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LEUKOCYTE CONTENT OF STALLION EJACULATES AND ITS RELATIONSHIP TO SEMINAL ATTRIBUTES AND FERTILITY

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SUMMARY

The relationship between seminal leukocytes and semen parameters of the Arab stallions has been examined. A total of seventy ejaculates were collected from fourteen Arabian horses (5-19 years old). Semen samples were evaluated and viability indices were calculated at 30°C and 5°C storage conditions. The concentrations of total and differential leukocyte counts were estimated by direct cell count and using stained smears. Eighteen biochemical constituents of seminal plasma were analyzed and fertility of the stallions was retrospectively evaluated. The results of the study revealed that all ejaculates harbored leukocytes and aged stallions had the lowest leukocyte concentrations in their semen. Ejaculates with high leukocyte counts had lower initial sperm motility, number of sperm with normal morphology and sperm viability indices. Moreover, most of biochemical constituents of seminal plasma were detracted and fertility was low in ejaculates containing high concentrations of total leukocyte counts.

INTRODUCTION

Throughout the foregoing decade, there was a controversy on the biological significance of leukocytes in semen (Wolff, 1995). While some researchers did not behold any sperm damage in the wake of the leukocyte existence in stallion ejaculates (Malmgren et al., 1998), others deduced a convincing evidence that activated blood neutrophils could impair equine sperm motility in vitro (Baumber et al., 2002).

The objectives of the current perusal were, therefore, contrived to scout 1) the effect of age and individuality on concentrations of leukocytes in stallion semen, 2) the impact of leukocytes on some parameters of stallion semen, and 3) the in-

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fluence of seminal leukocytes on the fertility potential of breeding stallions.

MATERIALS AND METHODS

Unless otherwise stated, all kits and chemicals used in this study were purchased from Sigma-Aldrich Co. Germany, Randox Co. UK and Stanbic laboratory Co.

The investigation was launched in March, 2002 and lasted for nine months thereafter. A total of fourteen fertile Arabian horses (5- 19 years old) belonging to El-Zahraa Arab Horse Stud, Cairo was used.

Seventy semen samples were collected from stallions by means of CSU model artificial vagina. Promptly after collection, semen samples were transferred to the laboratory and kept in a water bath at 30°C until evaluation by means of conventional methods. Sperm motility was assessed at hourly intervals after incubation of aliquots (5 ml) of gel-free semen at 30°C for 4 hours and viability index was calculated according to Milovanov et al. (1964). Moreover, a 0.90 ml aliquot of freshly ejaculated gel-free semen was mixed with 0.10 ml of 0.05% methylene blue solution (in 2.90% trisodium citrate), incubated at 46.5°C, and the time required for complete discoloration of methylene blue was recorded to the nearest 0.50 minute.

The concentration of total leukocytes in gel-free semen was estimated by direct cell count using the improved Neubauer hemocytometer counting chamber. In brief, 500 ul of semen was thoroughly mixed with 1000 ul of Leucoscreen solution as a diluent (Fertipro Co., Belgium) and the concentration of total leukocytes per ml semen was calculated by multiplying the number of cells counted in five large main squares by 6000. Also, smears from gel-free semen stained with Wright's stain (Morel, 1993) were examined (1000 x) for differential leukocyte counts as polymorphonuclear and mononuclear leukocytes per one million progressively motile sperm.

Within five minutes after collection, each semen sample was prepared for preservation in Tris-egg yolk extender (Samper, 1988) at 5°C for 72 hours. Progressive sperm motility was measured during the incubation period and the viability index was calculated.

Following initial evaluation, a 5 ml aliquot of freshly collected gel-free semen was centrifuged at 3000 x g for 15 minutes and the supernatant seminal plasma was harvested and stored at -20°C pending analysis. Glucose, protein, albumin, total lipids, cholesterol (free and esterified moieties), lactate, nitric oxide (as nitrates and nitrites), iron, total iron binding capacity, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, acid phosphatase, lactate dehydrogenase, superoxide dismutase, total α-glucosidase, neutral

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α-glucosidase and fumarase were assayed spectrophotometrically under the conditions specified by the commercial kit systems.

The fertility of stallions after natural services (throughout this study) was retrospectively evaluated from the available records kept in the stud. Fertility was defined as the total number of cycles mated over the total number of pregnancies achieved.

Statistical analysis of the data was carried out using a commercial software statistica for windows, Statsoft (1993).

