Slov Vet Res 2021; 58 (Suppl 24): 307–14
DOI 10.26873/SVR-1450-2021

Original Research Article

EFFECTS OF AGE AND SEASON ON SEMEN CHARACTERISTICS IN ARABIAN STALLIONS UNDER SUBTROPICAL CONDITIONS OF KUWAIT

Gamal Shawki[1], Tawfek Mohamed Barakat[1], Attia Mohamed Samy[2], Ahmed Alhamed AL-Mejren[2], Ayman Mesalam[1]*

[1]Department of Theriogenology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt, [2]Veterinary diagnostic lab center, Public Authority of Agriculture Affairs and Fish Resources (PAAFR), Kuwait City 13075, Kuwait

*Corresponding author, E-mail: aymanmesalam@gmail.com

Abstract: Semen analysis is a valuable approach to assess stallion fertility. Many factors, including the age of the stallion and the season of year have been shown to influence semen quality. Therefore, the current study aimed to evaluate the influence of stallion's age and season on the potential semen parameters and sperm morphology in Arabian stallions. A total of 108 ejaculates were collected using artificial vagina throughout the year from nine Arabian stallions aged 4-22 years, divided into three groups according to their ages; Group I (4-6 years), Group II (8-11years), and Group III (15-22 years). The ejaculates were analyzed immediately after collection for macroscopic and microscopic parameters using computer-assisted sperm analysis (CASA). Statistical analysis was applied to study the effect of the age of the stallion and season of semen collection on semen parameters. Our results showed a clear age-based differences ($P < 0.05$) with stallions aged from 4 to 11 years produce higher semen quality especially in sperm count, livability, motility and normal sperms compared with old stallions (15-22 years). Moreover, old stallions showed higher ($P < 0.05$) sperm abnormalities especially the secondary defects. The semen volume increased ($P < 0.05$) in the spring, and sperm concentration was higher ($P < 0.05$) in the winter and autumn, while, no significant variation in total sperm number between different seasons. Moreover, the livability, motility, normal sperms and progressive sperm motility were higher ($P < 0.05$) in hot season (breeding season) compared with cold season, but abnormal sperms were lower. These data suggest that, older stallions (15-22 years) produce ejaculates of larger volume but the motility, viability, and abnormalities percent were better in ejaculates collected from stallions aged 4-11 years. Ejaculates collected during spring and summer showed better characteristic than that collected during autumn and winter.

Key word: Kuwait; Arabian stallions; age; aeasons; aemen characters

Introduction

The breeding soundness examination (BSE) of a stallion, as proposed by the society for theriogenology, recommends that a stallion produce a minimum of one billion progressively motile, morphologically normal sperm in the second of two ejaculates collected 1 h apart to be classified as a satisfactory prospective breeder (1). Artificial insemination (AI) is the most valuable breeding management tool available to improve the fertility and genetics of equines (2). Collection of relatively large numbers of ejaculates containing the highest number of morphologically normal sperm is among the important factors determining the success of AI (3). Semen production is influenced by ejaculatory frequency, age, season, and nutritional status in horses (4, 5). Many factors, including service frequency and season of year have been shown to influence semen quality and sexual behavior of stallions (6) and could be

Received: 24 September 2021
Accepted for publication: October 2021

used as prediction tools to select animals for AI program.

Estimation of the sperm motility is one of the most commonly evaluated parameter used for analyzing the quality of each ejaculate used for AI (7). Evaluation of sperm morphology, using computed assisted sperm analysis (CASA) or light microscope, is an important part of stallion breeding soundness evaluation (8-10), evaluation of semen quality (11), and predicting fertility (12). Some authors reported that sperm morphology is related with fertility to various degrees (13), while others did not find any relationship between sperm morphology and fertility (14, 15). When more than 30% of the ejaculated spermatozoa have structural defects, reduced fertility may occur in domesticated animals (16). The sperms defects classification into primary defects and secondary defects (17), whereas, primary abnormalities are considered to originate during spermatogenesis, secondary abnormalities develop in the excurrent ducts (18).

