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Comparison of Ovine Spermatozoal Morphological Features After Staining of Fixation and Assessment of Morphological Abnormalities in Dead/Live Spermatozoa

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Abstract: The objectives of this study were to (i) compare the effects of two staining techniques and one fixative solution on the morphological features of ram spermatozoa and (ii) evaluate the morphological features of dead/live spermatozoa by using Eosin-Nigrosin staining. The morphological features of raw spermatozoa were evaluated (by Phase Contrast microscopy) after staining with Eosin-Nigrosin mixture or Nigrosin (alone), or after fixation with Hancock's buffered formol saline solution. 42 raw pooled semen samples from 6 Kıvırcık rams were used. The percentages of spermatozoa with abnormal acrosomes and total morphological abnormalities averaged 3.97% and 14.78% in Hancock's solution, compared with averages of 10.07% and 18.54%; 7.97% and 18.45% for smears stained with Eosin-Nigrosin or Nigrosin (alone), respectively ($P<0.05$). The percentages of the same features for dead spermatozoa averaged 15.38% and 24.42%, compared with averages of 4.21% and 10.80% for live spermatozoa, respectively ($P<0.05$).

These results suggest that staining procedures were detrimental to the acrosomes and total morphological features, thus increasing the total morphological and acrosomal abnormalities in dead ram spermatozoa.

Key Words: Ram, spermatozoa, fixation, staining

Koç Spermasında Morfolojik Özelliklerin Fiksasyon veya Boyama İşlemlerinden Sonra Karşılaştırılması ve Ölü/Canlı Spermatozoitlerde Morfolojik Anormalitelerin Değerlendirmesi

Özet: Bu çalışmada (i) koç spermatozoitlerinin morfolojik özellikleri üzerine iki ayrı boyama yönteminin ve bir fiksatif solusyonun etkisinin karşılaştırılması ve (ii) Eosin-Nigrosin boyama yöntemi kullanılarak ölü/canlı spermatozoada morfolojik özelliklerin araştırılması amaçlanmıştır. Taze spermatozoitlerin morfolojik özellikleri Eosin-Nigrosin, sadece Nigrosin ile boyandıktan veya Hancock'un tamponlu formol-tuz solusyonu ile fikse edildikten sonra Phase Contrast mikroskobu kullanılarak incelendi. 6 adet Kıvırcık koçun, 42 adet taze, "pooling" yapılmış sperma örnekleri kullanıldı. Hancock'un solusyonu ile fiksasyondan sonra akrozomal ve toplam morfolojik anormaliteler sırasıyla ortalama %3,97 ve %14,78 iken, aynı değerler Eosin-Nigrosin boyama yönteminde %10,07 ve %18,54 ve Nigrosin boyama yönteminde ise %7,97 ve %18,45 olarak saptandı ($P<0.05$). Eosin-Nigrosin ile boyamadan sonra ölü spermatozoitlerde aynı morfolojik anormaliteler sırasıyla %15,38 ve %24,42 olarak bulunurken, canlı spermatozoitlerde ise sırasıyla %4,21 ve %10,80 olarak kaydedildi ($P<0.05$).

Sonuç olarak, koç spermatozoitleri için boyama işlemlerinin akrozomal ve toplam morfolojik özellikler üzerine zararlı etkisi olduğu, dolayısıyla ölü spermatozoitlerde aynı morfolojik özelliklere ait anormalitelerin arttığı kanısına varılmıştır.

Anahtar Sözcükler: Koç, spermatozoa, fiksasyon, boyama

Introduction

It is necessary to examine the cytostructural detail of spermatozoa and to assess fertility potential (an indication of fertilising capacity of the sperm of an animal) of a semen sample (1-10). Several studies have shown that the fertility of an animal is related to the morphological features of its spermatozoa (1-11). Morphological abnormalities of spermatozoa are associated with decreased fertility in rams (12). The morphology can be assessed by using either conventional light microscopy or Phase Contrast microscopy, with or without staining (4,11). The acrosomal integrity and acrosomal degeneration have also been examined by using many other staining or fixation techniques (1-3,5,13-20). Since the acrosomal integrity is related to fertility, accurate evaluation of acrosomal defects is essential (4,11). While Eosin-Nigrosin staining is routinely used in Artificial Insemination programmes, Nigrosin (alone) may be used for morphological studies of spermatozoa (21,22). The wet fixative solutions are reliable and also commonly used for the evaluation of spermatozoal morphological features (3,4,9,23). The methods that are used in assessing the morphological characteristics (including the acrosomal integrity) must be accurate and reliable. While the staining procedures are detrimental to acrosomal integrity, wet mounts are the simplest and most reliable ones for acrosomal studies (3,4). Furthermore, several studies have shown that false (non-physiological) acrosome reactions occur in dead spermatozoa as a post-mortem change, which is characterised by random breakdown and/or loss of the acrosomal and plasma membranes (13,14,19). These reports postulate that staining procedures may also increase the percentage of acrosomal defects and dead spermatozoa. In particular, the evaluation of acrosomal integrity of frozen/thawed spermatozoa is more critical in rams than in the other domestic animals (9,24,25).

