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RESEARCH ARTICLE

Determination of alpha-napthyl acetate esterase activity of native and frozen-thawed bull sperm acrosome

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Taze ve dondurulup-çözdürülen boğa sperm akrozomlarının alfa-naftil aseteat esteraz aktivitelerinin belirlenmesi

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Öz

Amaç: Akrozomal enzimlerin seminal plazmaya sızıp sızmadığının belirlenmesi, suni tohumlama için sperma işlenmesi sırasında oluşan akrozom hasarının öncü bir göstergesi olabilir. Alfa-naftil asetat esteraz enzimi bu amaçla kullanılabilecek olan akrozomal enzimlerden biridir. Bu çalışmada, taze ve dondurup-çözdürülen boğa spermatozoonlarındaki alfa-naftil asetat esteraz enziminin pozitivite oranları ve lokalizasyonunun ışık mikroskobuyla belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: Çalışmada materyal olarak 7 boğadan alınan sperma örneği kullanıldı. Bu amaçla, taze ve dondurulup-çözdürülen sperma örneklerinden sürme frotiler hazırlandı. Bu frotilerde alfa-naftil asetat esteraz enzimi sitokimyasal metotla demonstre edildi. Hazırlanan preparatlar ışık mikroskobuyla incelenerek alfa-naftil-asetat esteraz pozitivite oranları, enzimin lokalizasyon ve dağılım tarzı belirlendi.

Bulgular: Alfa-naftil asetat esteraz enzimi pozitivitesi, spermatozoonun akrozomunda lokalize olan kırmızımsı-kahve renkli reaksiyon ürünü halinde gözlendi. Bazı hücrelerde enzimatik reaksiyon akrozom zarında dar bir band halinde gözlendi. Taze sperma örneklerinin alfa-naftil asetat esteraz pozitivitesinde belirgin bireysel farklılıklar gözlendi. Enzimatik reaksiyon, hücrelerin tamamında aynı şiddette değildi. Bazı hücrelerdeki reaksiyon nispeten daha zayıftı. Düşük spermatozoon vitalitesi ve motilitesi tesbit edilen taze sperma örneklerinin alfa-naftil asetat esteraz enzim pozitivitesi de düşüktü. Dondurup-çözme işlemi, örneklerin alfa-naftil asetat esteraz pozitivitesinde önemli düşüşe yol açtı.

Öneri: Akrozomal alfa-naftil asetat esteraz enzimi, boğa spermasının fertilite potansiyelinin ve dondurma işlemine uygunluğunun değerlendirilmesinde ve önemli bir parametre olarak dikkate alınabileceği sonucuna varılmıştır.

Anahtar kelimeler: Boğa spermatozoonları, akrozomal enzimler, dondurup-çözdürme, alfa-naftil asetat esteraz

Abstract

Aim: The assay of the leakage of acrosomal enzymes into seminal plasma might provide an early and sensitive evidence for membrane damage ocurred during freezing of semen for artificial insemination. Alpha-naphthyl acetate esterase is one of the acrosomal enzymes that can be used for this purpose. In the the present study, the positivity rates and localization pattern of alpha-naphthyl acetate esterase in the spermatozoa of both native and frozen-thawed bull semen samples were determined by light microscopy.

Materials and Methods: Semen samples of 7 bull were used as material. Smears were prepared from native and frozen-thawed semen samples. The enzyme was demonstrated by a cytochemical method. Specimens were observed under ligth microscope. Positivity rates of alpha-naphtyl acetate eseterase-positive spermatozoa, distribution and localization pattern of the enzymatic reaction were determined.

Results: The reddish-brown enzymatic reaction was peculiar to spermatozoon acrosome. In some cells, the enzymatic reaction was observed as a narrow band located on acrosomal membrane. The enzymatic reaction intesity was not similar in all cells and was weaker in some cells. The reaction product primarily located in the acrosome. There were large individual differences in alpha-naphthyl acetate esterase positivity rates of the native sperm samples. The native semen samples with low spermatozoon vitality and motility tended to display lower of alpha-naphthyl acetate esterase positivity rates. Freeze-thawing process significantly decreased alpha-naphthyl acetate esterase positivity rates of the samples.

Conclusion: Acrosomal alpha-naphtyl acetate esterase might be considered as a significant parameter in the asssessment of availibility for freezing and to determine fertility potential of the bull semen.