RESULTS

In general, all ejaculates harbored leukocytes of polymorphonuclear and mononuclear types (Fig.1). The overall mean (± SEM) concentrations of polymorphonuclear, mononuclear and total leukocytes in gel-free semen were 70.84 ± 7.73 x 10^3/ml, 58.50 ± 2.40 x 10^3/ml and 129.34 ± 6.71 x 10^3/ml, respectively.

As shown in Table 1, semen samples of stallions aged 16 to 19 years were characterized by presence of a significantly ($P<0.05$) lower count of leukocytes and a remarkable increase ($P<0.05$) in the number of progressively motile sperm with normal morphology. The minimum ratio (0.58 ± 0.16) of polymorphonuclear to mononuclear leukocytes as well as the maximum value (0.96 ± 0.10) of viability index was recorded in semen samples of 16 to 19 years old stallions. Upon calculation of correlation coefficients, significant ($P<0.01$) negative relationships were found between the viability indices of incubated spermatozoa at 30°C and number of polymorphonuclear ($r = -0.49$), mononuclear ($r = -0.47$) and total ($r = -0.53$) leukocytes per one million motile sperm.

Frequency distribution of total leukocyte concentrations in gel-free semen disclosed that 34.29%, 21.42% and 44.29% of the ejaculates fell in the ranges of 50-78, 80-96 and 100-240 X 10^3/ml, respectively. When semen samples were categorized according to these ranges (Table 2), it was found that higher concentrations of polymorphonuclear and mononuclear leukocyte counts (up to 115.94 ± 6.09 and 66.06 ± 3.84 x 10^3/ml, consecutively) in gel-free semen was associated with a perspicuous augmentation ($P<0.05$) in the volume of gel and methylene blue reduction time besides a pronounced decrease ($P<0.05$) in the initial sperm motility, number of progressively motile sperm with normal morphology per ml and the viability indices of incubated semen at 30°C and 5°C.

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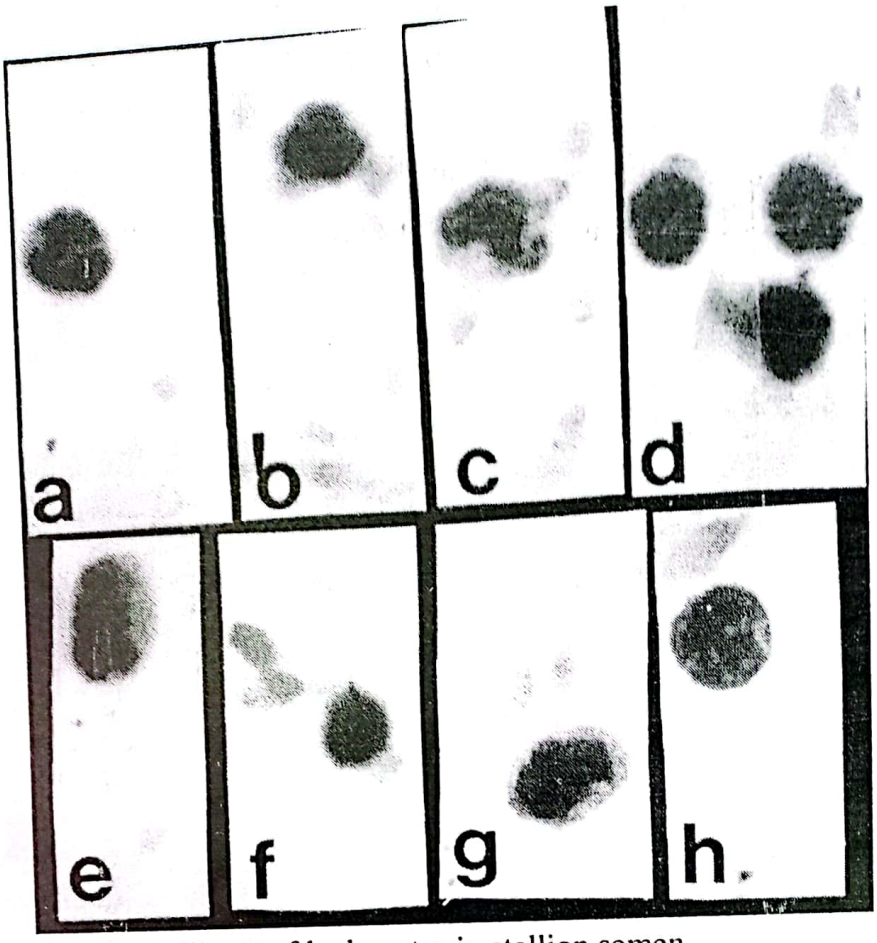