Therefore, the objectives of the present study were to:

(i) compare the two staining techniques and one fixation method for evaluation of morphological characteristics of ram spermatozoa in fresh semen and

(ii) evaluate the morphological abnormalities of dead/live spermatozoa.

Materials and Methods

Animals used

The animals used were 6 Kıvırcık rams from the experimental/research station in the Veterinary Faculty of İstanbul University. They were fed with 1 kg dried grass and 1 kg crushed barley per ram/day and with water *ad libitum* and were kept in indoor boxes under 15-25ºC temperature.

Semen collection

Ejaculates from 6 rams were collected by electroejaculation (9) twice in one week and pooled in a tube. In total, 42 pooled ejaculates were used. The first 10 ejaculates from each animal were also evaluated to assess their spermatological characteristics: volume (ml), mass activity (0-4), progressive motility (%), concentration (10^9/ml), viability (%) and morphological abnormalities (%). The Eosin-Nigrosin staining was used for assessment of live/dead spermatozoa, while morphological abnormalities were evaluated by using Hancock's buffered formol saline solution.

Experimental design

This experiment was conducted to compare the effects of two stains and one fixative solution on morphological characteristics (particularly the acrosomal integrity) of spermatozoa in fresh samples. The pooled ejaculates were kept in a water bath at 32ºC.

The following methods were compared (9,26):

1. Eosin B- Nigrosin, in bidistilled water (Figure 2),
2. Nigrosin, in bidistilled water (Figure 2),
3. Hancock's buffered formol saline solution (Figure 3).

Since the main objective of this experiment was only to compare the effects of the two staining solutions and fixative solution upon the morphological characteristics of spermatozoa, no additional control group was included.

All evaluations were performed for both individual and pooled semen samples.

Stained smears were prepared by touching a coverslip to a drop of the mixture of stain and semen, and then drawing the mixture across the surface of a clean slide at an angle of 45º. Smeared slides were similarly prepared with each of the 2 stains used. In the Eosin-Nigrosin staining technique, one drop of Eosin B and semen each and two drops of Nigrosin were placed closely

at one end of the slide and then mixed gently for 10 sec with a coverslip and smeared. In the Nigrosin staining technique, one drop of semen and two drops of Nigrosin were placed at one end of the slide and, then smeared in the same way (4,9,26).

Fixation with Hancock's buffered formol saline solution was accomplished by pipetting 0.5 ml of semen in to eppendorf glass vials containing 1 ml of fixative. Wet mounts were prepared by placing one drop of fixed semen upon the centre of a clean slide and covering with 22x22 mm coverslips. Morphological characteristics of dead/live spermatozoa were evaluated by using Eosin-Nigrosin staining. Experimental procedures are summarised in Figure 1.

Spermatozoa were classified as Laving abnormal acrosomes (i), abnormal heads (ii), abnormal mid-pieces (iii), tail defects (iv) and total morphological abnormalities (v). In all procedures, 200 spermatozoa were evaluated *per* slide. More than one abnormality in a single sperm cell was considered as one total abnormality. To avoid morphological artefacts due to cold shock, the stains and the fixative were kept at 35ºC before use. All smears and wet mounts were evaluated by Phase Contrast microscopy at a total magnification of 1500X under immersion oil. Data were analysed by Duncan's multiple range and F tests (27).

