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Research Article

Comparison of Flow Cytometric Analysis and Eosin-Nigrosin Staining Methods for Determining some Morphological Characteristics of Bull Epididymal Spermatozoa [1]

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Abstract

The aim of this study is to investigate necrosis and apoptosis in epididymal bull spermatozoa before freezing and after thawing using the flow cytometric method and to compare this with eosin-nigrosin dyeing, which is the conventional method used in assessing of spermatozoa. The testicles from fourteen bulls at local slaughterhouse were used for this study. The proportions of live spermatozoa, total apoptotic, necrotic and early necrotic spermatozoa levels were observed via flow cytometry. Annexin V/PI fluoruscence dyeing was used to investigate the proprotions of apoptotic, necrotic, early necrotic and live spermatozoa for flow cytometry. The proportion of dead spermatozoa and protoplasmic droplets were determined using the eosin-nigrosin conventional dyeing method in fresh and frozen-thawed spermatozoa. The average dead spermatozoa count with flow cytometry was less than with the eosin-nigrosin method ($P<0.05$). Some morphological characteristics such as protoplasmic droplets could be determined with the eosin-nigrosin method; however, sperm subpopulations entering the death process (apoptotic, necrotic and early necrotic) could be defined clearly only with the flow cytometric method. As a result, combination of eosin-nigrosin dyeing method and flow cytometric analysis of sperm morphological evaluation could give better results of bull epididymal semen in comparison to eosin-nigrosin dyeing method alone.

Keywords: *Flow cytometry, Eosin-nigrosin, Morphological characteristics, Epidydimal spermatozoa, Bull*

Epididimal boğa Spermatozoonlarının Bazı Morfolojik Özelliklerinin Belirlenmesi Amacıyla Akım Sitometri ve Eozin-Nigrozin Boyama Yöntemlerinin Karşılaştırılması

Özet

Bu çalışmanın amacı, epididimal kaynaklı boğa spermatozoonlarının taze ve dondurulup çözdürüldükten sonra akım sitometri (Flow Cytometri) yöntemiyle analiz edilmesi, nekroz/apoptoz düzeylerinin incelenmesi ve klasik boyama yöntemi olan eozin-nigrozin boyama metoduyla kıyaslanması amaçlanmıştır. Bu amaçla yerel mezbahada kesilen 14 boğanın epididimislerinden elde edilen spermatozoonlar kullanılmıştır. Canlı spermatozoa, toplam apoptotik, nekrotik ve erken nekrotik spermatozoa düzeyleri akım sitometri yöntemiyle tespit edilmiştir ve bu amaçla AnnexinV+PI (Propidium İyodür) floresan boyama yöntemi kullanılmıştır. Taze ve çözdürülmüş sperma içerisindeki, ölü spermatozoa ve protoplazmik damlacık taşıyan spermatozoonların oranları eozin-nigrozin boyama yöntemi ile belirlenmiştir. Taze spermada, akım sitometri ile ölü spermatozoon sayısı eozin-nigrozin boyamada bulunana göre daha az olduğu görülmüştür ($P<0.05$). Protoplazmik damlacık gibi bazı morfolojik parametrelerin mikroskopik muayenelerle belirlenebildiği ancak ölüm sürecine giren spermatozoonların alt kategorilerinin (apoptotik, nekrotik, erken nekrotik) akım sitometri ile net olarak tespit edilebildiği görülmüştür. Sonuç olarak, klasik yöntemlere göre spermatozoa canlılığı tespitinde akım sitometri yönteminin klasik boyama yöntemlerine destek olarak ve daha etkin bir şekilde kullanılabileceği düşünülmüştür.

Anahtar sözcükler: *Akım sitometri, Eozin-nigrozin, Morfolojik özellikler, Epididimal spermatozoa, Boğa*

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INTRODUCTION

There are many ways to investigate spermatozoa to find out their morphological intactness, and motility or diagnose whether they are dead or alive. The fertilization capability of spermatozoa largely correlates with their morphological structure and motility. Various conventional staining methods (such as eosin-nigrosin, papanicolaou, mygrunwald-giemsa, hematoxylin-eosin) are used to determine whether spermatozoa are dead or alive. There seems to be no difference among them regarding effectiveness [1].

