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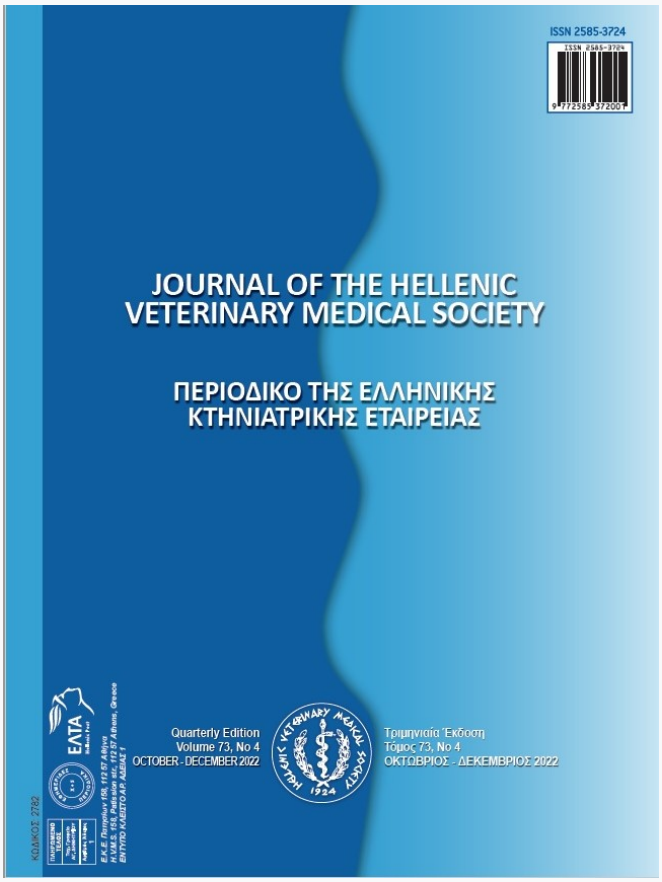

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Research article
Ερευνητικό άρθρο

A novel approach to sperm selection: Nanoparticle-based purification improves quality of Angora cryopreserved buck's semen

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ABSTRACT: The aim of the study was to investigate the applicability of an up-to-date method, sperm selection with nanoparticles at different temperatures (37ºC, 21ºC, and 4ºC), on Angora buck semen. Semen samples were collected from 3 Angora bucks (2-4 years old) with 7 repetitions and each sample was diluted with TRIS+egg yolk (TEYG) and divided into 4 groups. Nanopurification procedure was applied by using nanoparticles in the form of a cocktail of iron oxide nanoparticles coated with silica magnetite, Annexin-V and *Pisum sativum* agglutin (PSA) with a dose of 10 µg/ml to the three groups at 37ºC, 21ºC, and 4ºC while in the control group samples were cryopreserved without nanopurificiation treatment. Post-thaw total motility (TM) and morphology parameters of the sperm samples were analyzed by using computer-aided sperm analysis (CASA) and fluorescein microscopy in which, *Arachis hypogaea* (peanut) lectin (FITC-PNA, 1 mg lyophilized powder, Sigma) and JC-1 (5 mg, Invitrogen, Thermo Fischer Scientific) Fischer Scientific) stains were used. After nanopurification at 37ºC total motility, linearity (LIN) ($P<0.05$), curvilinear velocity (VCL), straight-line velocity (VSL), and acrosome integrity parameters ($P<0.001$) were higher than the control group; while the semen samples nanopurified at 21ºC were superior to the control group in terms of LIN, straightness (STR) ($P<0.05$), VCL, VSL, average path velocity (VAP), wobble and plasma membrane-acrosome integrity (PMAI) ($P<0.001$). There was no significant difference between the control and the 4ºC group. Also,there were no differences between the groups in terms of mitochondrial activation rates ($p=0.892$) by fluorescent staining.

In conclusion, nanopurification procedure on buck semen was useful in terms of parameters that affect fertility and the method can be performed at 21ºC, as well as 37ºC, to adapt the procedure to field conditions.

Keywords: Angora buck; Cryopreservation; Iron oxide nanoparticles; Sperm selection

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INTRODUCTION

High reproduction rates are of great importance for livestock since newborn animals have to reach and even exceed the number of animals in the herd to ensure profit. Unlike other goat breeds, in Angora goats, low-reproductive yield constitutes a well-known problem (Shelton, 1995). It is possible to benefit more from Angora goats and bucks with high quality mohair by improving semen freezing studies and spreading artificial insemination practices (Inanç et al., 2017; Tekin and Daşkın, 2019; Tirpan and Tekin, 2015).

