Relationship of Sperm Characteristics, in Reference with DNA Fragmentation, with Fertility of Some Sheep Breeds in Egypt

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ABSTRACT

The objective of this study was to adopt an accurate *in vitro* test (sperm DNA damage) to predict fertility through ram spermatozoa. Semen was collected once a week for 8 collection weeks from six rams of Rahmani and Romanov breeds, 3 rams from each breed. Semen was evaluated for ejaculate volume, sperm concentration, progressive motility, livability and abnormality of spermatozoa. DNA damage test was applied with Halosperm kit. Results showed significant (P<0.05) individual differences in percentages of abnormality and DNA damage in both breeds as well as in percentages of progressive motility, livability and total sperm output in Romanove breed. Breed had a significant (P<0.05) effect only on ejaculate volume and total sperm output, being higher in Rahmani compared to Romanove breed. Sperm abnormality, sperm cell concentration and DNA damage showed an opposite trend with respect to breed effect. Rams showed the best semen quality in both breed had the highest conception rate (CR). Mean of CR was higher (P<0.05) in Rahmani than Romanov breed (88.38 vs. 78.79%, respectively). Percentages of progressive motility and livability of spermatozoa were negatively correlated (P<0.05) with DNA damage and positively with CR only in Rahmani breed. In both breeds, DNA damage had (P<0.01) a positive and strong correlation with percentage of abnormal sperm where it was r= 0.557 in Rahmani and r=0.840 in Romanov. In addition, CR had a positive correlation with sperm cell concentration in Rahmani and Romanove rams. The correlation between CR and sperm DNA damage was strong and negative (P<0.01) in both breeds (r=-0.699 for Rahmani and -0.795 for Romanov). **In conclusion**, the present study indicated the strong negative correlations between sperm DNA damage percentage of abnormal spermatozoa and conception rates especially for Romanov rams which showed lower CR. Thus, *in vitro* sperm DNA damage test can used for evaluating fertility, in accompany with standard semen analysis assays.

***Keywords**: Ram, semen, DNA damage, conception rate.*

INTRODUCTION

Reproduction is one of the most important factors share the economics of livestock production **(Chenoweth, 1994)**. Evaluation of reproductive ability of rams is an integral part of managerial programs of sheep flocks. Specifically evaluation of male fertility prior to breeding is one of paramount factors to achieve breeding success **(Ford *et al*., 2009).**The breeding soundness examination of rams is used to evaluate and classify their breeding ability.

The differences in basic motility parameters among spermatozoa of sheep breeds were reported by **Kubovičová *et al*. (2011)**. Therefore, over years, several alternative methods of assessing semen quality have been sought to predict male fertility **(Larsson and Rodrıguez-Martınez, 2000)**. Standard semen analysis assays includes assessment of morphology **(Barth, 1992)**, motility **(Holt *et al*., 1997)**, intact acrosome **(Correa *et al*., 1997)** and membrane integrity **(Pérez *et al*., 1997)** of spermatozoa of livestock species. Several researchers reported relationships between sperm motility and *in vitro* and *in vivo* fertility **(Aitken, 1989; Vestergen *et al*., 2002)**. In this respect, **Kjaestad *et al*. (1993)** and **Correa *et al*. (1997)** found a significant correlation between motility and field fertility, while others **(Graham *et al*., 1980; Januskauskas *et al*., 1996)** did not prove this correlation. Also, **Farrel *et al*. (1998)** found a strong correlation between several motility characteristics and 59-day non-return to estrus rates of cows inseminated with frozen-thawed spermatozoa.

Defects in the DNA-chromatin packaging or fragmentation of DNA are likely to inhibit sperm motility as suggested by **Hourcade *et al*. (2010)**, as a result of changes in the overall sperm morphology, and eventually impede fertilization or subsequent embryo development **(Fatehi *et al*., 2006 and Eid e al., 2011)**. It also has been suggested that sperm DNA integrity may be a more objective marker of sperm function as opposed to the standard semen analysis **(Avdatek *et al*., 2010)**,

and it is more reliable to predict the potential fertility of semen using a combination of laboratory tests for prediction of different attributes of the sperm.