With the eosin-nigrosin staining methods eosin stains the spermatozoa with damaged membranes while nigrosin stains the background of the spermatozoa. A darker color thus, stained spermatozoa can be examined more easily due to the contrast between these dyes [2].

Some fluorescent probes are used for flow cytometric analysis and allow many spermatologic parameters to be investigated in a short time, quantitatively. In addition, the flow cytometrical method presents data with minimal statistical errors and enables dead and live spermatozoa to be determined spermatozoa subpopulations such as early necrotic, apoptotic can be singled out with this method [3,4].

There are some contradictory views concerning the correlation between apoptosis and some spermatologic parameters. Some researchers [5-10] showed negative correlation with sperm concentration, motility and normal sperm morphology [11]. It was reported that there was no relationship between sperm morphology and apoptosis [11,12].

A fluorescent probe which dyes all of the cells and propidiumiodude (PI), which dyes only dead cells, are used together for flow cytometric analysis to determine the percentages of live and dead spermatozoa [13]. Phosphatidyl serine (PS) allocated in the plasma membrane of the cytoplasmic side normally translocate from the inner face of the plasma membrane to the cell surface. This replacing occurs early period of apoptosis for most mammalian cell types. PS translocate to the cell surface can be detected by staining with fluorescein isothiocyanate (FITC) labeled annexine V (annexine V-FITC), a protein with an affinity for PS. Apoptotic cells can be defined by Annexine-V and PI in flow cytometry and this way is also reliable, simple and fast [14,15].

The aim of this study is to investigate necrosis and apoptosis in epididymal bull spermatozoa before freezing and after thawing using the flow cytometric method and to compare this with eosin-nigrosin dyeing, which is the conventional method used in assessing of spermatozoa.

MATERIAL and METHODS

Epididymal Sperm Collection

Testicles from 14 crossbreed bulls which did not have any morphological abnormalities were provided at the local slaughterhouse. Testicles were transported to the laboratory in styrofoam boxes within two hours. Semen was collected from each epididymis separately. Epididymal semen was recovered using the modified retrograde flushing (RF) method [16]. No semen extender was used for this process except air pressure. Cauda epididymis and ductus deferens were isolated from testicle tissues and the lumen of the ductus deferens was cannulated and perfused with air. The cauda epididymis was incised to recover pure epididymal semen after being swelled by air pressure.

Sperm Evaluation

Spermatozoa concentration was determined by Neubauer haematocytometer before further processing of semen. The sperm samples from all the groups were evaluated for total motility and morphology. Total, motility was assessed subjectively (to the nearest 5%) by phase contrast microscopy (magnification 200) after dilution with the semen extender at 37°C.

Sperm morphology was evaluated with a phase contrast microscope at 1000x magnification after eosin-nigrosin staining. Two separate smears were prepared for each epididymis. A total of 200 cells were counted and the results are presented as percentages (spermatozoa viability and spermatozoa with a proximal and distal protoplasmic droplet).

Sperm Freezing

Tris-citric acid extender containing egg yolk was used as a cryodiluent (pH 7.0; osmotic pressure, -300 mOsm/kg). The spermatozoa were diluted in this extender, fructose 0.2% (wt/vol; Merck, Germany), glycerol (7%; vol/vol; Merck, Germany) and egg yolk 20% (vol/vol).

The spermatozoa collected from each pair of testicls were pooled and diluted with extender in a single step ($40x10^6$ spermatozoa/ml). Diluted semen was packed in straws (0.25 ml, IMV, France) and they cooled to 4°C for 3 h. The straws were kept above liquid nitrogen vapours for 10 min and then plunged into liquid nitrogen. Semen was used after 24 h, semen straws were thawed at 37°C for 30 sec in water bath for assessment of postthaw evaluation.