Especially storage of frozen semen creates structural, biochemical and functional damage to spermatozoa; that results in decreased motility, viability and fertility (Fruend and Wiederman, 1966; Leboeuf et al., 2000). The main purpose of extenders is to provide energy to spermatozoa, protecting cells from heat-stress, and creating a suitable environment for temporary protection of spermatozoa (Purdy, 2006). Egg yolk which is commonly used in the extenders, interacts with the phospholipase A enzyme in the seminal plasma, creating negative effects on spermatozoa (Purdy, 2006; Ritar and Salamon, 1982).

In addition, there are other factors that negatively affect the *in vivo* progression of spermatozoa thus directly affecting fertility (Feugang et al., 2015). Since apoptosis of some spermatozoa in a sample lead to elevated production of the ROS, increased lipid peroxidation, and formation of harmful end products; it results with a decreased motility and viability of the live spermatozoa in the sample (Aitken et al., 2015). Elimination of unnecessary competition, between live and dead spermatozoa, allows more live spermatozoa to reach the fertilization site and may contribute to fertility (Feugang, 2017; Feugang et al., 2015). In order to eliminate these negativities, using selection methods such as; swim-up assay (Arias et al., 2017; Moritimer, 2000), density gradient centrifugation (DGC) (Morrell, 2016; Noguchi et al., 2014) filtration through columns (Galarza et al., 2018; Husna et al., 2016; Roach et al., 2016) single layer centrifugation (SLC) (Al-Essawe et al., 2018; Crespo-Felez et al., 2017; Nongbua et al., 2017) and microfluidic devices (Li et al., 2014; Niederberger, 2016; Suarez and Wu, 2017) before semen freezing can be beneficial in terms of removing damaged spermatozoa.

In recent years, with the development of the nanotechnology field, a novel approach referred to as nanopurification has been added to the previously defined sperm selection methods (Feugang et al., 2015; Odhiambo et al., 2014). The use of nanoparticles to target the physical and physiological characteristics of sperm can lead to successful fertilization (Sutovsky and Lovercamp, 2010). Iron oxide (Fe_3O_4) nanoparticles are the preferred material in biomedical technologies due to their higher biocompatibility and superparamagnetic properties compared to other magnetic components (Figuerola et al., 2010; Mohammed et al., 2017). Mammalian sperm are known to have variable levels of fertility. There is increasing evidence that certain types of abnormal spermatozoa produce unique cell surface components that are not present on the surface of normal spermatozoa capable of fertilizing oocytes (Baska et al., 2008; Ibrahim et al., 2000; Olson et al., 2004). This type of spermatozoon surface components are thought to be negative biomarkers of spermatozoon's quality and fertility (Odhiambo et al., 2004; Sutovsky and Lovercamp, 2010) which can be determined using special probes and targeted using nanoparticles for spermatozoa selection (Odhiambo et al., 2004; Sutovsky and Kennedy, 2013). Particularly, Fe_3O_4 coated with fluorescein isothiocyanate-labelled *Arachis hypogea* lectin (PNA) / fluorescein isothiocyanate-labelled Pisum sativum lectin (PSA) or Annexin-V, selectively bind to reacted acrosomes or apoptotic spermatozoa (Durfey et al., 2017; Feugang, 2017; Grunewald et al., 2006; Lee et al., 2010; Said et al., 2006; Said et al., 2005). Lectin and carbohydrate receptor systems that are located on the surface of spermatozoa are used for this purpose. The lectins cause agglutination by binding to carbohydrates on the plasma membrane of the spermatozoon (Feugang et al., 2015; Feugang et al., 2019).

In the nanopurification studies conducted so far, post-thawing nanopurification is the method used on various other species such as; bull (Odhiambo et al 2014), boar (Feugang et al., 2019) and human (Grunewald et al., 2006) sperm, but the procedure was performed only at 37ºC. However, it is thought that removing low-quality spermatozoa from the environment by selection before freezing and limiting the production of by-products that could have a detrimental effect on healthy spermatozoa during the freezing process play a significant role in the success of the fertilization (Durfey et al., 2017). In addition, the procedure of nanopurification has been found efficient, affordable without the need for expensive equipment, and easy to perform.

Considering all the information above, this study is aimed to investigate the effect of the selection of higher quality spermatozoa from Angora buck semen samples before cryopreservation. The nanopurification of buck

sperm would be a first to assess the feasibility. It was also aimed to facilitate the adaptation of the nanopurification procedure, which was previously only practiced at 37ºC, to field conditions or at least to equilibration process required for freezing by applying the procedure at different temperatures (37, 21, and 4ºC). Thus, our main objectives consisted of investigating the effects of nanopurification on Angora buck semen and determining the most suitable temperature for nanopurification procedures applied prior to the cryopreservation.