Several techniques had been also developed to detect DNA fragmentation levels including, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), comet assay, DNA Breakage Detection-Fluorescence, *in situ* Hybridization test, the chromomycin A3 test, Sperm Chromatin Structure Assay (SCSA) and Sperm Chromatin Dispersion (SCD) test **(Gorczyca, *et al*., 1993 and Fernández, *et al*., 2005)**. **Eid e al., (2011**) reported on Egyptian buffalo a high correlation between sperm motility morphology and DNA damage (indicated as comet value), when two groups of bulls with different potential in vivo fertility were compared. Also, the increased sperm DNA damage adversity affected embryonic development. The halo sperm is a new improved SCD test based on the principle that sperm with fragmented DNA does not produce halo of dispersed DNA loops, which characterize sperm with non-fragmented DNA **(Fernández *et al*., 2003)**. A number of tests are currently available for the measurement of sperm DNA fragmentation **(De Jonge, 2002)**. A negative correlation between sperm DNA fragmentation with fertilization rates and/or embryo development were reported **(Seli *et al*., 2004; Virro *et al*., 2004)**, however, no effect on pregnancy rate has been reported **(Seli *et al*., 2004)**.

Few reports studied the effect of DNA damage on fertility in sheep rams. Therefore, the objective of this work was to evaluate the potential influence of ram spermatozoa DNA damage, as an early test for predicting male fertility for Rahmani (Egyptian local breed) and Romanov (imported breed) sheep.

MATERIALS AND METHODS

The experimental work was conducted at Mehalet-Musa Experimental Station, belonging to Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt.

Animals:

Six rams (3 Rahmani and 3 Romanov) with an average ages 23.9±0.61 and 24.2±0.16 months and live body weight 48.12±4.57 and 43.71±3.89 kg, respectively were used for semen collection. Rams were fed 1 kg concentrate feed mixture (CFM) containing 15% crude protein (CP) and 70% total digestible nutrients (TDN), plus 0.400 kg rice straw and 0.250 kg Alfalfa hay during the collection period. Mineral blocks were freely offered Rams had free access to water all the day.

Semen Collection and Initial Assessment:

Semen was collected once a week from each ram using artificial vagina (using heated teaser ewe) for eight weeks during the period from August to September (24 ejaculates from each breed). Immediately after semen collection, each ejaculate assessed for volume, progressive motility and concentration of spermatozoa (Neubauer haemocytometer). Semen smears were prepared using nigrosin/eosin stain for determining the percentages of live and abnormal spermatozoa.

Sperm DNA Fragmentation Assessment

Sperm DNA fragmentation assessed in fresh semen with Halosperm kit (Indas Biotech, Madrid, Spain) according to **Fernandez *et al*. (2003)**. In brief, semen aliquots, purified by gradient separation, mixed with low melting point agarose, pipetted onto a pre coated glass slide with 0.65% standard agarose, covered with a coverslip, and left to solidify at 4°C. Cover slips removed, and the slides denatured with HCl 8% for 7 minutes and subsequently neutralized for 25 minutes with the neutralization solution provided by the kit. After washing in distilled water, the slides were dehydrated through an ethanol graded series (70, 90, and 100%), two minutes for each, at room temperature, then air dried. The cells stained with mix of Wright's staining solution (Merck, Germany) and PBS (1:1) (Merck, Germany) for 5-10 minutes. Slides washed in tap water and allowed to dry. A minimum of 200 spermatozoa per field were scored under the 100 ^{x} objective using bright field microscope.

Spermatozoa with large or medium size halo were classified as 'spermatozoa having no fragmentation'. Spermatozoa with small or without halo were classified as spermatozoa having DNA fragmentation (Fig. 1 *a* & *b*).

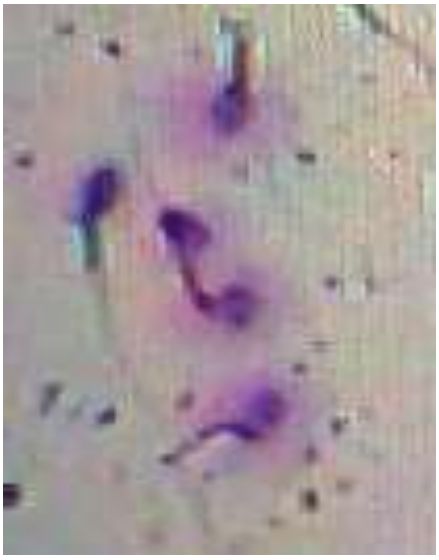

Fig. (1*a*): Spermatozoa having no DNA fragmentation with large or medium sized halo.

