnification under a fluorescence microscope (Leica DM 3000 Microsystems GmbH, Ernst-Leitz-Strasse, Wetzlar, Germany; excitation at 450–490 nm, emission at 520 nm) to assess sperm membrane integrity. Sperm cells displaying green-red or red colorization were considered as membrane dead, while those displaying green colorization were considered to have alive spermatazoon.

Assessment of sperm acrosome integrity

Sperm acrosome integrity was assessed by fluorescein isothiocyanate conjugated to Arachis hypogaea (peanut) (L7381 FITC-PNA, Sigma) and by PI staining as described by Nagy et al (2003) with modifications. Approximately 120 µg of FITC-PNA was added to 1 mL of PBS (15630056, Invitrogen) for the preparation of the staining solution. After being filtered, the solution was divided into equal aliquots (100 µL) and stored at -20°C. Each thawed straw was diluted 1:3 with a Tris stock solution without cryoprotectants and egg yolk, and then 60 µL of diluted semen was mixed with 10 µL of FITC-PNA and 2.5 µL of PI. The sample was gently mixed, incubated at 37°C in the dark for 20 min., and was added 10 µL of Hancock's solution (Schafer and Holzmann 2000) to stop sperm movement. A wet mount was made using a 2.5 µL drop of sample placed directly on a microscope slide and covered by a coverslip. At least 200 sperm cells were examined at 400x magnification under a fluorescence microscope (Leica DM 3000; excitation at 450–490 nm, emission at 520 nm) to assess sperm acrosome integrity. Spermatozoa displaying bright green or patchy green fluorescence were considered to have a non-intact or damaged acrosome, whereas cells that did not display green fluorescence in the acrosome cap were considered to have an intact acrosome.



Table 1. Dog sperm subjective, CASA and progressive motilities in semen supplemented with additives following freeze-thawing (Mean±SE).

Groups	Freeze-thawed motilities (%)		
	Subjective	CASA	Progressive
TGB2.5	57.1±2.40	53.6±7.28	19.31±1.52 ^{ab}
TGB10	53.5±2.60	52.2±7.35	23.19±1.61 ^b
TEB2.5	50.7±2.54	46.3±5.24	16.38±1.49 ^a
TEB10	52.8±2.85	53.5±3.53	15.09±1.35 ^a
TG	47.8±4.06	47.8±4.85	17.65±1.51 ^{ab}
TE	50.0±2.67	44.7±3.88	14.08±3.07 ^a
P	-	-	*

a, b: Different superscripts within the same column demonstrate significant differences (*:P<0.05).

Assessment of sperm mitochondrial activity

Sperm mitochondrial activity was assessed using the JC-1/PI staining protocol modified from Garner et al (1997). A stock solution of 5,5',6,6'-tetrachloro-1,1',3,3' tetraethyl-benzimidazolyl-carbocyanine iodide (T3168 JC-1, Invitrogen, 1.53 mM) was prepared in DMSO solution, divided into equal aliquots (100 µL) after being filtered, and was stored at -20°C. Each thawed straw was diluted 1:3 with a Tris stock solution without cryoprotectants and egg yolk, and then 300 µL of diluted semen was mixed with 2.5 µL of JC-1 and 2.5 µL of PI. The sample was gently mixed, incubated at 37°C in the dark for 20 min., and was added 10 µL of Hancock's solution (Schafer and Holzmann 2000) to stop sperm movement. A wet mount was made using a 2.5 µL drop of sample placed directly on a microscope slide and covered by a coverslip. At least 200 sperm cells were examined at 400x magnification under a fluorescence microscope (Leica DM 3000; excitation at 450–490 nm, emission at 520 nm) to assess sperm mitochondrial activity. A bright green/orange fluorescence associated with the sperm midpiece (where the mitochondria are located) indicated to mitochondrial activity. Mitochondria showing low activity stained pale green.