MATERIALS AND METHODS

This study was conducted at Ankara University, Faculty of Veterinary Medicine Education, Research and Practice Farm, and the study procedure was approved by Ankara University Local Ethics Committee on Animal Experiments (Ethical Committee Approval No: 2018-14-90).

Materials Used

The nanoparticles used in the study were purchased from Clemente Associates Incorporated, Madison, Connecticut, USA and consisted of a cocktail of silica magnetite, *Pisum sativum* agglutinin (PSA), and Annexin-V coated iron oxide nanoparticles.

The fluorochromes, *Arachis hypogaea* (peanut) lectin (FITC-PNA, L7381, Sigma-Aldrich Co., St. Louis, Mo, USA), and Propidium iodide (PI) and JC-1 (Invitrogen, Thermo Fisher Scientific, USA) were used for the sperm analysis.

Sample Collection and Nanopurification Procedure

The Angora bucks from which semen samples were obtained were andrologically examined and fertile. They were kept under uniform breeding conditions at Ankara University Faculty of Veterinary Medicine Practice and Research Farm. A total of 21 semen samples were collected from 3 Angora bucks (2-4 years old) by an electro ejaculator (12-14Volt) (e320, Minitube, Germany) two times a week during breeding season (November-December). The average measurement of the total 21 ejaculates; volume (1,93 ±0,16), pH (6,79 ± 0,07), mass movement (3,81 ± 0,24), concentration ($1,6\times10^9 \pm 0,3\times10^9$) and motility (%) (81,14 ± 2,62) parameters of the sperma were recorded prior to the dilution and the semen samples with normozoospermic properties were used. Each semen sample was diluted with TEYG (3,63 g tris, 1,82 g citric acid, 0,5 g glucose, 10% egg yolk and, 5% glycerol in 100 ml distilled water) to a final concentration of 800×10^6 sp/ml (Tirpan and Tekin, 2015).

After the dilution of semen samples, subjective motility percentages were recorded and the samples were divided into 4 groups.

The control group was directly filled into 0.25 ml straws and kept at 4°C for 1.5 hours for routine equilibration step of the freezing procedures. Nanoparticles in the form of a cocktail of iron oxide nanoparticles coated with silica magnetite, Annexin-V and Pisum sativum agglutin (PSA) (Clemente Associates Incorporated, Madison, Connecticut, USA) were used for this study. Since there is no study on the use of nanoparticles in the freezing process of goat semen, a preliminary study was carried out in field conditions considering the dose values used in studies in other species, and dose optimization was made considering these studies. As a results of preliminary studies, nanoparticles (10 µg per ml of extended semen) were added to the experimental groups which were placed in water baths at specific temperatures (37±1°C, 21±1°C, and 4±1°C). Semen samples containing nanoparticles were mixed every 5 minutes by rotational movements and kept in water baths for a total of 20 minutes (Clemente-Associate Inc, 1996). After this process, the samples were moved onto magnets (obtained from Clemente Associates with the nanoparticle cocktails) and were kept in the water baths on the magnets for another 10 minutes to ensure the accumulation of spermatozoa bound to the nanoparticles at the bottom of the tubes (Figure 1). At the end of the waiting period, the selected spermatozoa were collected into another tube, filled into 0.25 ml straws and placed at 4°C for routine equilibration step of the freezing procedures.

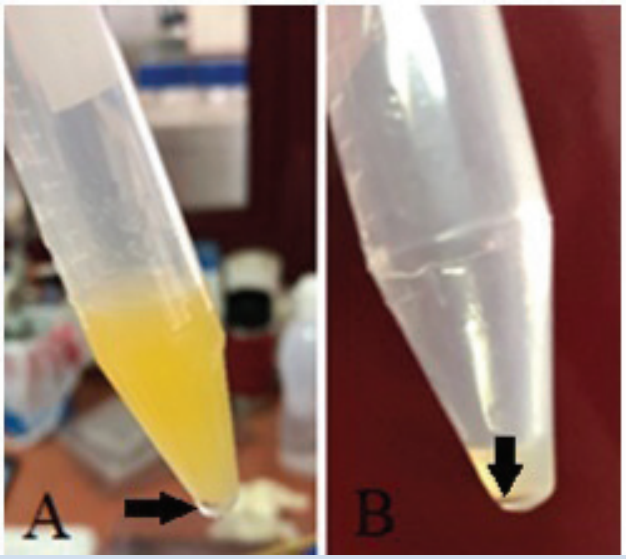


Figure 1. Separation of nanoparticles from the semen sample: A- Nanoparticles aggregated at the bottom of the tube after the sample was taken off the magnet; B- Nanoparticles left in the tube after the nanopurified semen was placed in straws