Fig. 1: Types of leukocytes in stallion semen.
a-c: polymorphonuclear leukocytes (neutrophiles).
d-g: mononuclear leukocytes (lymphocytes)
h: mononuclear leukocytes (monocytes)

Calculation of correlation coefficients revealed significant ($P<0.05$) negative relationships between initial sperm motility and each of polymorphonuclear ($r = -0.36$) and of total ($r = -0.42$) leukocyte concentrations. The number of normal progressively motile sperm per ml of gel-free semen was significantly ($P<0.05$) correlated with concentrations of mononuclear ($r = -0.37$) and total ($r = -0.33$) leukocytes. Also, there was a significant ($P<0.05$) negative relationship between viability indices of incubated semen at 30°C and the concentration of mononuclear leukocytes ($r = -0.30$). With respect to gel volume, significant ($P<0.05$) correlation coefficients were obtained between the latter and concentrations of polymorphonuclear ($r = 0.38$) and total ($r = 0.36$) leukocytes. Likewise, methylene blue reduction time was significantly ($P<0.05$) correlated with the concentration of polymorphonuclear leukocytes ($r = 0.53$). Furthermore, a significant ($P<0.05$) coefficient of correlation ($r = 0.32$) was demonstrated between the concentration of total leukocytes in gel-free semen and the percentage of total sperm abnormalities.

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Table 1: Effect of age on the quality of stallion semen.

Stallion semen quality	Stallion age (years)		
	5 - 10 (n=20)	11-15 (n=30)	16 -19 (n=20)
Total leukocytes concentrations ($x10^3$/ml)	144.00 ± 11.15^a	122.08 ± 7.05^a	86.76 ± 9.30^b
Number ($x10^3$) of total leukocytes per one million progressively motile spermatozoa	2.01 ± 0.66^a	0.59 ± 0.11^b	0.66 ± 0.20^b
Polymorphonuclear leukocytes concentrations ($x10^3$/ml)	80.39 ± 7.90^a	63.87 ± 5.21^a	32.26 ± 7.94^b
Number ($x10^3$) of polymorphonuclear leukocytes per one million progressively motile spermatozoa	1.13 ± 0.38^a	0.28 ± 0.06^b	0.27 ± 0.12^b
Mononuclear leukocytes concentrations ($x10^3$/ml)	63.61 ± 5.95	58.21 ± 3.68	54.50 ± 3.03
Number of mononuclear leukocytes per one million progressively motile spermatozoa	0.88 ± 0.28^a	0.31 ± 0.06^b	0.39 ± 0.11^b
Polymorphonuclear : mononuclear leukocytes ratios	1.45 ± 0.45^a	1.22 ± 0.38^{ab}	0.58 ± 0.16^b
Number ($x10^9$) of progressively motile sperm with normal morphology/ml of gel-free semen	0.09 ± 0.03^a	0.20 ± 0.04^b	0.23 ± 0.04^b
Number ($x10^9$) of progressively motile sperm with normal morphology/ejaculate*	1.31 ± 0.30^a	3.56 ± 0.64^b	7.86 ± 2.04^c
Viability indices (30°C)	0.75 ± 0.11^{ab}	0.68 ± 0.09^a	0.96 ± 0.10^b

Means ± SEM with dissimilar superscripts in the same row are significantly different at $P< 0.05$.
* Calculated per volume of gel-free semen
n = Number of ejaculates

Concerning the effect of individuality (Table 2) there were explicit ($P<0.05$) stallion-to-station variations in the concentration of polymorphonuclear and mononuclear leukocytes in gel-free semen, the number of progressively motile sperm with normal morphology per ml gel-free semen and in the viability indices of incubated spermatozoa at 30°C and 5°C.

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As can be seen from Table 3, seminal plasma of ejaculates containing the highest concentrations (100-240 x 10^3/ml) of total leukocytes was demarcated by significant (P<0.05) detraction in the concentrations of glucose, protein, cholesterol and iron as well as significant (P<0.05) decline in the activities of aspartate aminotransferase, total and neutral α-glucosidase and fumarase enzymes. Notwithstanding, none of the other biochemical constituents of the seminal plasma was significantly influenced by the diversity of total leukocyte concentrations among semen samples.

