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RESEARCH ARTICLE

Investigation of Relationships between DNA Integrity and Fresh Semen Parameters in Rams [1]

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Summary

The aim of the present study was to evaluate the correlation between the routine semen analyses and sperm DNA integrity, as assesed by TUNEL in fresh semen of rams in Kivircik and Awassi breeds in breeding season. Semen was collected by electro-ejaculation five times every other day. For that purpose, 50 fresh ejaculates from 6 Kivircik and 4 Awassi rams were evaluated. The mean percentages of semen volume, concentration, mass activity, motility, acrosomal defects, HOST and DNA fragmentation of Kivircik and Awassi rams were 1.3 ml, 1.9 x109, 3.3 (0-5 scale), 72.6%, 13.2%, 73.6%, 16.2% and 1.0 ml, 1.9 x109, 3.3 (0-5 scale), 72.5%, 11.1%, 77.9%, 15.9%; respectively (P>0.05). Sperm DNA fragmentation was correlated adversely with semen volume (r = -0.329, P<0.05), concentration (r = -0.188, P>0.05), mass activity (r = -0.349, P<0.05), motility (r = -0.448, P<0.01), acrosomal defects (r = -0.103, P>0.05) and HOS test (r = -0.513, P<0.01). This study provided clear evidence that most of the parameters evaluated herein related to DNA fragmentation at one point or the other. Hence, DNA structure evaluation of sperm might be a useful tool for accurate prediction of the male fertility in individual rams.

Keywords: *Ram, Fresh semen, DNA fragmentation*

Koçlarda Taze Sperma Parametreleri ve DNA Bütünlüğü Arasındaki İlişkilerin Araştırılması

Özet

Bu çalışmanın amacı, Kıvırcık ve İvesi ırkı koçlardan alınan spermaların, sezon içerisinde rutin sperma analizleri ve TUNEL ile yapılan sperm DNA bütünlüğü arasındaki korelasyonu değerlendirmektir. Sperma elektro-ejakülasyon yöntemiyle 5 kez ve birer gün aralıklarla alındı. Bu amaçla; 6 baş Kıvırcık ve 4 baş İvesi ırkı koçtan alınan 50 ejakülat değerlendirildi. Kıvırcık ve İvesi ırkı koçların ortalama sperma hacmi, konsantrasyonu, mass aktivitesi, motilitesi, akrozomal bozukluğu, HOST ve DNA fragmantasyonu yüzdeleri sırasıyla 1.3 ml, 1.9 x109, 3.3 (0-5 skala), %72.6, %13.2, %73.6, %16.2 ve 1.0 ml, 1.9 x109, 3.3 (0-5 skala), %72.5, %11.1, %77.9 ve %15.9 olarak bulundu. Sperm DNA fragmantasyonu; sperma hacmi (r = -0.329, P<0.05), sperma konsantrasyonu (r = -0.188, P>0.05), mass aktivite (r = -0.349, P<0.05), motilite (r = -0.448, P<0.01), akrozomal bozukluk (r = -0.103, P>0.05) ve HOS testi (r= -0.513, P<0.01) ile negatif korelasyon gösterdi. Bu çalışma, değerlendirilen çoğu parametrenin DNA fragmantasyonu ile bağlantılı olduğunu açıkça gösterdi. Dolayısıyla, DNA yapısının incelenmesi her bir koç ejakülatının fertilitesinin doğru olarak tespit edilmesi konusunda faydalı olabilir.

Anahtar sözcükler: *Koç, Taze sperma, DNA fragmantasyonu*

INTRODUCTION

In livestock breeding, the impact of male infertility upon the reproductive efficiency of farms is high, since a male animal can serve a large number of females, either by artificial insemination or by mating. Therefore, male infertility is observed commonly as increased return to oestrus rate or decreased lambing rate in ewes [1]. The widespread use of artificial insemination in domestic animals has encouraged the development of laboratory tests that accurately predict the individual fertility of rams.

In order to assess the potential fertility of rams, semen evaluation, complementary to the clinical examination, is useful [2,3]. Semen quality and its relationship to fertility are of major concern in animal production. The fertilization

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process involves complex biochemical and physiological events that cannot be measured solely by routine semen evaluation. The general methods for evaluation of semen quality have been primarily based on routine semen analyses (i.e., motility, morphology and acrosomal integrity), however such routine examinations have a narrow capacity for predicting the potential fertility of a given ejaculate [4-7]. Numerous studies were conducted on semen freezing [8-11], and rutine ram semen evaluation [7,12,13]. Also most of the studies explain the routine semen parameters and DNA integrty in man [14-16], especially in patients with miscarriage history. However, there appears no study available on the relation between rutine semen parameter and DNA integrity of fresh semen in rams.