Equilibration, Freezing, and Thawing

Equilibration and freezing protocols were modified from a study by Leboeuf et al., (2000). All groups were treated with the same equilibration, freezing, and thawing procedures: the straws were equilibrated at 4°C for 1.5 hours, and kept 4-5 cm above liquid nitrogen level in liquid nitrogen vapor for 10 minutes and plunged into liquid nitrogen. Frozen semen samples were kept in liquid nitrogen tanks and were thawed in a water bath at 37°C for 30 seconds.

Kinetic and Morphological Analysis

Total motility (TMOT) and kinetic parameters were evaluated by computer assisted semen analyzer (CASA) using Sperm Class Analyzer software (SCA® v.4.2; Microptic S.L., Spain) and a connected phase-contrast microscope (Nikon Eclipse 50i; Japan). From each frozen-thawed sample, 5 µl were taken and placed between slide and cover slide. Analyses of TMOT and kinetic parameters were done with 100x magnification, green filtered negative phase-contrast (Ph-). Motile spermatozoa were identified as rapid (>75 µm/s), medium (45-75 µm/s), slow (10-45 µm/s), and static (<10 µm/s) due to their curvilinear velocity. Spermatozoa which have ≥75% straightness were determined as progressive. Total motility (TMOT, %), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), amplitude of lateral head displacement (ALH, µm/s), beat-cross frequency (BCF, Hz), linearity (LIN %) [(VSL / VCL) × 100], wobble (WOB %) [(VAP / VCL) × 100], straightness (STR %) [(VSL / VAP) × 100] were evaluated. A total of 200-400 spermatozoa per sample were evaluated in six microscopic zones and were recorded.

The plasma membrane-acrosome integrity (PMAI), which were analyzed using triple stain FITC-PNA kit to determine the PMAI together by fluorescence staining, were evaluated with a modification to the protocol defined by Nagy et al., (2003). Since the FITCH-PNA stain was in the form of a combined stain kit, both plasma membrane and acrosome integrity results could be obtained together. Briefly, thawed semen samples were diluted 1:2 with phosphate buffer saline (PBS). 10 µl of FITC-PNA (1 mg/mL) and 2.5 µl of PI were mixed with 60 µl of diluted semen and incubated for 15 minutes at 37°C, followed by the addition of 10 µl of Hancock's solution for fixation (Schaafer and Holzmann, 2000). At least 200 spermatozoa were examined with fluorescence microscopy from each sample. The spermatozoa with green-stained acrosome were regarded as damaged, and the non-stained acrosome part reflected the acrosome intact spermatozoa.

The mitochondrial activation was evaluated by a modified version of the protocol defined by Garner et al., (1997). Thawed semen samples were diluted 1:5 with PBS. 2.5 µl of JC-1 (ThermoFischer Scientific, USA) and 2.5 µl of PI were mixed with 300 µl of diluted semen and incubated for 15 min at 37°C, followed by the addition of 20 µl of Hancock's solution for fixation (Schaafer and Holzmann, 2000). At least 200 spermatozoa were examined with fluorescence microscopy (100x magnification) (LeicaDM2500, Germany) from each sample. The presence of bright yellow/orange fluorescence in the midpiece of spermatozoa where mitochondria are located was regarded as sperm with high mitochondrial membrane potential, while low mitochondrial activity was reflected by the green fluorescence in the midpiece.

Statistical Analysis

All of the data were examined by the Shapiro Wilk test for normality and with the Levene test for homogeneity of variance. One-way analysis of variance (ANOVA) was used to analyze normally distributed variables and when a significance was obtained post-hoc Tukey test was applied. For the variables which did not have a normal distribution Kruskal Wallis test was used and when a significance was obtained post-hoc Mann Whitney U test was applied with Bonferroni adjustment. The findings were presented as mean ± SEM. All statistical analyzes were analyzed with a minimum 5% margin of error and the SPSS Statistics Subscription program was used.

RESULTS

The mean values of the fresh sperm characteristics, which include semen volume, pH, mass activity (before dilution), and density - subjective motility traits (after dilution), collected from 3 Angora bucks were presented in Table 1. The cryopreservation process led to a 20% of motility loss.