Keywords: Bull spermatozoa, acrosomal enzymes, freeze-thaw

Introduction

Acrosomal cap of the spermatozoon plays a vital role in the fertilization as it contains hydrolytic enzymes which enable the spermatozoon to penetrate through corona radiata cells and zona pellucida (Kakar and Anand 1984). During the early phases of acrosom formation, glycoprotein-rich acrosomal substances are synthesized in the granular endoplasmic reticulum and transported to the Golgi complex, where they become small vesicles and then form a single acrosomal vesicle (Jin et al 1995). This acrosomal vesicle associates with the anterior pole of the condensing nucleus of the spermatid during the cap phase (Susi et al 1971, Clermont and Rarnbourg, 1978).

Numerious acrosomal enzymes have been characterized from seminal plasma and spermatozoa of different animal species. They are mainly hydrolytic enzymes with optimum activity mainly at acid pH (Allison and Hartree 1970). Because that the acrosome developes from Golgi complex during spermatozoon metamorphosis, it contains large majority of hydrolytic enzymes, such as acid phosphatase (de Duve and Wattiaux 1966), neuraminidase (Horvat and Touster 1968), proteases and aryl sulphatase (Rowden 1967), β-N-acetyl glucosaminidase (Sellinger et al 1960), phospholipase A (Blaschko et al 1967, Reinauer et al 1968) proacrosin acrosin and hyaluronidase (McRoe and Williams 1974). Trypsin activity of rabbit (Yamane 1930), rat and horse spermatozoa (McRorie and Williams 1974) was also demonstrated. Neutral and alkaline enzymes are also found in the acrosome.

Hyaluronidase is a water soluble enzyme playing crucial roles in both acrosome reaction and fertilization. Following ejeculation, the enzyme is the first enzyme leaking from acrosome into the seminal plasma and digesting hyaluronic acid between cumulus oophoricus and corona radiata cells (McRorie and Williams 1974). The most exposed and loosely bound enzyme is needed first to start spermatozoon penetration of the egg components. Since, hyaluronidase is the most readily released enzyme from spermatozoon, seminal plasma from fresh and frozen-thawed bull semen is rich in hyaluronidase (Yang 1972).

Acid phosphatase (ACP-ase) and alkaline phosphatase (ALP-ase) have been demonstrated (Wislocki 1950) in different regions of human spermatozoa. Experiments of Allison and Hartree (1970), in different vertebrate spermatozoa, have revealed that the ACP-ase located in the acrosome. In the bull spermatozoa, ACP-ase positivity was observed in the galea capitis of spermatozoa, but Mann (1969) concluded that both acid and alkaline phosphatase activities might arisen from a possible contamination by seminal plasma phosphatases, but not resulted from phosphatases originally present in the spermatozoa.

Glucose-6-phosphatase (G-6-P-ase) is specifically distributed in the head, midpiece and the tail of the spermatozoa in all of the different mice strains. Adenosine triphosphatase (ATP-ase) is present in the head, midpiece and the tail regions of some mammalian spermatozoa (Mann 1969, Quinn and White 1968).

Monoamine oxidase (MAO) is an relatively stable enzyme and it has been observed in the head, midpiece and the proximal part of the spermatozoon tail (Zeller and Joel 1941). In the mouse, midpiece and tail of the spermatozoon contain α-mannosidase and β-N-acetylglucosaminidase (Mann 1969). Neuraminidase activity (Hartree and Srivastava, 1965, Srivastava et al 1965) and trimetaphosphatase which is different from the ACP-ase (Seguchi et al 1992) have been demonstrated in the acrosome.

Hyaluronidase and protease activities were observed in acrosomal lipoglycoprotein complex (Allison and Hartree 1970). They are lysosomal enzymes (Filipovic and Buddecke 1968) and show activity in dispersing the follicular cells and disrupting the zona pellucida (Srivastava et al 1965). Hence, these enzymes are also involved in the acrosomal reaction during fertilization (Jin et al 1995).

Cholinesterase activity has been observed in the head, midpiece (Nelson 1964) and in the tail (Mann 1969) of the bovine spermatozoa. Esterases are the lysosomal enzymes breaking ester groups. The non-specific esterase is is mainly found in the midpiece of the mouse spermatozoon (Mathur 1971). Their existence in acrosome shows the relation between lysomal and acrosomal enzymes. For the first time, 5-bromo-4-chloro indoxil acetate esterase was demonstrated in new guinea pig spermatozoa by Dalcq (1967). There is not detailed information on the activity and localization of alpha-naphthyl acetate esterase (ANAE) in the bull spermatozoa.