The percentage of normal sperm and primary sperm defects were affected significantly by age of the bulls, with middle age bulls exhibited better sperm morphology than young and old ones (19). Some studies stated that the season of the year affect the percentage of normal sperm, primary and secondary sperm defects (3, 20), while other studies reported that the age did not affect the percentage of secondary sperm defects (21). It has been reported that the morphology of spermatozoa, sperm concentration, semen motility and the volume of the ejaculate was improved with an increase in the age of animal (22).

Environmental temperature and seasonal variations may also play a role in semen output. It has been reported that the semen quality does not remain the same throughout the year, especially as the animal advances in age (23). Considerable attention has been paid to the effect of age (24) and the season of collection (22) on sperm morphology. Like other countries of the world, Kuwait is anticipated to be potentially influenced by the global climate warming, especially its location in subtropical area. Therefore, the current study aimed to evaluate the influence of the age of the stallion and the seasons on ejaculate characteristic of Arabian stallions selected for natural and AI programs under subtropical conditions in Kuwait.

Material and methods

Ethics statement

All of the methods and experimental procedures were conducted according to the Ethics for Humane Treatment of Animal Use in Research Guidelines.

Animals

The present study was performed during 2019 and 2020 in the Arabian Equine Farm, the Public Authority for Agriculture Affairs in Kuwait city, Kuwait between latitudes 28.45 ° and 30.05 ° North and longitude between 46.30 ° and 48.30 ° East. A total of nine Arabian stallions aged (4-22 years) were used, the stallions were housed in closed stables with open yard for exercise and were fed on balanced ration consisted of barley and rice straw with green fodder. The stallions were noted to be in good body condition, had an excellent disposition, the external genitalia revealed no abnormalities, and didn't receive any doping drugs.

Semen collection

A total of 108 ejaculates were collected throughout the study using an artificial vagina (Missouri AV models, IMV International Co., France) as previously described (25-27). Briefly, with the help of teaser mare displaying estrous behavior ejaculates were collected using an AV pre-warmed at 45-48°C, lightly lubricated with a non-spermicidal lubricant (NETTEX, UK) and fitted with an inline filter to separate the gel fraction (28). Semen samples were collected twice monthly throughout the study period and two successive ejaculates were collected within 1 hour a part during each collection session (1st and 2nd ejaculate) (29, 30).

Semen analysis

The total volume of the ejaculate (TSV/ml), volume of the gel fraction (GV/ml), and the volume of the gel-free ejaculate (GFV/ml) were measured in a graduated cylinder. The pH of the ejaculate was measured using a pH tester. The percentage of the motile sperm and progressively motility sperms (Pms) were estimated using CASA (31). The sperm cell concentration (Spcc/ml) was estimated using SpermaCue® (semen analyzer, Minitube) by measuring the optical density and comparing with a calibration curve

(10) and the total sperm count per ejaculate (Con/ej) was calculated by multiplying the semen volume and the sperm concentration. The percentage of live sperm (Liv %) was estimated after preparing a stained film using eosin-nigrosin stain (minitube, GmbH, Germany) as previously described (32, 33).

The sperm morphology was assessed after applying the haematoxylin staining technique using CASA (34). Sperm cell abnormalities were grouped into primary and secondary defects and expressed as a percentage. Primary defects include certain types of abnormal heads, acrosome defects, loosed abnormal heads, mid-piece defects, proximal droplets and pouch formation. Secondary defects include loosed normal heads, detached acrosomes, abaxial implantation, distal droplet and single bent tails (13, 15).