Evaluation of sperm DNA damage can satisfy the expectations on the prediction of the outcome of assisted reproductive techniques (ART) than conventional sperm parameters. The most commonly used tests to measure sperm DNA damage are the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and the sperm chromatin structure assay (SCSA). The TUNEL assay measures both single- and double-strand DNA integrity, measures a definitive end point (presence of free 3 = hydroxyl groups), and can provide more meaningful information on the implantation potential of an embryo.

Therefore, the aim of this study was to evaluate the correlation between routine semen analyses and sperm DNA integrity as assesed by TUNEL in fresh semen of rams in Kivircik and Awassi breeds in breeding season.

MATERIAL and METHODS

Chemicals

PBS tablets and poly-$_L$-lysine were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Triton X-100 (10% stock solution) (11332481001) and an *In Situ* Cell Death Detection Kit were purchased from Roche (Roche Diagnostics GmbH, Mannheim, Germany). Proteinase K (003011) and antibody diluents were purchased from Zymed (Zymed, San Francisco, California, USA). Bovine anti-rabbit fluorescein (FITC) (Sc: 2365) and mounting medium (Sc: 24941) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Semen Collection and Preparation

A total of ten rams (6 Kivircik and 4 Awassi breeds) aged 3-5 years kept at Uludag University, Faculty of Veterinary Medicine in Bursa, Turkey, were used during the breeding season. Semen was collected five times every other day by an electro-ejaculation with 12 cm probe length, 2.5 cm in diameter and 12 V (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand) [17]. To collect semen, rams were restrained physically and a lubricated probe was inserted into the rectum with downward pressure upon the front of the probe, so the electrodes rested on the upper portion of the ampullary region. An electrical stimulation was applied for 4-8 sec. The electrostimulation was stopped briefly (3-4 sec) while further massage was applied with the probe. This cycle was repeated until a 1-2 ml of semen sample was collected (usually 3-4 electro-stimulations). After the collection, each ejaculate was placed in a warm water bath (30°C) and immediately assesed for the volume, concentration, mass activity (0-5 scale), motile spermatozoa (zero to 100%), acrosomal defects, plasma membrane integrity, and DNA fragmentation rates (%).

Semen Evaluation

All semen parameters were measured by the same person throughout the study. Sperm motility was evaluated subjectively using a phase-contrast microscope (Olympus BX 51) (400x) on a warm slide (38°C) [17].

Fluorescein Lectin Staining Assay (Florescein Isocyanate-conjugated Pisum Sativum Agglutinin [FITC-PSA]): Acrosomal integrity was assessed by using FITC-conjugated *PSA* [9]. Briefly, 20 µl of diluted semen was re-suspended in 500 µl PBS and centrifuged at 2.000 rpm for 20 min; the supernatant was then discarded. The spermatozoa pellet was re-suspended in 250 µl PBS. One drop of resuspended spermatozoa was smeared on a glass microscope slide and dried in the air. Air-dried slides were fixed with acetone at 4°C for 10 min, and the slides were covered with FITC PSA solution (50 µg/ml in PBS solution) in the dark for 30 min. Stained slides were rinsed with PBS solution, covered with glycerol, and examined under a fluorescence microscope. At least 100 spermatozoa per smear were evaluated for acrosomal integrity.

Hypo-osmotic Swelling Test (HOST): Sperm membrane integrity was evaluated using a method as described by Nur et al.[18] with minor modifications. The semen was submitted to HOS test. A volume of 20.0 µl of semen was added into 1 ml of warmed hypo-osmotic swelling solution (100 mOsm/l) containing sodium citrate (25 mmol/l) and fructose (75 mmol/l) and incubated at 37°C for 60 min. Immediately after the incubation, one drop of semen was placed on a clean glass slide, covered with onother slide and assessed within 5 min under phase-contrast microscopy (400 x). For each sample, a total of 100 spermatozoa were counted per slide and the percentages of swollen and curled tailed spermatozoa were recorded.