Flow Cytometric Analysis

To detect apoptosis, sperm cells were stained and analyzed by BD FACS ARIA II flow cytometry. For staining firstly epididymal semen was centrifuged at 500 g twice for 10 minutes after being diluted in PBS (FITC/Annexin V-BD Pharminge-US). The binding solution was added after removing the supernatant, and the spermatozoa concentration was adjusted to $1x10^7$ cells/ml. 100 µl of extended semen were transferred from the last mixture to 5 ml tubes and 5 µl *fluorescein* isothiocyanate (and 5 µl

propidium iodide (PI) were added to this mixture. Aliquots of semen were diluted in annexin-V-binding buffer [10 mMHepes/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$] to a concentration of $1x10^6$ cells/ml. These tubes were incubated for 15 min at room temperature in the dark after being gently mixed. An additional 400 µl of binding buffer was added and the final concentration of $1x10^5$ cells/ml was inserted into each tube prior to flow cytometric evaluation, which was conducted within 30 min.

The total spermatozoa population was detected for each sample prior to detailed evaluation, following which necrotic (Q2-1), early necrotic (Q1-1), apoptotic (Q4-1) and live spermatozoa (Q3-1) were evaluated in the charts *(Fig. 1)*.

Statistical Analysis

The data were expressed as arithmetic means and standard error (X ± SEM). Statistical analyses were performed using the SPSS 13.0 version for Windows (SPSS Inc., Chicago, IL, USA). Independent Samples *t*-test was performed to estimate the statistically significant difference between the percentages of fresh and frozen-thawed spermatozoa concentrations with total motility. Independent Samples *t*-test was performed to estimate the statistically significant difference between the percentages of dead spermatozoa with eosin-nigrosin staining and necrotic spermatozoa concentrations with flow cytometric analysis in fresh spermatozoa. The differences were considered significant when the P value was less than 0.05.

RESULTS

The average motility was 75.36% and 41.07% in fresh and frozen thawed semen samples (n=14) respectively. The difference of between fresh and frozen thawed semen was significant (P<0.05). The percentage of dead spermatozoa was 16.71% with the eosin-nigrosin staining method but 11.18% dead (necrotic), 27.36% early necrotic and 3.01% apoptotic spermatozoa were found with flow cytometric analysis in fresh semen *(Table 1)*. The percentage of dead spermatozoa was 14.00% with the eosin-nigrosin staining method after thawing. In the thawed semen, 13.62% dead (necrotic), 29.14% early necrotic and 4.89% apoptotic spermatozoa were found by flow cytometric analysis. The difference in the rates of dead sperm was statistically significant between the flow cytometric analysis and eosin-nigrozin staining methods (P<0.05) *(Table 1)*.

Dead sperm rates were found to be statistically different between fresh and frozen with eosin-nigrosin staining (P<0.05). Flow cytometric examination indicated that the number of necrotic, early necrotic and apoptotic sperm were higher in the frozen semen, but these differences were not statistically significant (P>0.05) *(Fig. 2)*.

DISCUSSION

The present study compared the findings obtained from two methods widely used for the detection of live

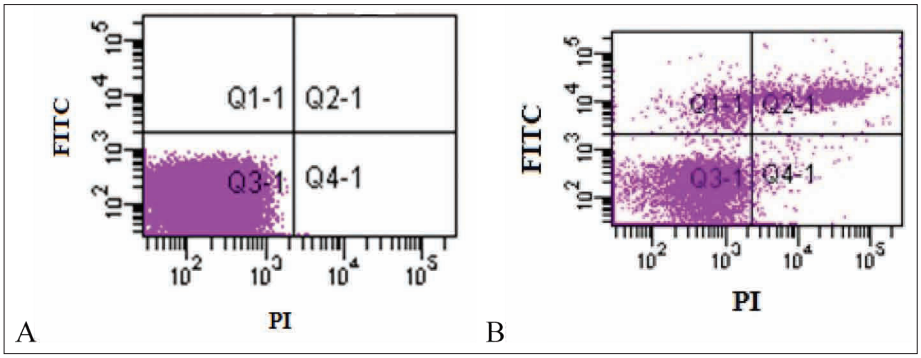
Table 1. *Comparison of eosin-nigrosin and flow cytometric analysis for fresh and after thawing epididymal spermatozoa*
Tablo 1. *Taze ve çözdürme sonrası epididimal spermatozoanın eosin-nigrozin boyama ve akım sitometrik analiz ile kıyaslanması*