Statistical analysis

All data were analyzed using the SPSS software (IBM, SPSS Statistics, Version 22, USA). The data were expressed as mean ± standard error of means (SEM). To study the effect of the season, season of the ejaculate collection was classified into breeding (from March to August) and non-breeding (from September to February) in one analysis and in another analysis the season was classified into four year seasons (spring, summer, autumn, and winter). To study the effect of the stallion's age, the stallions were categorized into three groups each included three stallions: Group I (4- 6 years), Group II (8 - 11years) and Group III (15-22). To investigate the significance of the difference between the breeding and non-breeding season independent sample t-test was used. While, one-way ANOVA was used to compare the effect of age (4 – 6, 8 – 11 and 15 – 22 years) and four year seasons (spring, summer, autumn and winter). Duncan Multiple Range test was used to differentiate between significant means at $P < 0.05$. Two-way ANOVA was used to assess interaction effect between the stallion's age and the order of the ejaculate as well as the stallion's age and season.

Results

Effect of the of the age of the stallion on the ejaculate characteristics

Data in Table 1 showed that the TSV and GFV in stallions aged 15-22 years were significantly ($P < 0.05$) higher compared with other groups. Moreover, the GV in stallions aged 4-6 years was significantly ($P < 0.05$) lowers compared with other groups. However, there was no detectable significant ($P > 0.05$) difference in the pH of the ejaculates between different age groups. Additionally, the Spcc/ml in stallions aged 4-6 years was significantly ($P < 0.05$) higher compared with that in other groups. However, the total sperm count/ejaculate in stallions aged 8-11 were significantly ($P < 0.05$) higher compared with other groups. Similarly, the livability and motility % in stallions aged 4-6 years were significantly ($P < 0.05$) higher than that in other age groups. In addition, the Psm % did not differ significantly ($P > 0.05$) between different age groups. Further, the percentage of abnormal sperms and the secondary abnormalities in stallion aged 15-22 years was significantly ($P < 0.05$) higher compared with other groups. There were non- significant ($P > 0.05$) differences between different groups in the primary abnormalities %. There was no effect for the order of the ejaculate on the seminal parameters ($P > 0.05$) and there was no interaction between the effect of the age of the stallion and the effect of the order of the ejaculate.

Effect of the seasons on the ejaculate characteristics in Arabian stallions

Data in Table 2 showed that the TSV and GFV of the ejaculates collected during spring season were significantly ($P < 0.05$) higher compared with that collected during summer, autumn and winter seasons. Moreover, the GV of the ejaculates collected in summer was significantly ($P < 0.05$) higher compared with that collected in winter and autumn. Similarly, the Spcc/ml of the ejaculates collected in winter and autumn seasons were significantly ($P < 0.05$) higher compared with that collected in spring and summer seasons.

Table 1: The effect of the age of the stallion on the seminal parameters of the ejaculates

Parameters	4 – 6 years	8 – 11 years	15 – 22 years
Total volume of the ejaculate (ml)	38.4 ± 0.8^{c}	52.48 ± 1.13^{b}	58.47 ± 0.32^{a}
The volume of gel fraction (ml)	3.64 ± 0.19^{b}	6.52 ± 0.23^{a}	6.04 ± 0.36^{a}
The volume of gel free ejaculate (ml)	34.76 ± 0.75^{c}	45.97 ± 0.98^{b}	52.43 ± 0.4^{a}
Sperm concentration $x10^{6}$ /ml	261.63 ± 3.87^{a}	246.74 ± 5.84^{b}	197.24 ± 4.93^{c}
Total sperm number x 10^{9} /ejaculate	9.09 ± 0.29^{c}	11.34 ± 0.36^{a}	10.33 ± 0.26^{b}
pH	7.37 ± 0.03^{a}	7.38 ± 0.03^{a}	7.44 ± 0.03^{a}
Livability (%)	76.64 ± 0.34^{b}	80.81 ± 1.02^{a}	78.58 ± 0.74^{b}
Motility (%)	73.23 ± 0.47^{b}	78.13 ± 1.09^{a}	73.41 ± 0.74^{b}
Progressive sperm motility (%)	68.51 ± 0.78^{a}	68.94 ± 0.7^{a}	69.02 ± 1.03^{a}
Abnormal sperms (%)	25.83 ± 0.33^{b}	25.67 ± 0.44^{b}	29.5 ± 0.29^{a}
Primary sperm abnormalities (%)	19.67 ± 0.6^{a}	19.83 ± 0.6^{a}	21.33 ± 0.33^{a}
Secondary sperm abnormalities (%)	6.17 ± 0.6^{b}	5.83 ± 0.17^{b}	8.17 ± 0.6^{a}