Retrospective assessment of fertility after natural services evinced that the overall mean number of cycles mated per pregnancies achieved was 1.72 ± 0.19. Stallions with high numbers (1.55 to 7.24 X 10^3) of total leukocytes per one million motile sperm significantly (P<0.05) required a greater number of mating to achieve pregnancies (Fig. 2).

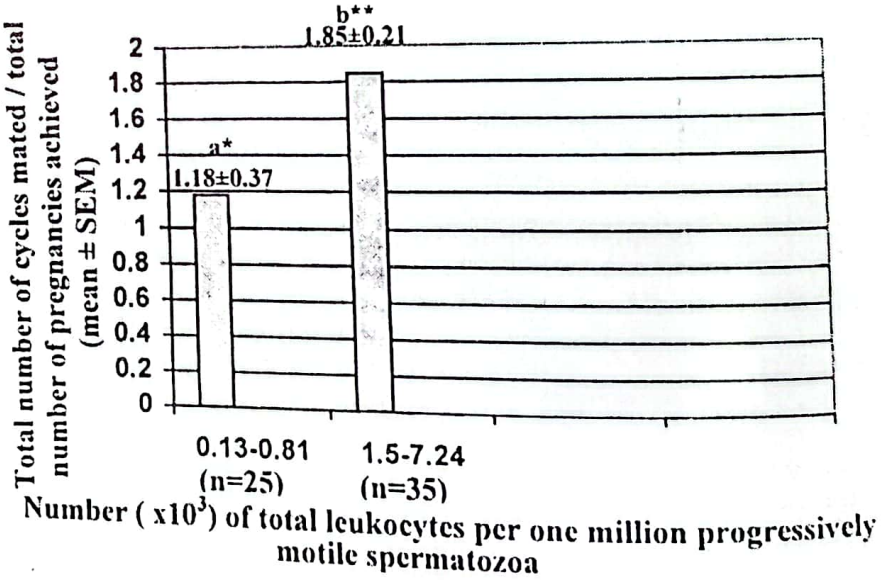

Fig. 2: Effect of seminal leukocytes on fertility of 5* and 7** stallions. Different letters above bars denote significance at P<0.05. n = Number of ejaculates.

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Table 2: Effect of seminal leukocytes on some biological characteristics of stallion semen.

Semen parameters	Concentrations of total leukocytes in gel-free semen		
	50-78X10^3/ml (n=24)	80-96X10^3/ml (n=15)	100-240X10^3/ml (n=31)
Polymorphonuclear cells (x10^3/ml)*	16.48 ± 1.09^a	31.17 ± 2.52^b	115.94 ± 6.09^c
Mononuclear cells (x10^3/ml)*	48.66 ± 1.26^a	58.66 ± 1.01^b	66.06 ± 3.84^b
Polymorphonuclear : mononuclear cells ratios	0.34 ± 0.02^a	0.54 ± 0.05^b	1.91 ± 0.32^c
Volume of gel (ml)	4.77 ± 1.21^a	3.50 ± 1.12^a	16.84 ± 3.75^b
Volume of gel-free semen (ml)	28.11 ± 5.72	31.50 ± 3.65	24.10 ± 4.01
Sperm concentrations (x10^9/ml)*	0.38 ± 0.04	0.34 ± 0.07	0.30 ± 0.04
Initial pH values	7.40 ± 0.05	7.50 ± 0.01	7.49 ± 0.08
Initial sperm motility (%)	71.07 ± 3.32^a	62.50 ± 5.16^{ab}	55.71 ± 4.59^b
Live sperm cells (%)	81.36 ± 4.04	74.67 ± 5.30	73.95 ± 3.22
Number (x10^9) of progressively motile sperm with normal morphology/ml of gel-free semen*	0.22 ± 0.04^a	0.17 ± 0.07^{ab}	0.10 ± 0.02^b
Methylene blue reduction time (minutes)	10.83 ± 1.03^a	15.33 ± 1.15^{ab}	21.67 ± 1.79^b
Viability indices (30°C)*	0.94 ± 0.07^a	0.71 ± 0.11^{ab}	0.66 ± 0.10^b
Viability indices (5°C)*	52.21 ± 4.62^a	44.04 ± 4.50^{ab}	40.14 ± 3.76^b

Means ± SEM with dissimilar superscripts in the same row are significantly different at P< 0.05.
n = Number of ejaculates
* Significant (P<0.05) effect of stallion on these parameters.

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Table 3: Effect of seminal leukocytes on biochemical properties of stallion semen.