After nanopurification at 37ºC TM, LIN ($P< 0.05$), VCL, VSL, and acrosome integrity parameters ($P<0.001$) were higher than the control group while the semen samples nanopurified at 21ºC were superior to the control group in terms of LIN, STR ($P<0.05$), VCL, VSL, VAP, WOB and PMAI($P < 0.001$) (Fig 2). There was no significant difference between the control group and the 4ºC group ($P > 0.05$) (Table 2). Besides, the percentage of sperm with high mitochondrial membrane potential was similar among temperature groups ($P> 0.05$). Figure 3 and Figure 4 show the images obtained for (PMAI) plasma membrane- acrosome integrity and mitochondrial activity in this study.

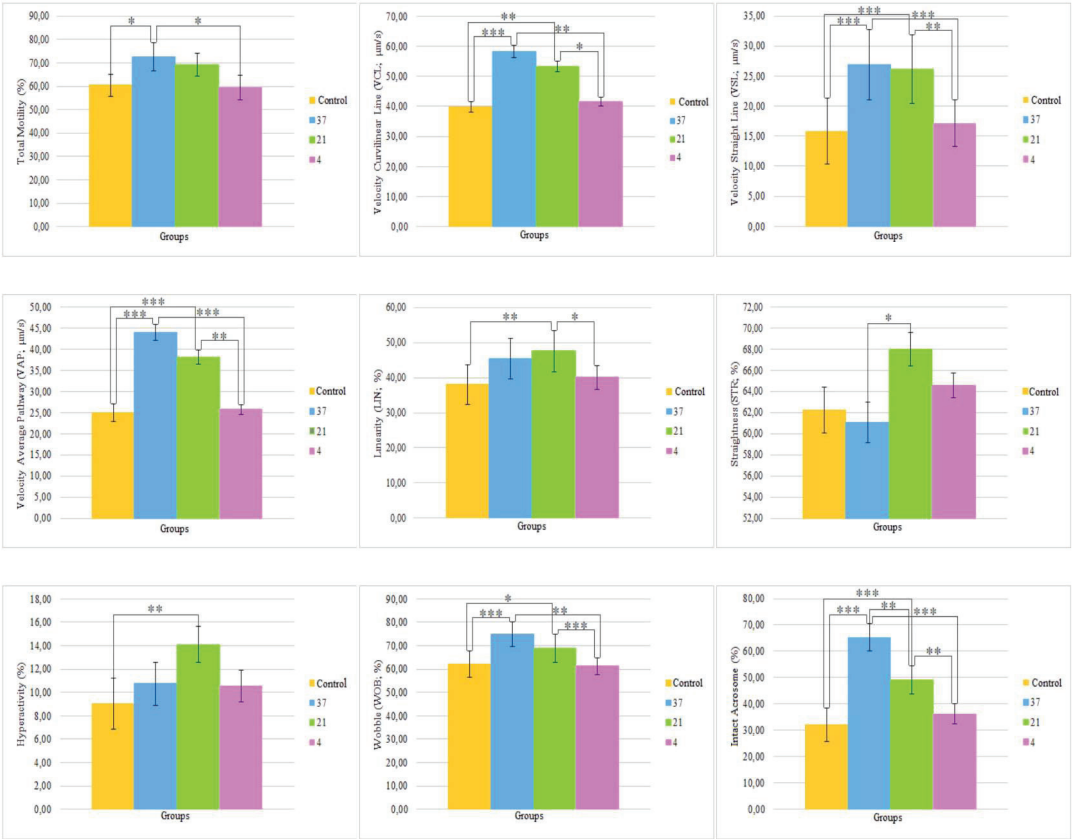
Table 1: Mean ± SEM values of fresh sperm characteristics in Angora bucks

The number of semen samples	Semen Volume (ml)	pH	Mass Motility Score (0-5)	Total Sperm Motility (%)	Sperm Concentration ($\times 10^9$ sperm/ml)
21	1.93 ± 0.16	6.79 ± 0.07	3.81 ± 0.24	81.14 ± 2.62	1.6 ± 0.3

Table 2. Spermatological parameters of Angora buck semen frozen after nanopurification at different temperatures