Sperm DNA Integrity by Terminal Deoxynucleotidyl Transferase-Mediated Fluorescein-TUNEL Assay: For the TUNEL technique, we used the *In Situ* Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with slight modifications. In brief, one drop of re-suspended spermatozoa was smeared on a glass slide and fixed with 10% formaldehyde for 20 min at room temperature. The slides were washed in PBS and stored at 4°C. Upon the

removal from storage, the samples were washed again in PBS (for three times, 5 min each). They were then treated in a humidified chamber with proteinase K for 10 min at room temperature, washed with PBS, treated with 3% H_2O_2 in distilled water for 10 min at room temperature and washed again with PBS. The slides were permeabilized with 0.1% Triton X-100 for 5 min on ice.

The permeabilized slides were incubated in the dark at 37°C for 1 h with the TUNEL reaction mixture, that contained terminal deoxynucleotidyl transferase (TdT) plus dUTP label. After the labeling, the samples were washed with PBS and analyzed immediately via fluorescence microscopy. Negative (omitting TdT from the reaction mixture) and positive (using DNase I, 1 mg/ml, for 10 min at room temperature) controls were included in each trial. At least 100 spermatozoa were evaluated for determining the percentage of TUNEL-positive sperm. Each microscopic field was evaluated first under fluorescence microscopy (40x) for determining the number of reactive sperm and then under phase-contrast microscopy) for determining the total number of sperm per field.

Statistical Analyses

Data were analyzed by independent samples T test. Spearman's correlation coefficient was used to assess the relationship between sperm volume, concentration, mass activity, motility, acrosomal defects, plasma membrane integrity and DNA fragmentation (TUNEL-positive) rate. All data were analyzed using the SPSS statistical package (SPSS 20.0 for Windows; SPSS, Chicago, IL, USA). Differences were considered significant when P<0.05.

RESULTS

The general means of the spermatological parameters in Kivircik and Awassi rams were summarized in *Table 1*. There were no significant differences in sperm volume, concentration, mass activity, motility, HOST, acrosomal defects and DNA fragmentation between the two breeds (P>0.05).

The correlation analyses between the semen parameters studied were presented in *Table 2* and *Fig. 1*. TUNEL assay demonstrated that spermatozoa with DNA fragmentation exhibited a green fluorescence *(Fig. 2)*. Sperm DNA fragmentation was correlated adversely with sperm volume (P<0.05), concentration (P>0.05), mass activity (P<0.05), motility (P<0.01), acrosomal defects (P>0.05) and swollen tailed spermatozoa obtained by HOS test (P<0.01).

Also, sperm functional plasma membrane integrity (HOS test) was correlated favourably with mass activity (P<0.01) and motility (P<0.01). Furthermore, the acrosomal defects were correlated with volume (P<0.01), while mass activity was correlated favourably with volume (P<0.05) and sperm concentration (P<0.05). Besides, there was a positive relationship between sperm concentration and volume (P<0.01).

DISCUSSION

Semen quality and its relation to fertility are of major concern in animal production. Quality tests are routinely used for determining the acceptability of processed semen

Table 1. *General means of the spermatological parameters in Kivircik and Awassi rams*
Tablo 1. *Kıvırcık ve İvesi ırkı koçların ortalama spermatolojik parametre değerleri*

Rams	Volume (ml) X±Sx	Sperm Concentration ($x10^9$) X±Sx	Mass Activity (0-5) X±Sx	Motility (%) X±Sx	Acrosomal Defects (%) X±Sx	HOST (%) X±Sx	DNA Fragmentation (%) X±Sx
Kivircik	1.34±0.16	1.90±0.17	3.31±0.19	72.59±2.24	13.16±0.93	73.59±1.75	16.19±1.87
Awassi	0.96±0.16	1.87±0.09	3.25±0.19	72.50±1.77	11.11±0.82	77.94±2.36	15.86±2.63

The values are the mean ± standard error of mean (SEM); There was no significant difference (P>0.05)

Table 2. *Correlation coefficients (r) between the results of semen characteristics and TUNEL*
Tablo 2. *Spermatolojik değerler ve TUNEL arasındaki korelasyon katsayıları (r)*

Spermatological Parameters	Sperm Concentration ($x10^9$)	Mass Activity (0-5) Scale	Motility (%)	Acrosomal Defects (%)	HOST (%)	DNA Fragmentation (%)
Volume (ml)	0.415**	0.301*	0.059	0.455**	0.073	-0.329*
Sperm concentration ($x10^9$)		0.536*	0.222	0.098	0.183	-0.188
Mass activity (0-5)			0.702**	-0.251	0.557**	-0.349*
Motility (%)				-0.086	0.601**	-0.448**
Acrosomal defects (%)					0.004	-0.103
HOST (%)						-0.513**