Assessment of sperm DNA damage

Sperm DNA damage was investigated using the single cell gel electrophoresis (COMET) assay, which is generally performed under neutral conditions. Our method was similar to several protocols applied in previous studies and involved few modifications (Duty et al 2002, Li et al 2008). The straws were thawed by gentle shaking in a 37°C water bath for 10 s, and centrifuged once at 600 g for 10 min at 4°C. The remaining sperm cells were washed with PBS (Ca⁺⁺ and Mg⁺⁺ free). Each pre-cleaned slide was pre-coated with a layer of 1% normal melting point agarose in PBS (Ca⁺⁺ and Mg⁺⁺ free) and then dried at room temperature. Approximately 100 000 sperm cells (18 µL) were mixed with 0.75% low melting point agarose (50 µL) at 37°C, and this suspension was poured

onto the first agarose layer. The slides were allowed to solidify for 20 min at 4°C. The coverslips were removed, and the slides were immersed in freshly prepared cold lysis buffer. The slides were then incubated at 37°C in lysis buffer with 20 µg/mL of proteinase K for 2 h. The slides were removed from the lysis buffer, drained and placed into a horizontal electrophoresis unit filled with fresh neutral electrophoresis buffer at 4°C for a 20 min incubation process to allow the DNA to unwind. Electrophoresis was performed at room temperature, at 25 V for 20 min. Following electrophoresis, the slides were air-dried, stained with 50 µL of 8 µL/mL ethidium bromide, and covered with a coverslip. The images of 200 cells randomly chosen nuclei per sample were analysed visually. Observations were made at a magnification of 400x using a fluorescence microscope (Olympus, Japan). Damage was detected by a tail of fragmented DNA observed to have migrated from the sperm head, causing a 'comet' pattern, whereas whole sperm heads, without a comet, were not considered as damaged.

Statistical analysis

The study was replicated six times. Results were expressed as mean±SEM. Means were analysed by one-way analysis of variance, followed by Duncan's post-hoc test to determine significant differences in all parameters between all groups using the SPSS/PC computer program (Version 15.0; SPSS, Chicago, IL). Differences with values of P<0.05 were considered to be statistically significant.

Results

As shown in Table 1, TGB10 group (23.19±1.61) resulted in a higher progressive motility, in comparison to TEB2.5 (16.38±1.49), TEB10 (15.09±1.35), and TE (14.08±3.07) groups (P<0.05) following the freeze-thawing process. As shown in Table 2, no significant differences were observed between the groups for sperm motion characteristics (P > 0.05). As shown in Table 3, TGB2.5 (62.74±2.50), TGB10





Table 2. Dog sperm motion parameter (VAP, VSL, VCL, ALH, LIN) results in semen supplemented with additives following freeze-thawing (Mean±SE).

GROUPS	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	LIN (%)
TGB2.5	40.35±3.59	30.07±3.39	63.45±5.01	4.18±0.18	47.01±2.74
TGB10	37.35±3.76	26.60±3.30	61.61±4.73	4.41±0.27	42.58±2.84
TEB2.5	33.32±2.53	22.50±1.82	57.72±3.87	4.68±0.23	39.10±1.96
TEB10	36.30±3.09	25.58±2.44	59.72±5.40	4.25±0.46	42.74±0.98
TG	39.71±3.96	28.91±4.22	64.18±4.27	4.45±0.10	43.75±4.49
TE	37.70±2.94	27.57±2.50	62.00±4.37	4.82±0.14	44.28±2.03
P	-	-	-	-	-

-: The same column shows no significant differences among proportions (P>0.05).

Table 3. Dog sperm viability, acrosome integrity, mitochondrial activity and DNA integrity results in semen supplemented with additives following freeze-thawing (Mean±SE).

Groups	SYBR-14/PI %	FITC/PI %	JC-1/PI %	COMET %
TGB2.5	59.46±2.55	44.99±4.97 ^b	62.74±2.50 ^b	93.83±0.35
TGB10	58.98±2.15	42.46±4.02 ^{ab}	63.31±2.15 ^b	94.00±0.59
TEB2.5	60.10±7.90	38.63±2.38 ^{ab}	57.96±2.53 ^{ab}	94.83±0.91
TEB10	53.82±2.32	39.94±1.83 ^{ab}	59.69±2.71 ^b	93.66±1.30
TG	54.60±2.46	33.63±4.05 ^a	57.10±0.56 ^{ab}	94.75±0.54
TE	55.02±1.95	35.46±2.41 ^{ab}	52.50±2.45 ^a	94.08±0.66
P	-	*	*	-

a, b: Different superscripts within the same column demonstrate significant differences (*:P<0.05).