The cleavage of acrosomal membranes which results in leakage of the acrosomal enzymes earlier than the time is one of the earliest morphological signs of spermatozoon damage. Because that membrane cleveage severely decreases penetration and fertilization capability of the spermatozoon, integrity of the acromal membranes has vital importance in the spermatozoon quality. There is a close relationship between increased enzymatic activity of seminal plasma and poor semen fertility (Foulkes and Watson 1975). Thus, Gould and Bernstein (1973) have suggested that increase of concentrations in seminal plasma of hyaluronidase, which is a major componet of acrosomal enzymes, and oxaloacetic transaminase which is a non-acrosomal enzyme (Foulkes and Watson 1975) might provide an early and sensitive indication of membrane damage ocurred during semen processing for

artificial insemination (Foulkes and Watson 1975, Ganguli et al 1980). However, Breeuwsma (1972) concluded that such results are not sufficent in perdicting the semen fertility of a bull.

In the present study, positivity rates and localization of ANAE in the spermatozoa of both native and frozen-thawed bull semen samples were compared.

Material and Methods

Semen samples

Semen samples were collected by using an artificial vagina from 7 bulls (4 Simmentals, 3 Holsteins). The semen samples were frozen and thawed with the method of Bucak et al (2010). In the study, directives of Selcuk University Veterinary Faculty Experimental Animals Production and Research Center Ethics Committee (SÜVDAMEK 2017/60) were strictly followed.

The semen samples were analyzed for quality measures with a commercial computer assisted spermatozoon analysis system (CASA, Version 12 IVOS; Hamilton-Thorne Biosciences, Beverly, MA, USA). Spermatozoon motiliy and vitality, spermatozoon number per ml of the semen, frequencies of head and acrosomal anomalies plus midpiece and tail anomalies were determined of the samples. For enzyme histochemistry 0,5 ml of semen sample of each bull was taken prior to freezing process.

For enzyme histochemistry, the native samples were washed in a sperm washing solution containing 0,1% polyvinyl alcohol (PVA, MW: 30,000-70,000), 0,1%0,1 poly ethylene glycol and 275 mM sucrose. Following third washing, supernatant was discarded and each sample was diluted to 3 ml with the washing solution. From each semen sample 5 smears were prepared soon after the collections were made and air dried for 6 hrs at room temperature (22°C) before fixation. Frozen semen samples in plastic straws were thawed in a water bath (39°C) and processed for enzyme histochemistry as processed the native semen samples.

The smears were fixed in the glutaraldehyde-acetone solution (9 ml glutaraldehyde, 21 ml distilled water and 45 ml acetone) at -10°C for 3 min, rinsed 3 times in distilled water, then air dried.

ANAE histochemistry

Hexazotization of pararosaniline with sodium nitrite, dissolving alpha-naphthyl acetate was performed according to Çelik et al (1994). Final incubation solution was prepared according to (Maiti et al 1990). The smears were incubated for 2 h at 37°C, and then rinsed in distilled water. The cell nuclei were stained for 20 min with 1% methyl green solution at pH 4,2 prepared in acetate buffer according to Maiti et al (1990). Control specimens were prepared by incubating the smears in an incubation solution without substrate (Çelik et al 1994).

The specimens were observed under a light microscope (Nikon Eclipse, E-400 equipped with Nikon DS-5M DS5 Camera Head and DS-L1 Control Unit). The spermatozoa with a reddish-brown reaction porduct was regarded as ANAE-positive. Arithmetic means, standard deviations, variation coefficients of both native and frozen-thawed semen samples were calculated.

Results

ANAE reaction was observed in the spermatozoon acrosome as a reddish-brown staining with different intensities and localization patterns. In some cells acrosomal area displayed from weak to strong intensity, homogenenous and diffuse ANAE staining. The reaction was relatively weaker and displayed granular reaction at the acrosomal membrane border of some cells (Figure 1). ANAE positivity rates of the native semen samples were higher than those of the frozen-thawed animals (Figure 5).