*Correlations are significant when * P<0.05, ** P<0.01*

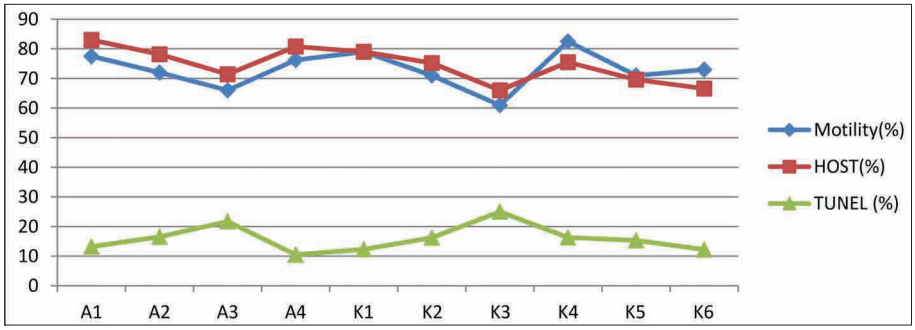
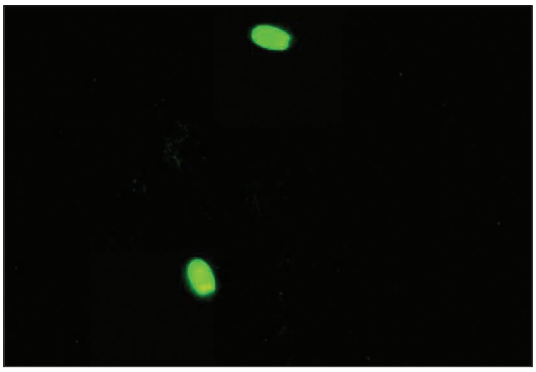


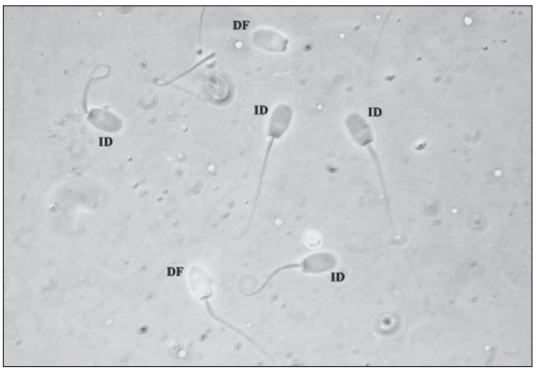
Fig 1. Correlation between the motility, HOST and DNA fragmentation rates (TUNEL)

Şekil 1. Motilite, HOST ve DNA fragmantasyon (TUNEL) oranları arasındaki korelasyon

Fig 2. DNA fragmentation (DF) and intact DNA (ID) detected by TUNEL assay

Şekil 2. DNA fragmantasyonu (DF) ve DNA bütünlüğünün (ID) TUNEL ile belirlenmesi





for breeding purposes. Thus, the accurate measurement of the quality is a major importance. The widespread semen evaluation generally includes the measurement of semen volume, sperm concentration, mass activity and the percentage of motile and morphologically normal spermatozoa [19]. Although some of these parameters are correlated with fertility in rams [7], several authors suggest that this information does not accurately predict whether a male is truly fertile [20,21].

The process of fertilization involves complex biochemical and physiological events that are not completely reflected in the conventional measures of concentration, motility, and morphology. Since the functional activity of the nuclear structure is crucial for the viability and fertilizing ability of spermatozoa, it is important to assess the DNA integrity of sperm [14]. A number of tests are currently available for the measurement of sperm DNA fragmentation. These include the TUNEL assay [7], the comet assay [14] and the SCSA test [7]. To the best of our knowledge, only very limited information is available for the correlation between semen parameters and DNA integrity in rams. In the present study, we evaluated the relationship between the routine semen analyses and sperm DNA integrity, as assesed by TUNEL in fresh Kivircik and Awassi ram semen during breeding season.

Semen collection methods, season, age and breed of rams may all affect the ejaculate volume. The mean semen volume varies between 0.6-2 ml in fertile ejaculates in different breeds [22]. Present study demonstrated that the general mean ejaculate volumes were 1.34 and 0.96 ml in Kivircik and Awassi rams, respectively (P>0.05).