(63.31±2.15), and TEB10 (59.69±2.71) led to higher sperm mitochondrial activity when compared to the extender TE (52.50±2.45) (P<0.05). No significant differences were observed between the groups for sperm viability (Table 3, P>0.05). A better sperm acrosome integrity was achieved in TGB2.5 (44.99±4.97), when compared to the TG (33.63±4.05) (Table 3, P<0.05). The comet assay demonstrated no significant differences between the groups (Table 3, P > 0.05).

Discussion

This study was designed to determine which BME concentration and particular cryoprotectant would protect the Kangal dog sperm against cold shock damage during the freeze-thawing stages. The motility of the spermatozoon depends on the flagellar movement of the tail and the frequency of this movement (Coyan et al 2002). Another major factor, which determines sperm motility, is the concentration of cAMP (Garner and Hafez 1993). In the present study, cryopreservation with TGB10 significantly improved sperm progressive motility when compared to cryopreservation with TE. The results obtained in the present study for progressive motility diverge from those previously reported for frozen-thawed buck sperm, where BME supplementation provided improvement in post-thaw sperm subjective, CASA total and progressive motilities (Farshad and Hosseini 2013). The different

results obtained in these studies were attributed to differences in the investigated species, extender composition, and antioxidant dose. On the other hand, results similar to those obtained for post-thaw subjective and CASA motilities and sperm motion parameters in the present study have been reported by Bucak et al (2009b) in rams and by Taşdemir et al (2014) in bulls.

It has been reported that enzymes contained by the acrosome are involved in the penetration of the zona pellucida by the spermatozoon and that the rate of acrosome integrity is correlated with the rate of fertility (Garner and Hafez 1993). In this respect, the investigation of the effects of the additives used in the trial groups established in the present study on the post-thaw acrosome integrity of Kangal dog sperm demonstrated better results to have been achieved in Group TGB2.5 than in Group TG. The results obtained in the present study showed that the BME essential amino acid solution provided the protection of the acrosome structure during the freeze-thawing of spermatozoa, owing to the antioxidant activity of this solution. No statistically significant differences were observed between the groups for post-thaw sperm viability.

Axoneme and fibrillar structures embraced by the sperm mitochondrial sheath were responsible for the production



of ATP needed for sperm motility as a result of that sperm could reach the oocyte (Garner and Hafez 1993). Oxidative damage to mitochondrial DNA and membrane architecture may be of major importance to explain the impaired fertility and motility of cryopreserved semen, when spermatozoa were cooling its mitochondrial membrane potential dramatically decreasing (Cummins et al 1994). As a result of this condition was reflected in impaired fertility and motility (O'Connell et al 2002). Reports suggest a close correlation between sperm motility and high mitochondrial activity (Garner and Johnson 1995, Nagy et al 2003). In this study, cryopreservation with TGB2.5, TGB10 and TEB10 resulted in higher sperm mitochondrial activity, when compared to cryopreservation with TE. This result was in agreement with previous reports for frozen-thawed buck sperm (Pena et al 2003), which demonstrated antioxidant supplementation to improve both post-thaw sperm motility and mitochondrial activity. It is reported that the COMET assay is a rather efficient method in determining intracellular oxidative stress, and it is suggested that this assay can be used to detect DNA strand breakage (Anderson et al 1994). This enables not only the demonstration of the oxidant/antioxidant properties of additives in the sperm cell, but also the protective effect of additives on the genetic material of the sperm cell (Anderson et al 1999). Reports indicate that spermatozoon DNA integrity is correlated with functional spermatozoon parameters (Rajesh et al 2002).

In this study, the BME did not provide a better protection of the DNA integrity of Kangal dog sperm, when compared to the extenders without antioxidants. The results received in this study for DNA integrity are agreed with those obtained in previous research on bull sperm following incubation (Taşdemir et al 2014), where DNA integrity was provided with BME supplementation. It can be hypothesized that the dose-dependent effect of BME suggest to different results.

Conclusion

This study investigated ameliorating effect against the cryopreservation of dog sperm in vitro. The results of this study suggest that supplementation with BME offers protection of sperm motility, as well as high mitochondrial activity and acrosome integrity. Our study highlights the effect of BME on Kangal dog sperm survival and contributes to efforts aimed at the improvement of Kangal dog semen freezing and establishes sperm banks.

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