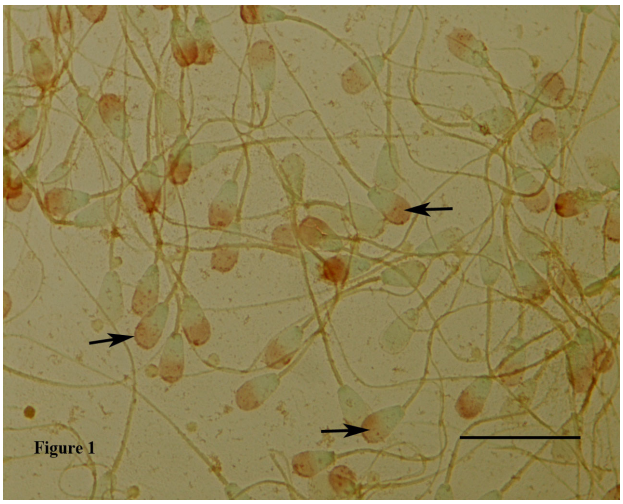

Figure 1. ANAE positivity of a native semen sample. The positivity is peculiar to the acrosomes (arrows). Enzyme histochemical ANAE demonstration. Magnification bar: 100 µm.

Frozen-thawed semen samples displayed weak ANAE intensity (Figure 2) and low level ANAE positivity rates. In some cells of the frozen-thawed semen samples, the ANAE reaction was seen as a narrow band located on the acrosomal membrane (Figure 3). Interestingly, in the specimens prepared from frozen-thawed semen samples with weak ANAE reaction, a weak ANAE positivity was also observed as pale brownish regions among spermatozoa clumps (Figure 4).

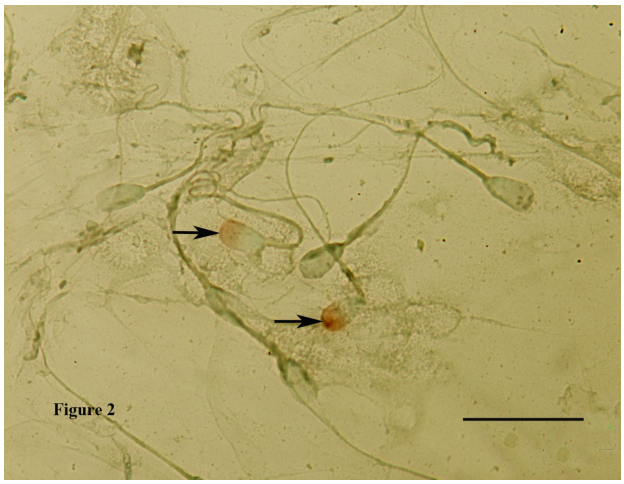

Figure 2. ANAE positivity of a frozen-thawed semen sample. Arrows depict ANAE positive spematozoa. Enzyme histochemical ANAE demonstration. Magnification bar: 100 µm.

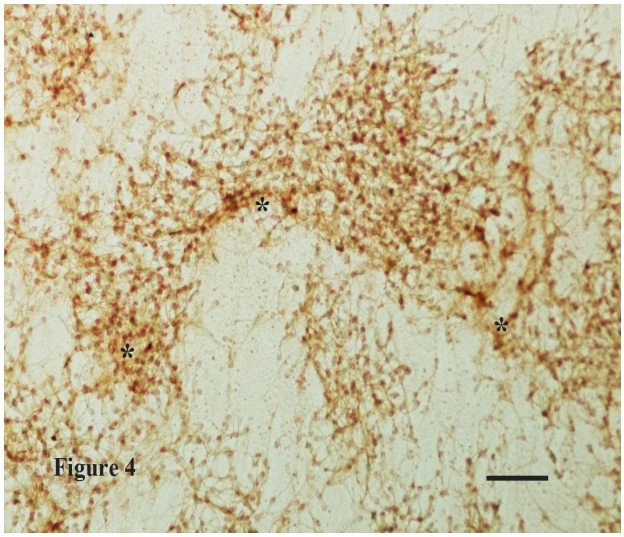

Figure 4. ANAE positivity of a frozen-thawed semen sample. ANAE positivity is also seen as pale brownish regions (asterisks) among sperm clumps. ANAE demonstration. Magnification bar: 500 µm. he