Hafez [23] reported that the sperm motility is a prerequisite for sperm transportation to the fertilization area, but it is not indicative of the fertilizing ability. Also, the sperm acrosome has an effect on the fertilizing ability. Fresh ram sperm motility may vary between 70% [9,11] to 90% [22] and fresh acrosome defect may vary between 3.8% [11] to 6.7% [24] after different fixation and staining assays. The general means of motility and acrosomal defects were 72.59% *vs.* 13.16% and 72.50% *vs.* 11.11% in Kivircik and Awassi rams, respectively. The increasing rates of acrosomal defects may be related to the staining of assay. There are no studies documented on fresh ram semen about the acrosomal defects evaluated by FITC-PSA staining assay.

Sperm membrane integrity is a crucial parameter for the evaluation of sperm quality, because the intact plasma membrane is an essential borderline for survival of sperm cell [25]. Hypo-osmotic swelling test has recently been shown to be useful in detecting subtle changes in the functional integrity of ram sperm membranes [26]. Ollero et al.[27] reported that fresh semen exhibited 72% HOS test response. Similar results were also obtained for both breeds herein.

DNA damage may originate from improper packaging and ligation during spermatogenesis and epididymal sperm maturation [28]. The ram sperm DNA has the most degredation response under the similar experimental conditions when compared to other mammalian species [29]. Therefore, the evaluation of individual fresh semen DNA integrity could give important information related to the fertilizing ability. Therefore, we used TUNEL assay to

determine the DNA integrity. Nur at al.[7] reported that the mean percentage of spermatozoa with damaged DNA were 1.8% in Tris diluted ram semen. In men, the fertile percentage of fresh spermatozoa with damaged DNA was 12.9 % while in infertil men it was 48.8% obtained with commet assay [14]. Semen collection time, breeding season, ejaculation frequency, sexual arrest, age, breed, body condition and nutritional regime may all have an effect on semen quality and fertility [12,30]. The mean percentages of TUNEL positive spermatozoa were 16.19% and 15.86% in Kivircik and Awassi breeds, respectively.

Comparation of the semen parameters studied revealed that most of the parameters correlated to other paramaters at one point or the other. In this study, swollen tailed spermermatozoa corelated to motility (r: 0.601) and mass activity (r: 0.557). These findings are not suprising because the motility partly depends on transport of compounds across the membrane of spermatozoa [13]. Similar findings have been reported for sperm motility and HOST values earlier [13,31].

There exist remarkable numbers of motile spermatozoa from fertile donors containing fragmented DNA [32,33]. The degree of DNA damage in sperm cells leads to impairment of fertilization, embryo development [34-36], and reduced chance of producing live offspring [37-39]. The energy source of motilty that plays critical roles for sperm to reach to the fertilization site is provided by mitochondria, as controlled by sperm nucleus [40]. The failure of nuclear integrity also affects the sperm motility. In our study, the relationship between motilty and DNA integrity supports our theory. These relationships also present for the sperm plasma membrane functional integrity and the mass activity. There is an increasing interest in the use of DNA integrity related assays as a predictor of fertility potential [7]. A previous study showed that there is a positive relationship between sperm motility and DNA damage [41]. However, a markedly inverse correlation has been found between sperm motility and DNA integrity (r: -0.448, P<0.01). These findings were in agreement with results reported by Piasecka et al.[42] and Sheikh et al.[14]. Undoubtedly, poor-quality semen has a greater percentage of spermatozoa with DNA fragmentation than that of superior quality semen [32,33].

Functional integrity of sperm plasma membrane is a sign of healthy substance exchange needed for the viability of cells. The percentages of spermatozoa with damaged plasma membrane were higher than those sperm with DNA defects in both breeeds. Balasuriya et al.[15] reported that the sperm plasma membrane damage was higher than that of nuclear damage. It is expected that, the higher motility rates can be found in those spermatozoa that have solid membranes [43]. This study showed that the functional membrane integrity of sperm has a strict relationship with the motility. This condition was also proved in the earlier studies [18]. The increase in failure of the plasma membrane integrity results in a decrease in DNA integrity [44]. Sperm DNA fragmentation rate was correlated adversely with functionally active sperm population (P<0.01). However, according to Fatehi et al.[45], the cells with DNA damage did not show signs of functionally affected integrity of membranes and motility.

In summary, we have demostrated that sperm DNA fragmentation correlated unfavourably with the ejaculate volume (P<0.05), mass activity (P<0.05), motility (P<0.01), and HOST values (P<0.01). Also, there were positive correlations among HOST values, motility and mass activity (P<0.01). According to the results, we can conclude that testing DNA damage, in addition to standard methods may be a useful tool for the accurate prediction of the fertility in ram semen.

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