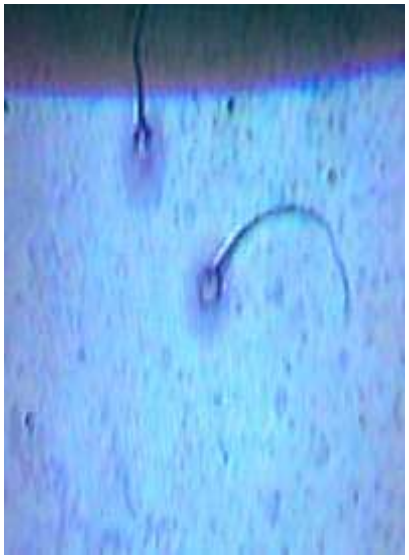

Fig. (1*b*): Spermatozoa having DNA fragmentation with small halo.

Fertility Trial:

Total of 67 clinically healthy, non-lactating ewes (34 Rahmani and 33 Romanov) were naturally mated during breeding season. Each ram mated naturally 11-12 ewes. Fertility rate determined as conception rate (proportion of ewes mated to that conceived) after pregnancy diagnosis using ultrasonography on day 56 post-mating according to **Martinez *et al*. (1998).**

Statistical Analysis

The obtained data were statistically analyzed using General Liner Model (GLM) procedures adapted by **SPSS (2008).** Duncan Multiple Range Test within SPSS was used to compare differences between means. Chi-square test used to determine the differences in conception rate.

RESULTS AND DISCUSSION

Semen physical characteristics:

Evaluation of semen physical characteristics (Table 1) revealed significant (P<0.05) individual differences in percentages of abnormality and DNA damage in both breeds as well as in percentages of motility, livability and total sperm output in Romanove breed. As shown in able (1), Rahmani breed and Romanov breed had good semen quality.

The present results showed that breed had significant (P<0.05) effect only on ejaculate volume and total sperm output, being higher in Rahmani than Romanove breed, while sperm abnormality, sperm cell concentration and DNA damage showed significantly (P<0.05) an opposite trend between the two breeds (Table 1). These results may reflect better semen quality in Rahmani than in Romanove breed. Similar trend on semen quality was reported comparing Egyptian Awassi and Ossimi rams **(Abdel-Hakeam *et al*., 1978; Abou-Ahmed *et al*., 1986; Seida *et al*., 1986)**. In a study performed by **Abdel-Rahman *et al*. (2000)**, live sperm percentage were significantly higher in Najdi and Naemi breeds than in Merino, Somalian and Sudanese breeds. Moreover, the effect of breed on sperm motion characteristics was reported by **Kumar *et al*. (2010)** on Malpura and Barat Merino rams. In goats, **Taha *et al*. (2000)** and **Ayoub *et al*. (2013)** found significant difference in sperm cell concentration between Awassi (imported from Syria) and local Awassi bucks in Egypt. On the other hand, **Hassanin *et al*. (2013)** found that overall mean of ejaculate volume, sperm

motility, live sperm percentages and sperm cell concentrations were not significantly affected for Najdi and Harri sheep breeds. Also, **Aller *et al.* (2012)** reported no effect of ram breed on the semen volume.

Table (1): Semen physical characteristics of Rahmani and Romanov rams.

Ram (no.)	Semen volume (ml)	Progressive motility (%)	Live sperm (%)	Abnormal sperm (%)	Sperm concentr. (x10^6/ml)	Total sperm output (x10^6/ml)	DNA Damage (%)
Rahmani rams:							
1	0.68±0.067	77.5±2.11	78.6±2.07	8.0±0.46^a	250.5±14.88	158.2±13.68	15.0±0.53^a
2	0.65±0.067	86.9±3.77	86.5±3.97	6.1±0.64^b	260.4±12.51	159.4±15.96	9.1±1.3^b
3	0.82±0.126	78.1±4.53	77.4±3.68	7.6±0.56^{ab}	245.8±15.89	181.8±22.31	14.3±1.03^a
Mean± SE	0.72±0.055^A	80.83±2.18	80.83±2.03	7.3±0.35^B	252.0±8.10^B	166.5±10.02^A	12.8±0.78^B
Romanov rams:							
1	0.39±0.017	82.5±5.43^a	80.4±5.11^a	6.5±0.43^b	311.1±3.07	119.8±5.48^b	13.5±0.82^b
2	0.49±0.023	67.5±1.89^b	67.9±2.28^b	14.0±0.76^a	305.5±6.07	150.9±7.45^a	20.6±1.1^a
3	0.39±0.015	75.6±5.3^{ab}	74.8±5.21^{ab}	7.6±0.32^b	310.5±4.03	122.9±6.04^b	15.6±0.56^b
Mean± SE	0.42±0.015^B	75.8±2.79	74.3±2.66	9.4±0.75^A	309.0±2.57^A	131.2±4.57^B	16.6±0.78^A