Results and Discussion

The first 10 ejaculates from each of 6 rams were used to assess the fertilising capacity (fertility potential) of all individuals (Table 1). These results agree well with those of İleri et al. (9), who reported that the averages of volume, concentration, motility, mass activity and the percentage of abnormal spermatozoa in ram semen were 1 ml, 2-3 $x10^9$ sperm/ml, 80-90%, 4 (0-4), and 5-15%, respectively. Additionally, although the studies on Kıvırcık ram semen are quite limited, the present findings are also highly similar to those of Ak et al. (28,29), who showed that the averages of volume, motility, the percentage of live spermatozoa, concentration, and the percentage of total abnormal spermatozoa were 1.03 ml, 87.50%, 88.73%, 2.01 $x10^9$ sperm/ml and 12.05%, respectively. They also reported that the averages of volume, mass activity, motility, concentration, and the percentages of abnormal head, mid-piece, tail and total spermatozoa were 0.89 ml, 3.45 (0-4), 87.25%, 1.87 $x10^9$ sperm/ml, and 8.90%, 1.90%, 5.75% and 16.50%, respectively.

In raw, pooled semen, significantly lower ($P<0.05$) percentages of acrosomal, head and total morphological abnormalities were obtained on samples fixed in Hancock's formol saline solution than those on stained smears (Table 2). These low figures of morphological abnormalities (particularly those of acrosome) are of high importance, since the assessment of acrosomal integrity is essential for assessment of the fertilising capacity of spermatozoa (2). Therefore, the data from the acrosomal status must be considered a valuable one. Several studies have also shown that staining procedures are more detrimental on the acrosomal integrity compared to wet preparations (2-4). Additionally, the fixation procedure of

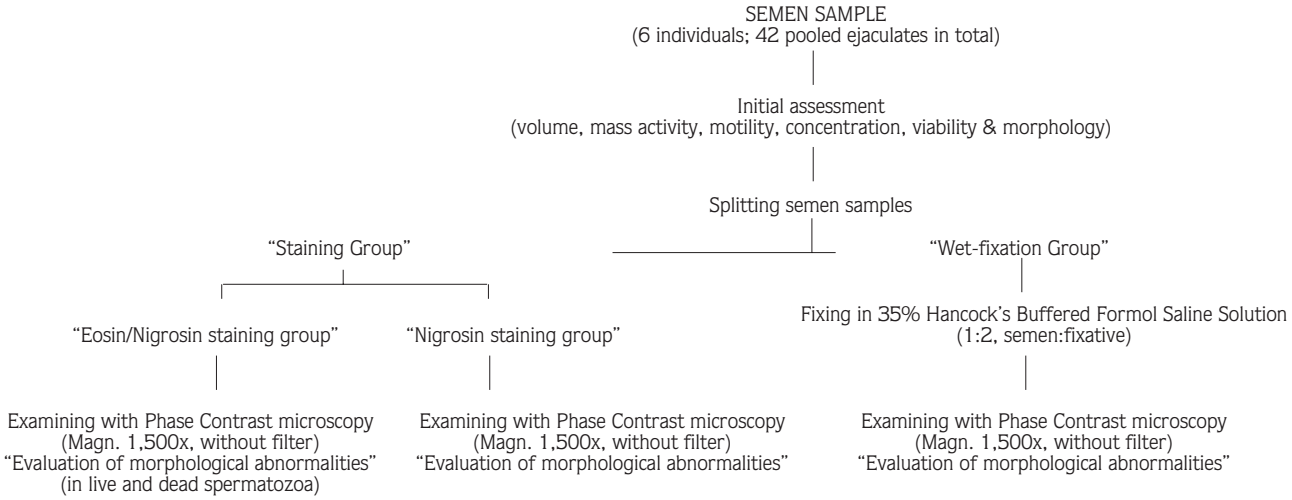

Figure 1. Experimental design.

Eosin stain, 5%	
Eosin B	5.0 g
Bidistilled water	to 100 ml
Nigrosin stain, 10%	
Nigrosin	10.0 g
Bidistilled water	to 100 ml

Figure 2. Composition of Eosin-Nigrosin stain (9).

Formalin (35%)	62.5 ml
Stock NaCl solution	150 ml
Stock buffer solution	150 ml
Bidistilled water	to 500 ml
Stock sodium chloride solution:	
NaCl	9.01 g
Bidistilled water	to 500 ml
Stock buffer solution:	
a-$Na_2HPO_4.2H_2O$	21.682 g
Bidistilled water	to 500 ml
b-K_2HPO_2	22.254 g
Bidistilled water	to 500 ml

To prepare stock buffer solution, 200 ml of (a) and 80 ml of (b) solutions were mixed.