A total of 36 ejaculates were used per each age group. Values with different superscripts in the same row are significantly different ($P < 0.05$)

Table 2: The effect the seasons on the ejaculate characteristics of Arabian stallions

Parameters	Spring	Summer	Autumn	Winter
Total volume of the ejaculate (ml)	49.04 ± 1.1^{a}	45.3 ± 1.26^{b}	41.85 ± 0.98^{c}	39.85 ± 0.61^{c}
The volume of gel fraction (ml)	5.93 ± 0.27^{ab}	6.46 ± 0.33^{a}	5.02 ± 0.3^{c}	5.37 ± 0.28^{bc}
The volume of gel free ejaculate (ml)	43.11 ± 1.09^{a}	38.83 ± 1.03^{b}	36.83 ± 1.06^{bc}	34.48 ± 0.5^{c}
Sperm concentration $x10^{6}$ /ml	255.56 ± 9.37^{b}	267.41 ± 9.53^{ab}	290.0 ± 12.67^{a}	292.96 ± 11.01^{a}
Total sperm number x 10^{9} /ejaculate	10.98 ± 0.44^{a}	10.28 ± 0.34^{a}	10.78 ± 0.64^{a}	10.05 ± 0.35^{a}
pH	7.39 ± 0.02^{b}	7.5 ± 0.02^{a}	7.54 ± 0.03^{a}	7.53 ± 0.03^{a}
Livability (%)	75.52 ± 0.66^{a}	75.22 ± 0.79^{a}	72.59 ± 0.71^{b}	73.04 ± 0.88^{b}
Motility (%)	70.44 ± 0.59^{a}	71.11 ± 0.66^{a}	68.07 ± 0.57^{b}	68.59 ± 0.67^{b}
Progressive sperm motility (%)	65.93 ± 0.58^{a}	66.96 ± 0.67^{a}	62.0 ± 0.48^{b}	62.33 ± 0.5^{b}
Abnormal sperms (%)	26.11 ± 0.51^{bc}	24.33 ± 0.62^{c}	27.56 ± 0.65^{b}	30 ± 0.85^{a}
Primary sperm abnormalities (%)	18.11 ± 0.23^{b}	17.28 ± 0.44^{b}	21.89 ± 0.49^{a}	22.94 ± 0.59^{a}
Secondary sperm abnormalities (%)	8 ± 0.35^{a}	7.06 ± 0.45^{a}	5.67 ± 0.6^{a}	7.06 ± 0.79^{a}

A total of 27 ejaculates were used per each season. Values with different superscripts in the same row are significantly different ($P < 0.05$)