Values in the same column for each breed with different superscripts are significantly different (P<0.05).

Male fertility declines when DNA Fragmentation Indices (DFI) exceed 10-20% in bulls, and 8% in boars **(Evenson and Wixon 2006)**. The magnitude of the level of sperm DNA damage found in the endangered species (gazelles) with high levels of inbreeding is enormous when compared to out bred populations **(Lopez *et al.*, 2010)**. Comparable with the results of DNA damage found in the present study, found highly significant differences in the percentage of DNA damage of sperm between inbred and outbred group of rams. In current study the value of sperm chromatin damage of rams ranged from 1.93 to 2.37% in the outbred group, and 13.76 to 37.67% in the group of inbred rams. These findings confirm the link that inbreeding and semen quality might be mediated by the effects of inbreeding upon sperm DNA damage. Such high levels of sperm DNA fragmentation are thus likely to have a considerable impact upon male fertility. Generally, **Chavarro *et al.* (2010)** reported that spermatozoa with high DNA damage were significantly more numerous in obese than in normal weight men. **Kort *et al.* (2006)**.

Fertility Trial:

Results of conception rate (CR) after natural mating (Table 2) revealed significant (P<0.05) differences among individuals within breeds. As ram No 2 shown in table 2 of Rahmani and ram No. 1 of Romanov breed had the highest CR compared to other rams, with CR being 100% and 90.91%, respectively. However, the mean CR was significantly (P<0.05) higher in Rahmani than in Romanov breed (88.38 vs. 78.79%, respectively), however the small number of rams within groups might not enough, with that high individual differences.

It is of interest to note that results of CR as individual or mean values are in more association with percentages of sperm abnormality and DNA damage rather than with other semen characteristics in each breed.

Correlation coefficients:

Results of correlation coefficients (Table 3) indicated that both percentage of progressive motility and livability of spermatozoa correlated significantly (P<0.05) in negative pattern with percentage of sperm with DNA damage and in positive pattern with CR only in Rahmani breed. In both breeds, percentage of sperm with DNA damage had significantly (P<0.01) positive and strong

correlation with percentage of abnormal sperm and in Rahmani (r= 0.557) and strong correlation moderate in Romanov (r=0.840). Also, CR had positive correlation with sperm cell concentration.

Table 2: Conception rate of ewes inseminated by Rahmani and Romanov rams.

Breed	Number of mated ewes	Number of conceived ewes	Conception rate (%)
Rahmani breed:			
1	11	9	81.82[b]
2	11	11	100.00[a]
3	12	10	83.33[b]
Mean ± SE	34	30	88.38[A]
Romanov breed:			
1	11	10	90.91[a]
2	11	7	63.64[c]
3	11	9	81.82[b]
Mean ± SE	33	26	78.79[B]

Values in the same column for each breed with different superscripts are significantly different (P<0.05).

Table 3: Correlation coefficients between percentage of sperm with DNA damage or conception rate and each of progressive motility, livability and sperm cell concentration in Rahmani and Romanov breeds.