Figure 3. Composition of Hancock's buffered formol saline solution (23).

wet samples is simpler than the preparation of stained smears (4). In the present study, it was also observed that the percentage of acrosomal abnormalities were higher in smears stained with Eosin-Nigrosin than those of Nigrosin alone ($P<0.05$). For the percentage of head and total morphological abnormalities, there was no significant difference between the two staining methods. It seems that both the use of a greater amount of stain and also the preparation technique may deteriorate the acrosomal integrity. Indeed, it has been reported that staining or preparation methods may alter the morphology of canine spermatozoa, artefactually (11). Comparing the stained smears, a higher percentage of tail defects and a lower percentage of mid-piece abnormalities were seen on smears stained with Nigrosin (alone) than those of Eosin-Nigrosin. Also, there was no significant difference between Nigrosin staining and Hancock's method for tail defects and mid-piece abnormalities ($P<0.05$). Differences of mid-piece or tail defects that were seen among the methods may be due to cold shock, hypoosmolality of the stain used or the skill of the investigators (3,11).

In dead or live spermatozoa, morphological features were also evaluated (Table 3). A higher percentage of

Table 1. Total average (mean ± SE) of spermatologic characteristics in fresh ram semen (n=10).

	Vol. (ml)	Mass Act. (0-4)	Motility (%)	Concentr. (10^9/ml)	Live/dead sperm (%)		Type of abnormality					
					Live	Dead	Acrosome (%)	Head (%)	Mid-piece (%)	Tail (%)	Total abn.(%)	Total norm.(%)
Total	0.75 ±0.33	3.63 ±0.68	80.33 ±11.11	2.39 ±1.11	77.45 ±10.81	22.55 ±5.16	5.81 ±5.16	1.24 ±1.14	1.98 ±3.38	5.46 ±5.38	14.39 ±8.08	85.61 ±4.01

Table 2. Effects of three methods of slide preparation on the percentage (mean ± SE) of abnormal morphological characteristics of spermatozoa (n=42).

Preparation	Type of abnormality					
	Acrosome (%)	Head (%)	Mid-piece (%)	Tail (%)	Total abn.(%)	Total norm.(%)
Eosin-Nigrosin	10.07[a] ±3.94	3.14[a] ±2.15	2.11[a] ±1.53	3.26[b] ±3.53	18.54[a] ±5.67	81.46[a] ±2.94
Nigrosin	7.97[b] ±2.90	2.76[a] ±2.56	1.52[b] ±1.17	6.16[a] ±4.54	18.45[a] ±6.58	81.55[a] ±3.25
Hancock's buff. form. saline	3.97[c] ±2.90	1.30[b] ±1.19	2.11[b] ±3.95	7.38[a] ±7.24	14.78[b] ±9.24	85.22[b] ±4.58

[a, b, c] Values with different superscripts in the same column are different ($P<0.05$).

Table 3. The percentage (mean ± SE) of morphological abnormalities of dead/live spermatozoa (n=42).

Sperm	Type of abnormality					
	Acrosome (%)	Head (%)	Mid-piece (%)	Tail (%)	Total abn. (%)	Total norm. (%)
Dead	15.38[a] ±5.72	3.80[a] ±3.59	2.38[a]±2.71	2.88[a] ±2.62	24.42[a] ±7.64	75.58[a] ±3.80
Live	4.21[b] ±3.00	1.54[b] ±1.76	1.61[a] ±1.44	3.19[a] ±4.68	10.80[b] ±5.11	89.20[b] ±2.45

[a, b] Values with different superscripts in the same column are different (P<0.05).

abnormal acrosomes, head and total morphological abnormalities were seen in dead spermatozoa than in those of live ones (P<0.05). Amongst these, the difference in the percentage of acrosomal abnormalities was more marked. As a matter of fact, it has been reported that there is an inverse relationship between the percentages of live spermatozoa with intact acrosomes and dead spermatozoa with detached acrosomes (13). Furthermore, it has also been shown that 29-81% of spermatozoa that had lost their acrosomal contents were, in fact, dead (14). Our results also agree with these reports.

Consequently, these results suggest that staining procedures were detrimental to total morphological features, particularly to the acrosomes, and thus increasing the total morphological and acrosomal abnormalities in dead ram spermatozoa.

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