Methods	Parameters (%)	Fresh (Mean±S.E.M)	After Thawing (Mean±S.E.M)
Eosin-nigrosin	Total motility	75.36±2.37	41.07±2.73
	Dead spermatozoa*	16.71[a]±1.18	14.00±0.91
	Proximal droplet	3.89±0.45	3.26±1.41
	Distal droplet	19.54±0.67	17.91±1.07
Flow cytometry	Live spermatozoa	58.24±5.18	52.37±4.42
	Necrotic sp.*	11.18[b]±1.17	13.62±1.79
	Apoptotic sp.	3.01±0.70	4.89±0.76
	Early necrotic sp.	27.36±4.31	29.14±2.72

** Means within same columns with different superscripts differ significantly (P<0.05)*

Fig 1. A- Number of total sperm cell of flow cytometric measurement, **B-** Necrotic (Q2-1), early necrotic (Q1-1), apoptotic (Q4-1) and live spermatozoa (Q3-1)

Şekil 1. A- Akım sitometrisinde toplam sperm sayıları, **B-** Nekrotik (Q2-1), erken nekrotik (Q1-1), apoptotic (Q4-1) ve canlı spermatozoa (Q3-1)



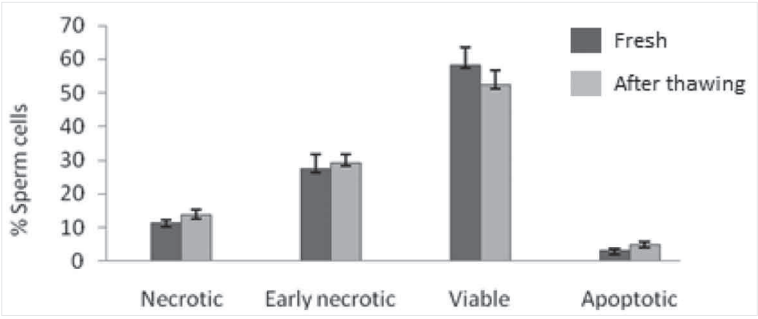


Fig 2. Different sperm subpopulations averaged fourteen bulls (mean ± SEM), fresh semen and after thawing

Şekil 2. 14 farklı boğaya ait taze ve çözdürülmüş sperm alt populasyonlarınının ortalamaları (mean ± SEM)

and dead spermatozoa (eosin-nigrosin staining and flow cytometry). The eosin-nigrosin staining method accounted for statistically higher percentage of dead sperm than the flow cytometry analysis ($P<0.05$).

Flow cytometric analysis were quantitatively clearly identified phase of cell dead processing for example early necrotic, necrotic, apoptotic sperm and live spermatozoa rates although dead spermatozoa could be determined subjectively with eosin-nigrosin staining. Some morphological characteristics, such as distal and proximal droplets on spermatozoa, can be determined with the eosin-nigrosin staining method; however, this observation was not possible with flow cytometry.

There is no significant difference among staining methods (Eosin-nigrosin, Hematoxylin-eosin, Caserette stains etc.) of spermatozoa based on the findings [17]. The eosin-nigrosin staining method is widely used for the evaluation of sperm because it is simple and rapid, but this conventional staining method for the evaluation of sperm is very subjective because only a very limited number of dead sperm can be counted [18].

Dyeing time, sperm concentration, characteristics of the semen extenders or some factors affecting the stability of the sperm membrane intactness are involved in eosin dyeing processes could alter the ratio of intact sperm [2,19].