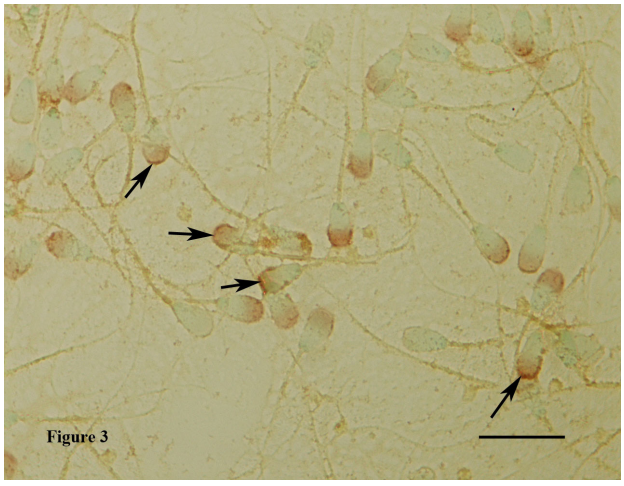

Figure 3. ANAE positivity of a native semen sample. In some cells, the positivity is seen as narrow band (arrows) located at the acrosomal border. Enzyme histochemical ANAE demonstration. Magnification bar: 100 µm.

Mean ANAE positivity of the native semen samples displayed individual differences and this finding was also observed in the frozen-thawed samples. Mean ANAE positivity value of the native semen samples was 73,5%, whereas the frozen-thawed samples had lower ANAE positivity (49,9%). There were large individual differences between ANAE positivity values of both the native and the frozen-thawed semen samples of the animals. The frozen-thawed semen samples of the animals with low spermatozoon vitality and motility tended to display lower ANAE positivity than their unfrozen semen samples (Figure 5).

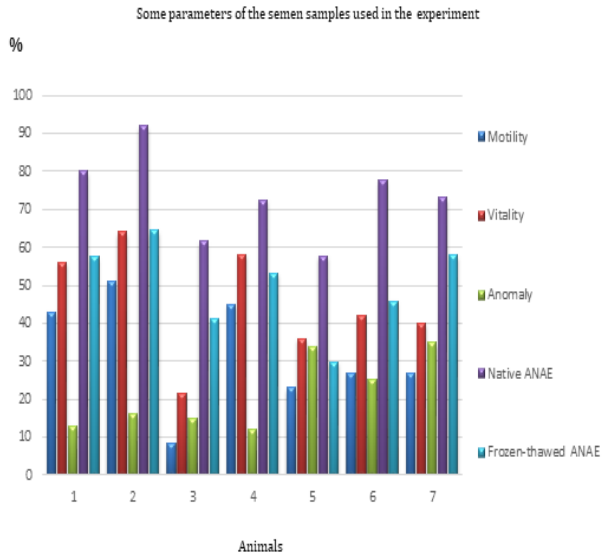

Figure 5. Some parameters of the semen samples used in the experiment.

Discussion

Acrosome of the mammalian spermatozoon covers the anterior half of the spermatozoon (Tulsiani et al 1998) and it is surrounded by plasma membrane. It has outer and inner acrosomal membranes. The mammalian acrosome contains a variety of proteolytic enzymes of which proacrosin/acrosin is best characterized (Yu et al 2006). Acrosome reaction is necessary just prior to penetrating spermatozoon into the zona pellucida. The acrosome reaction involves progressive vesiculation of acrosomal membranes and fusion with overlying plasma membrane (Almadaly 2012). Following, acrosomal enzymes are released and spermatozoa penetrates the zona pellucida, and spermatozoon membrane fuses with the egg plasma membrane (Diaz-Perez et al 1988). Spermatozoon damage has always been one of the hotspots in the field of reproductive biology. Spermatozoon fertilizing potential mainly depends on the integrity and functionality all of the cellular structures (Kumar et al 2016). Specifically, integrity of the acrosomal membranes is essential for fertilization becau-

se that they play crucial roles in acrosome reaction and oocyte plasma membrane fusion (Flesch and Gadella 2000). The acrosome membranes are also responsible for the preservation of acrosomal enzymes. Since the acrosome integrity and functionality are essential for fertilization, evaluation of the acrosomal status is important for semen quality control both in artificial insemination programs and fertilization studies (Almadaly 2012).