Item	Breed	Progressive motility (%)	Live sperm (%)	Abnormal sperm (%)	Sperm cell concentration	DNA damage (%)
DNA damage	**Rahmani**	-0.422*	-0.453*	0.557**	0.162	----
	Romanov	-0.284	-0.230	0.840**	-0.170	-----
Conception rate	**Rahmani**	0.409*	0.406*	-0.476*	0.148	-0.699**
	Romanov	0.452*	0.398	-0.552**	0.199	-0.795**

*** Significant at P<0.05.** **** Significant at P<0.01.**

The present results indicate positive and strong correlation between sperm DNA damage and sperm abnormality (P<0.01) in both breeds, but CR correlated negatively with sperm abnormality in Rahmani (P<0.05) and Romanov (P<0.01). On the other hand, the correlation between CR and sperm DNA damage was strong and negative (P<0.01) in both breeds. In this respect, some authors reported that males with high levels of sperm DNA damage may fertilize under optimum conditions (for example, given enough time and repeated sexual access to females) and in the absence of competition from other males **(Evenson *et al.* 1994)**, as in the case of captive breeding programmers. In these cases, the damage in sperm DNA may result in deleterious effects upon offspring **(Aitken *et al.* 2004, Lewis and Aitken 2005)**. Many studies suggested that inbreeding in sheep and other populations has an impact on the vitality of the offspring **(Overall *et al.* 2005)**.

In conclusion, the present study indicate a strong negative correlation between DNA damage or abnormal spermatozoa with conception rates) especially in Romanov rams which had lower CR. Thus, *in vitro* sperm DNA damage test can used for accurate judge on rams fertility as well as stander semen analysis assays.

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علاقة خصائص الحيوانات المنوية وخصوصاً تشوه الحامض النووي للحيوانات المنوية بخصوبة بعض سلالات الأغنام في مصر

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الهدف من هذه الدراسة هو استخدام بعض الاختبارات المعملية، وخاصة تشوه الحامض النووي للحيوان المنوي، كوسيلة دقيقة للتنبؤ بخصوبة الحيوانات المنوية للكباش.

تم جمع السائل المنوي مرة كل أسبوع لمدة 8 أسابيع من 6 كباش (رحماني وررومانوف) 3 كباش من كل نوع.

تم تقييم كل قذفة من حيث (الحجم والتركيز والنسبة المئوية لكل من الحركة التقدمية والحي والشواذ) للحيوانات المنوية. وتم إجراء اختبار تشوه الحامض النووي باستخدام المجموعة الكيماوئية (Halosperm).

وقد أظهرت النتائج أن هناك اختلافات فردية معنوية عند مستوي (P<0.05) في النسبة المئوية لكل من الشواذ وتشوه الحامض النووي للحيوانات المنوية في السلالاتين بينما كانت هناك اختلافات فردية في النسبة المئوية للحركة التقدمية والعدد الكلي للحيوانات المنوية في سلالة الرومانوف فقط.

وقد اتضح ان حجم القذفة أعلى معنوياً في الرحماني عن الرومانوف بينما كان تركيز الحيوانات المنوية والنسبة المئوية لتشوهات الحامض النووي أعلى معنوياً عند مستوي (P<0.05) في الرومانوف عن الرحماني.

كما لوحظ أن الكباش التي أظهرت أعلى معدل خصوبة كان لها أعلى جودة سائل معنوي، وكان متوسط معدل الخصوبة أعلى معنوياً ($p < 0.5$) في الرحماني عن الرومانوف 88.38 مقابل 78.79 % على التوالي.

وقد ارتبطت النسبة المئوية لتشوهات الحامض النووي ارتباطاً سالباً ومعنوياً مع الحركة التقدمية والشواذ للحيوانات المنوية وارتباطاً سالبا مع معدل الخصوبة في الرحماني.

وقد وجد أن النسبة المئوية لتشوهات الحامض النووي للحيوانات المنوية في كل من السلالتين ترتبط ارتباطاً قوياً موجباً مع نسبة الشواذ للحيوانات المنوية في الرحماني r=0557 وفي الرومانوف r=840. أما النسبة المئوية لتشوهات الحامض النووي للحيوانات المنوية فقد اظهرت ارتباطاً معنوياً سالباً مع معدل الخصوبة في كل من النوعين في الرحماني(0.699-) ، و الرومانوف (0.795-) .

ومن النتائج السابقة نستخلص أن هناك ارتباط قوي وسلبي بين تشوه الحامض النووي ومعدل شواذ الحيوانات المنوية والخصوبة خاصة مع كباش الرومانوف و التي اظهرت إنخفاض في معدل الخصوبة لذلك يمكن استخدام اختبار تشوه الحامض النووي للحيوان المنوي للحكم بدقة على خصوبة الكباش.

Relationship of Sperm Characteristics, in Reference with DNA Fragmentation, with Fertility of Some Sheep Breeds in Egypt