Table 3: The interaction effect between the ages of the stallions and the season

Parameters	4-6 years				8-11 years				15-22 years				*P* value
	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	
TSV/ml	36.44 ± 0.41	36.11 ± 0.39	43 ± 0.82	38.67 ± 0.73	41.22 ± 0.7	40.38 ± 0.53	48.33 ± 0.47	44.44 ± 0.93	47.89 ± 0.61	43.11 ± 0.35	55.78 ± 0.8	52.78 ± 1.23	0.000
GV/ml	4.94 ± 0.54	4.5 ± 0.22	6.17 ± 0.47	4.72 ± 0.26	5.5 ± 0.57	5.31 ± 0.46	5.39 ± 0.38	6.89 ± 0.45	4.61 ± 0.45	6.22 ± 0.58	6.22 ± 0.55	7.78 ± 0.43	0.002
GFV/ml	31.5 ± 0.74	31.61 ± 0.41	36.83 ± 0.68	33.94 ± 0.6	35.72 ± 1.11	35.06 ± 0.58	42.94 ± 0.55	37.56 ± 0.93	43.28 ± 0.6	36.89 ± 0.49	49.56 ± 0.86	45 ± 1.04	0.000
Con/mlx10^6	283.89 ± 19.72	315 ± 22.44	274.44 ± 19.52	293.33 ± 17.24	281.11 ± 22.99	301.88 ± 20.7	244.44 ± 12.81	271.11 ± 15.04	305 ± 24.59	264.44 ± 13.14	247.78 ± 15.61	237.78 ± 12.89	0.369
Con/ej x10^9	8.94 ± 0.64	9.96 ± 0.72	10.16 ± 0.83	9.93 ± 0.54	10.19 ± 1.06	10.58 ± 0.76	10.54 ± 0.66	10.16 ± 0.57	13.23 ± 1.12	9.73 ± 0.44	12.23 ± 0.68	10.74 ± 0.7	0.076
pH	7.52 ± 0.05	7.52 ± 0.05	7.4 ± 0.06	7.51 ± 0.03	7.51 ± 0.05	7.5 ± 0.06	7.41 ± 0.04	7.47 ± 0.06	7.59 ± 0.05	7.56 ± 0.04	7.37 ± 0.04	7.53 ± 0.02	0.834
Liv %	72.56 ± 1.23	75 ± 1.41	74.11 ± 1.22	75.67 ± 1.15	71.33 ± 1.42	73.38 ± 1.21	76.44 ± 1	74 ± 1.58	73.89 ± 0.98	70.78 ± 1.63	76 ± 1.18	76 ± 1.37	0.130
Mo %	68.78 ± 1.08	68.56 ± 0.91	69.44 ± 0.67	71.44 ± 0.94	66.67 ± 0.82	68.5 ± 1.18	71.22 ± 0.7	70.44 ± 1.54	68.78 ± 0.97	69 ± 1.53	70.67 ± 1.49	71.44 ± 0.94	0.738
Psm %	62.33 ± 0.87	63 ± 0.94	64.56 ± 0.65	67 ± 0.94	61 ± 0.78	61.5 ± 0.73	66.78 ± 0.83	66.56 ± 1.47	62.67 ± 0.83	62 ± 0.88	66.44 ± 1.37	67.33 ± 1.11	0.518

TSV; Total semen volume, GV; Gel volume, GFV; Gel-free semen volume, Con/ml; Concentration/ml x 10^6, Con/ej; Concentration/ejaculate x 10^9, Liv; Livability, Mo; Motility, Psm; Progressive sperm motility

Table 4: The seminal parameters of the ejaculates collected during breeding and non-breeding seasons in Arabian stallions

Parameters	Breeding season	Non-breeding season
Total volume of the ejaculate (ml)	47.17 ± 0.87*	40.85 ± 0.59
The volume of gel fraction (ml)	6.19 ± 0.22*	5.19 ± 0.2
The volume of gel free ejaculate (ml)	40.97 ± 0.8*	35.66 ± 0.61
Sperm concentration x10^6 /ml	261.48 ± 6.67	291.48 ± 8.32*
Total sperm number x 10^9 /ejaculate	10.63 ± 0.28	10.42 ± 0.37
pH	7.45 ± 0.02	7.54 ± 0.02*
Livability (%)	75.37 ± 0.51*	72.81 ± 0.56
Motility (%)	70.78 ± 0.44*	68.33 ± 0.44
Progressive sperm motility (%)	66.44 ± 0.44*	62.17 ± 0.34
Abnormal sperms (%)	25.22 ± 0.45	28.78 ± 0.6*
Primary sperm abnormalities (%)	17.69 ± 0.26	22.42 ± 0.39*
Secondary sperm abnormalities (%)	7.53 ± 0.3	6.36 ± 0.51

A total of 36 ejaculates were used per each season.Values with asterisk in the same row are significantly different ($P < 0.05$)

However, there were no detectable significant ($P > 0.05$) differences between ejaculates collected in different seasons in the total sperm number/ejaculate. The pH of the ejaculates collected in spring season was significantly ($P < 0.05$) lower compared with that collected during other seasons. Additionally, the livability, motility and Psm % of the ejaculates collected in spring and summer were significantly ($P < 0.05$) higher compared and winter seasons. However, the percentage of abnormal sperms in ejaculates collected during spring and summer were significantly ($P < 0.05$) lower compared with that collected during autumn and winter. The percentage of the primary defects in the ejaculates collected during winter and autumn was significantly ($P < 0.05$) higher compared with that in the ejaculates collected during spring and summer.