Parameter	Groups				P value
	Control (n=21)	37ºC (n=21)	21ºC (n=21)	4ºC (n=21)	
TM (%)	60.48 ± 2.79^a	72.75 ± 3.43^b	69.13 ± 2.83^{ab}	59.57 ± 3.06^a	0.005
VCL (µm/s)	39.80±1.72^a	58.31±1.97^b	53.19±1.75^b	41.61±1.52^a	< 0.001
VSL (µm/s)	15.84±5.47^a	26.90±5.81^b	26.17±5.72^b	17.15±3.92^a	< 0.001
VAP (µm/s)	24.97±2.18^a	44.05±1.88^b	38.05±1.64^b	25.72±1.20^a	< 0.001
LIN (%)	38.08±5.65^a	45.42±5.69^{ab}	47.61±5.87^b	40.09±3.35^a	0.003
STR (%)	62.24±2.17^{ab}	61.09±1.92^a	68.03±1.59^b	64.60±1.18^{ab}	0.019
WOB (%)	62.10±5.77^a	74.92±5.25^b	69.00±5.97^b	61.24±3.62^a	< 0.001
Hyperactivity (%)	9.05±2.17^a	10.74±1.83^{ab}	14.10±1.54^b	10.53±1.35^{ab}	0.007
Plasma membrane and acrosome integrity (%)	32.02±6.25^a	65.29±5.08^c	49.10±5.28^b	36.17±3.82^a	< 0.001
High Mitochondrial membrane potential (%)	59.78±3.29	60.12±4.79	60.61±5.49	63.56±3.29	0.892

a, b, c: Lower-case letters on the same line indicate significant differences. TM: Total motility, VCL: Curvilinear velocity, VSL: Straight line velocity, VAP: Average path velocity, LIN: Linearity, STR: Straightness WOB: Wobble



Total motility: *p<0.001, VCL: *p<0.05; **p<0.01; ***p<0.001, VSL: **p<0.01; ***p<0.001, VAP: **p<0.01; ***p<0.001, LIN:*p<0.05; **p<0.01, STR: *p<0.05, WOB: *p<0.05; **p<0.01; ***p<0.001, Hyperactivity: **p<0.01, Intact Acrosome: **p<0.01; ***p<0.001

Figure 2: The detailed evaluation of significance in results of spermatological parameters of Angora buck semen frozen after nanopurification at different temperatures

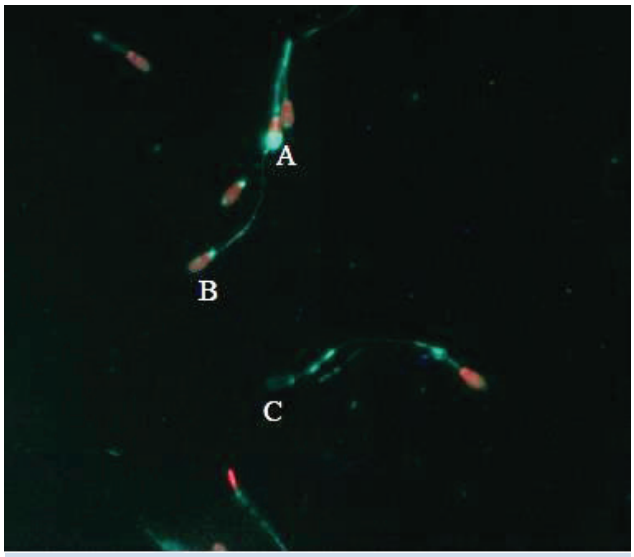


Figure 3. Analysis of semen samples obtained from Ankara bucks in terms of plasma membrane-acrosome integrity (PMAI) using FITC-PNA and PI stains after nanopurification at different temperatures: A- dead spermatazoa with Acrosome damaged with red head (PI) and green acrosome part (FITC-PNA); B- All dead spermatozoa with red head (PI) and an acrosome without colour by staining ; C- viable spermatazoa with acrosome integrity, whose head or acrosome is not stained

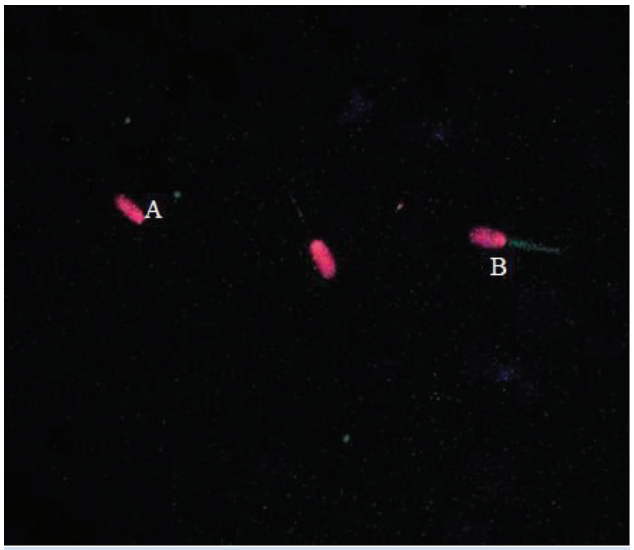


Figure 4. Analysis of semen samples obtained from Ankara bucks in terms of mitochondrial activity using JC-1 and PI stains after nanopurification at different temperatures: A- Dead spermatozoa without a mithocondrial activity, whose head is stained red (PI) ; B- Dead spermatozoa with mitochondrial activity, whose head is stained red (PI) and mitochondria recieved green or orange stain (JC-1)