Biochemical constituents of seminal plasma	Concentrations of total leukocytes in gel-free semen		
	50-78X10^3/ml (n=24)	80-96X10^3/ml (n=15)	100-240X10^3/ml (n=31)
Glucose (mg/ml)	0.71 ± 0.13^a	0.60 ± 0.07^{ab}	0.50 ± 0.03^b
Protein (mg/ml)	15.66 ± 2.84^a	20.17 ± 3.74^a	8.81 ± 1.98^b
Albumin (mg/ml)	6.49 ± 0.83	5.77 ± 0.99	4.85 ± 0.49
Total lipids (mg/ml)	7.85 ± 0.27	7.37 ± 0.27	7.23 ± 0.33
Cholesterol (mg/ml)	0.48 ± 0.03^a	0.46 ± 0.05^{ab}	0.38 ± 0.03^b
Lactate (mg/ml)	0.24 ± 0.05	0.18 ± 0.02	0.23 ± 0.03
Nitric oxide (mg/ml)	0.04 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
Iron (ug/ml)	2.01 ± 0.83^a	1.09 ± 0.43^{ab}	0.89 ± 0.11^b
Total iron binding capacity (ug/ml)	4.37 ± 1.02	4.46 ± 0.49	3.18 ± 0.19
Aspartate aminotransferase (IU/ml)	0.25 ± 0.03^a	0.26 ± 0.04^a	0.11 ± 0.02^b
Alanine aminatransferase (IU /ml)	0.06 ± 0.01	0.05 ± 0.01	0.10 ± 0.02
Alkaline phosphatase (IU /ml)	1.74 ± 0.09	1.63 ± 0.10	1.64 ± 0.08
Acid phosphatase (U/ml)	0.05 ± 0.01	0.07 ± 0.01	0.05 ± 0.01
Lactate dehydrogenase (U/ml)	0.19 ± 0.05	0.23 ± 0.07	0.23 ± 0.04
Superoxide dismutase (U /ml)	8.23 ± 3.20	7.72 ± 2.85	6.90 ± 01.86
Total ∝-glucosidase (m U /ml)	25.04 ± 1.64^a	29.00 ± 2.41^a	12.22 ± 1.90^b
Neutral ∝-glucosidase (m U /ml)	9.23 ± 0.61^a	10.69 ± 2.36^a	2.45 ± 0.70^b
Fumarase (U /ml)	1134.53 ± 11.14^a	1018.25 ± 10.49^a	790.27 ± 8.74^b

Means ± SEM with dissimilar superscripts in the same row are significantly different at P< 0.05.
n = Number of ejaculates

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DISCUSSION

As disclosed in the present work, leukocytes were present in all semen samples studied. This is in accordance with the previously reported data in human semen (Barratt et al., 1990). Nevertheless, there is little published information regarding the importance of leukocytes in stallion semen.

In this study, there was a strong relationship between increasing leukocyte concentrations in semen and poor semen parameters. Increased concentration of leukocytes in semen samples contributes to inhibition of sperm motility, normal sperm morphology and viability indices. The same results were reported by Wolff et al. (1990); Arata de Bellabarba et al. (2000); Baumber et al. (2002).

Our study is the first to declare the negative relationship between the age of stallions and mean concentration of leukocytes in their semen samples. Increased leukocyte concentrations in semen was accompanied an increase in gel volume and methylene blue reduction time. Dowsett and Knott (1996) found that gel volume decreased with increased stallion age. Consequently, increased stallion age is amenable to the decrease in both gel volume and total leukocyte concentrations. The increased methylene blue reduction time in semen samples with high leukocyte concentrations may be attributed to the hazard effect of leukocytes on semen parameters (Yanushpolsky et al., 1996).

In the current study, stallion-to-stallion variation in leukocyte concentrations and viability indices was attributed to variation in stallion age and semen parameters. Besides, this study elucidated the hazard effect of increased seminal leukocyte concentrations on most of biochemical constituents of seminal plasma.

The detrimental effect of seminal leukocytes on semen parameters was indicated by decreased fertility in stallions possessing high numbers of total leukocytes in their ejaculates. The same was reported by Wang et al. (1994) and was ascribed to oxidative stress raised by elevated leukocyte concentrations (Sukcharoen et al., 1995; Sharma et al., 2001).

In conclusion, identification and evaluation of leukocyte numbers should be performed in future routine stallion semen evaluation to help achieving proper clinical decisions.

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