Chalah and Brillard [20] reported that in terms of sperm membrane integrity flow cytometric analysis was a more effective method compared to eosin-nigrosin staining. Foster et al.[21] reported that number of live sperm with eosin-nigrosin was about 12.5% more compared to flow cytometric measurement. Johansson et al.[18] datas' were compatible with Foster's finding [21].

In the present study, the number of dead sperm with eosin-nigrosin staining method was higher than the data obtained by flow cytometric analysis. The reason for this was that early necrotic sperm cells were not considered to be dead sperm. When the numbers of early necrotic and necrotic sperm are accepted together as dead cell, our findings were similar to those of Foster et al.[21] and Johansson et al.[18]. The live sperm ratio can be determined inaccurately since the early necrotic sperm cell counts could vary depending on the amount of dye with eosin-nigrosin staining method.

Recent research shows apoptosis as an important mechanism that regulates spermatogenesis. Spermatozoa potential is greatly reduced during spermatogenesis and this continues throughout life. The number of germ cells can be supported by the sertoli-cells by eliminating abnormal spermatozoa via apoptosis [15,22-24].

Apoptotic cells, early necrotic and necrotic cell can be detected by flow cytometry, and but cannot be detected with conventional dyeing methods. In contrast, with the eosin-nigrosin method, morphologically abnormal spermatozoa can be distinguished. Relationships between abnormal morphological characteristics and the low fertilization ability of spermatozoa can be versatile. In this respect, the relationship between fertility capabilities and the morphological assessment of spermatozoa is difficult to define [25].

The main purpose of sperm analysis is to evaluate the sperm sample in a realistic, inexpensive, objective and quick way in terms of fertility. However, these criteria cannot be achieved in many laboratories and the findings cannot demonstrate clearly the impact on fertility [26]. Cell shape and structural features, such as morphological characteristics labeled with a fluorescent dye, can be determined by flow cytometry. Moreover, spermatozoa can be evaluated for many parameters and fertility properties simultaneously and objectively [27,28]. In addition, it is possible to evaluate a sample with tens of thousands of sperm in a few minutes by flow cytometry and it can offer a high level of reliable information compared to fluorescent microscope evaluation [29]. The subjective methods used to evaluate spermatozoa with flow cytometry may be a better estimate of fertility. In addition, conventional laboratory tests for predicting fertility in spermatozoa along with the use of flow cytometry can give better results [30].

In a study that investigated the sperm membrane using the eosin-nigrosin staining method, flow cytometric analysis, the samples with high levels sperm membrane integrity gave compatible results with these three methods. However, in cases where a high level of sperm membrane damage is observed, eosin-nigrosin staining gives more variable results compared to flow cytometric analysis [21].

In this study, the number of dead sperm obtained with eosin-nigrosin staining was higher than that found by flow cytometry (P<0.05). Our findings were similar to Foster et al.[21]. These findings may indicate that the eosin-nigrosin technique has many limitations, including a relatively low number of sperm (often only 100 or 200 cells) typically counted and subjectivity in the sperm sample evaluation as reported by Johanson et al.[18].

Klimowics-Bodys et al.[31] reported that the eosin-nigrosin staining method used in the morphological assessment of spermatozoa was cheap and practical, but this technique allows the assessment of a limited number of spermatozoa. Thousands of spermatozoa can be examined by flow cytometry in a short time; thus, it is a valuable and objective method compared to conventional methods.

In the present study, using the eosin staining method for the detection of live or dead spermatozoa was a subjective assessment that, in this respect, may result in some errors in sperm assessment depending on different levels of dye uptake. However, the apoptotic or necrotic phases of spermatozoa with flow cytometric methods can be clearly identified and the information can be obtained in more detail by this method compared to eosin-nigrosin staining method.

In this respect, determining the viability of spermatozoa with flow cytometry is more effective than conventional staining methods such as eosin-nigrosin. Flow cytometric analysis can be used to support conventional methods. As a result of this study, eosin staining method plus flow cytometry can be considered an advantage for the morphological assessment for epididymal bull spermatozoa. Further studies are necessary to determine the relationships between the morphological characteristics and motility of early necrotic and apoptotic spermatozoa.

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