DISCUSSION

Nowadays, semen selection procedures are frequently performed before various assisted reproduction techniques (semen freezing, IVF, artificial insemination, etc…) in many animal species in order to benefit in terms of fertility rate and success of ART (Al-Essawe et al., 2018; Barbas et al., 2018; Crespo-Felez et al., 2017; Galarza et al 2018; Liu et al., 1991; Martinez-Alborcia et al., 2012). The data obtained in the presented study are also similar to the data obtained in the studies on semen selection before other forms of assisted reproduction techniques. In this concept, it can be said that nanopurification of semen is an alternative semen processing method to increase the success of ART.

In addition to these, when the semen processing studies, before freezing of Angora Goat semen, are examined, it has been determined that dilution of buck semen with larger volumes before centrifugation, facilitates the removal of seminal plasma and that removal of seminal plasma improves the quality of post-thaw spermatological parameters results (Ritar and Salamon, 1982). In another study, it was concluded that washing the Ankara goat semen sample or removing the seminal plasma would be beneficial. And also it has been shown that adding bull or ram seminal plasma to the semen samples increase success rate of the results (Ari and Daşkın, 2010). In the presented study similar to these studies in the sense that the semen was processed and separated before freezing procedure, it was observed that the nanopurification procedure applied before the freezing of Ankara bucks semen had a positive effect on the post-thaw spermatological parameters. Accordingly, it can be said that nanopurification procedure could be a useful way of selection before freezing of buck semen.

It is known that the fertilization ability of the spermatozoa depends highly on the motility of the sperm cells, thus it is one of the most important criteria in determining sperm quality (Holt et al., 1997; Inanç et al., 2014). Parinaud et al., (1996) explained that there is a correlation between motility parameters and certain abnormalities. It was concluded that some risk factors lead to significant changes in sperm motility characteristics, which in turn indicate early changes in abnormal sperm quality (Lu et al., 2014). Even with the optimization of the protocols, decrease in motility and sperm kinetic parameters tend to occur during the cryopreservation, potentially due to the change in the temperature, the toxic and osmotic stress of cryoprotectants, and formation and dissolution of ice crystals (Watson, 2000). It has been reported in various stud-

ies that sperm motility and kinetic parameters can be used to predict the success of ART and fertility (Arias et al., 2017; Liu et al., 1991; Noguchi et al., 2014; Rodriguez-Martinez, 2007). Besides, some nanopurification studies stated that nanopurification process have positive effect on spermatological parameters (Durfey et al., 2017; Feugang et al., 2015; Grunewald et al., 2006; Lee et al., 2010; Nadalini et al 2014). Similiarly, in the present study, results of nanopurification at 37ºC TMOT, LIN VCL, and VSL parameters were higher than the control group while the semen samples nanopurified at 21ºC were superior to the control group in terms of LIN, STR, VCL, VSL, VAP and WOB. The reason for the higher progression obtained in the group nanopurified at 21°C could be caused by the gradual decrease of the temperature prior to the equilibration, due to the processing of the sperm nanopurification at room temperature. In this study, it can be concluded that nanopurification process is a suitable method for increasing the quality of buck semen in terms of motility and kinetic parameters and the nanopurification method could possibly be integrated within the cryopreservation steps.

In order to achieve successful fertilization, the acrosomal cap of the spermatozoa has a major significance. It has been shown that a variety of lectins, including PNA (*Arachis hypogaea*/peanut agglutinin) and PSA (*Pisum sativum* agglutinin), can bind to the glycoconjugates of the outer acrosomal membrane or the acrosomal matrix (Nagy et al., 2003). In the present study, PSA lectin-coated nanoparticles were able to bind to acrosome reacted sperm, both at 37ºC and 21ºC, resulting in a significantly higher percentage of the plasma membrane and acrosome intact sperm populations than in the control group. Similarly, in a recent study, PNA coated nanoparticles were used to purify the fresh semen on boar spermatozoa and it was concluded that the removal of defected spermatozoa increased acrosome intact and live spermatozoa as well as motility and progression. As a result of the increase in these parameters used for predicting the success of insemination and breeding performance, the researchers stated that the use of nanoparticles would significantly increase the insemination success (Feugang et al., 2015). In a comprehensive study, in which a 2-year field trial using semen from 4 bulls, semen purification with PNA nanoparticles and ubiquitin-binding nanoparticles were compared to non-treated samples, although no significant effect on sperm was observed by assessing the fluorescently tagged lectin PNA (P = 0.73) the combined pregnancy rates from AI trials (n=798) were found as 64.5%, 51.3% and 53.7% respectively (Odhiambo et al., 2014).