Although, cryopreservation is detrimental process for spermatozoa and causes to many undesirable structural and functional damages in the biological membranes and mitochondria (Kadirvel et al 2012, Kumar et al 2016), this technic became a valuable tool for large cattle industry. Freezing process, which exposes the spermatozoa to common effects of dilution, cooling, glycerolization and equilibration, freezing and thawing, cause damage to spermatozoon acrosome. Thus, the leakage of enzymes from spermatozoa is also used as the method employed for monitoring harmfull effects of cold shock and deep freezing in most domestic and laboratory animals (Kakar and Anand 1984). In an earlier report by Foulkes and Watson (1975), assay of the hyaluronidase activity in the seminal plasma has been suggested to provide an early and sensitive indication of spermatozoon damage during processing of semen for artificial insemination.

The cryopreservation also decreases the spematozoon vitality and increases the acrosome reaction (Medeiros et al 2002). However an intact acrosome with hydrolytic enzymes has vital importance for fertilization and its integrity is highly correlated with fertilization potential of the frozen semen (Chinnaiya et al 1980, Medeiros et al 2002). The integrity of the sperm plasma membrane has utmost importance in spermatozoon quality parameters (Kumar et al 2016).

In the past, the researchers substantially focused on the most commonly known parameters of semen, such as spermatozoon density, vitality, motility and morphology which partially uncover functional ability of the spermatozoon (Kumar et al 2016). The importance of the acrosome has increased because of its high sensitivity to cryogenic effects exposing during the deep-freezing processes (Chacarov and Mollova 1976). Acrosomal membranes of a number of animal species are very sensitive to deleterious effects occur during thawing of spermatozoa (Kumar et al 2016), becasuse that spermatozoa plasma membranes are destroyed by low temperature and high salt concentration being exposed during cryopreservation (Holt and North 1991). Moreover, toxic fatty acid peroxides are formed through peroxidation of membrane phospholipids, during storage of spermatozoa (Sinha et al 1996). Mammalian spermatozoa are seriously affected by lipid peroxidation, and then structural damage occurs in spermatozoon that lowers its motility and metabolism (Mann 1969).

There are significant differences between the response to deleterious effects of cryopreservation in different animal specieces (Azam et al 1998). Acrosome of some animal species, even of some inidividuals of the same species is more resistant to the cryopreservation than the other members (Kumar et al 2016). These researchers (Kumar et al 2016) also reported that the percentage of damaged spermatozoa increased significantly in frozen-thawed semen samples due to deteriation of plasma membrane during cryopreservation, and as a result of these process the viability of the semen samples declined (Kadirvel et al 2012).

This is the first study dealing with positivity rates and localization pattern of ANAE in both native and frozen-thawed bull spermatozoa. In the study, the ANAE reaction was mainly observed as diffuse granular reddish-brown reaction product peculiar to the acrosome and the reaction zone did not extend beyond the acrosomal border of the spermatozoon. The reaction intensity changed from weak to strong ANAE staining. A few cell displayed a band-shaped reaction pattern located in acrosomal membrane. A weak ANAE positivity was also observed in the seminal plasma surrounding spermatozoa clumps of the both native and frozen-thawed semen specimens with weak ANAE reaction. Nevertheles, the reaction in the seminal plasma was more intense in the frozen-thawed samples. This may be a significant indication of ANAE-leakage from acrosome into the seminal plasma. There were large individual differences between ANAE positivity percentages of the both native and frozen-thawed semen samples of the animals. The animals with low spermatozoon vitality and motility also tended to display lower ANAE positivity percentages in native, unfrozen semen samples. Similar tendeny was also observed in the frozen-thawed samples. Previous researchers (Januškauskas and Henrikas 2002) have suggested that there is a close relation between the fertility of semen and its measurable properties. The most important aim of the semen assessment is to determine one or a few parameters to evaluate the fertilizing ability of the semen. Many different methods have been attempted throughout the last decades, but only a few of them have been found to be practicable in the field of artificial insemination. In most of these studies, light microscopic evaluations of classical spermatozoon parameters, such as spermatozoon concentration, motility, morphology and viability have been used (Januškauskas and Henrikas 2002).

Conclusion

Based on the results of the present study, it was concluded that activity of acrosomal ANAE enzyme might be considered as an indicator of acrosomal membrane entity and a parameter for male fertility potential. The authors also strongly stress that further studies, including other acrosomal enzymes on larger populations of semen samples, should be performed.

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Conflict of Interest

The authors did not report any conflict of interest or financial support.

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