Mitochondria, located in the middle piece of mammalian spermatozoa, provide the energy required for flagellar movement (Grudzinskas and Yovich, 1995). Therefore, it is accepted that with the increase of motility, speed parameters and mitochondrial activation will increase (Volpe et al., 2009). JC-1 has been used to assess the mitochondrial membrane potential of spermatozoa in various species (Garner et al., 1997; Gravance et al., 2000). Generally, the spermatozoa with high potential have intact acrosome function and high fertilizing capacity as well as normal motility and morphology (Gallon et al., 2006; Grunewald et al., 2008). It has been reported that the mitochondrial integrity and membrane potential can be enhanced with the use of nanopurification combined with DGC in human sperm (Grudzinskas and Yovich, 1995). Besides, following the nanopurification, DNA integrity was determined to be in direct relation with the ratio of acrosome reacted spermatozoa (Lee et al., 2010). However, while nanopurification of boar semen, using nanoparticles coated with either Annexin V or PNA/PSA has led to an increase in kinetic parameters of sperm, no significant difference in terms of mitochondrial activation value was reported (Durfey et al., 2017). Similarly, in the present study, no significant difference was observed regarding the percentage of sperm with high mitochondrial membrane potential among the groups. The fluorescent microscopy might not be an effective method to objectively analyze this parameter as flow cytometry, considering the time needed to evaluate a good number of sperms from different fields, the fluorescein emission may shift or fade during the analysis. However, further detailed studies may aid in understanding the reason.

While the number of dead and damaged spermatozoa increases over time, there is more probability of interaction with the contemporary live spermatozoa. This fact is supported by the finding that the number of dead spermatozoa in the cryopreserved semen is both a function of non-viable spermatozoa counts in the fresh ejaculate as well as the time (Brinsko et al., 2003; Martinez-Alborcia et al., 2012). The plethora of dead and damaged spermatozoa in the fresh ejaculates of the bull are a consistent source of harmful H_2O_2 further giving rise to more harmful free radical species (Roca et al., 2016). In addition to that, dead spermatozoa negatively affect the motility characteristics and membrane integrity of live spermatozoa (Brinsko

et al., 2003; Shannon and Curson, 1972). Thus the current study included nanopurification as a way to eliminate the dead spermatozoa and their detrimental effects before further processing with cryopreservation.

Nanopurification before cryopreservation is the method used in studies on other species (Feugang et al., 2019; Grunewald et al., 2006; Odhiambo et al 2014). The objective of the nanopurification process is to remove the already low-quality spermatozoa from the environment by selection before freezing and to limit the production of by-products that could damage healthy spermatozoa during the freezing process (Durfey et al., 2017; Said et al., 2008; Said et al., 2005; Urbano-Bojorge et al., 2018). From this point of view, when the results of the sperm selection process with the nanopurification method in other species are examined, it has been reported that the nanopurification application has a positive effect on the research success. In the present study, similar to the studies mentioned, positive results were obtained in terms of post-thaw spermatological parameters. In the light of these data, it is thought that nanopurification process in sperm freezing can be used as a sperm selection method in the field of ART.

In conclusion, the current study, in which we aimed to determine whether nanopurification works and at what temperature gives the best results have shown that buck semen can be successfully nanopurified using iron oxide nanoparticles coated with Annexin-V, PSA, and silica, both at 37°C and 21°C, resulting with the selection of highly motile and acrosome intact sperm population. However, the adaptation of the nanopurification procedure into the equilibration process (4°C) did not provide an effective selection of the defective and immotile sperm. Repetition of the present study using a higher number of semen samples and supporting the results with other spermatological parameters like DNA integrity along with integrating the novel method of sperm selection with insemination protocols to obtain fertility proportions could aid in resolving the limitations of a preliminary study such as the current one. Further studies including artificial insemination programs of nanopurified buck semen at 37°C and 21°C are needed to develop a standard integration of nanopurification and cryopreservation protocols.

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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