The interaction between stallion's age and the seasons significantly ($P < 0.05$) affect the TSV, GV and GFV, however, it did not affect ($P > 0.05$) the Spcc/ml, total sperm number/ejaculate, livability %, motility % and Psm % (Table 3).

Additionally, the seminal parameters of the

ejaculates collected during breeding and non-breeding seasons (Table 4) showed that the TSV, GV, GFV, livability %, motility % , and Psm % were significantly ($P < 0.05$) higher in the ejaculates collected during the breeding season compared with that collected during non-breeding season, while, the Spcc/ml, pH, percentage of abnormal sperm and the proportion of the sperm cells with primary defects were significantly ($P < 0.05$) lower in the ejaculates collected during the breeding season compared with that collected during non-breeding season. There was no detectable significant ($P > 0.05$) variation between the ejaculates collected in breeding and non-breeding seasons in the total sperm number/ejaculate and in the secondary sperm abnormalities.

Discussion

Artificial insemination (AI) is being used more and more frequently in equine breeding (35) and the pregnancy rate after inseminations is greatly dependent on the sperm quality (36). Global warming has complex influences in both domestic and wild animals, however, the direct influence of climate change on animals fertility is not fully clarified (37). Several species restrict their sexual and reproductive activity to a certain seasons (38), as the spring and summer in temperate regions (39). Stallions show less reproductive seasonality under tropical conditions than that in temperate zones (40). Kuwait is characterized by a long dry hot summers and frigid short sometimes rainy winters (a desert-type climate). Therefore, we started deciphering the influence of season and age on Arabian stallion's seminal characteristics and sperm morphology.

Our results showed that although that stallion aged 15-22 years produced ejaculates of higher volume, stallion aged 4-11 years produce ejaculates showed better sperm count, proportions of livability, motility, and normal sperms than that produced by old stallions. This was comparable with previous reports showed that stallions aged 5-6 years produce ejaculates that have a higher proportions of sperm motility and normality, and lower abnormalities (41). Similarly, another study reported that the stallions between 3 and 11 years of age demonstrated the best semen characteristics (21). The reason behind decline in ejaculate quality is assumed to be due to aged stallions becomes more susceptible to substandard spermatogenesis and testicular degeneration due to the increase in workload required for optimum performance.

Equines are a long-day seasonal breeders. However, the effect of season on stallions' reproductive physiology is deemed controversial (41). The seasonal changes in sperm production and seminal pH have been recorded (42). Our results showed that ejaculates collected during spring and summer showed higher volumes, better motility and better viability, and ejaculates collected in winter and autumn showed higher sperm concentration. It has been reported that the seminal parameters such as volume, pH, sperm concentration, motility, and normal sperms morphology increase during the breeding season (4, 34, 43). We found that the livability, motility, normal sperms, and progressive sperm motility were higher in breeding season. These results are in line with previous studies reported that seasonality can produce alterations in sperm quality such as motility, and sperm structure in livestock animals (44, 45). Our results showed that no detectable significant differences between season groups in the total sperm number per ejaculate, which is in agreement with previous a study reported that ejaculates have to contain one billion morphologically normal, progressively motile sperm regardless of the season (46). Furthermore, the gel-free volume was reported to be highest in spring and summer and lowest in winter (34), which is matching with our finding reported that the gel-free volume in spring season were significantly higher compared with summer, autumn and winter seasons. Such effects is expected to be via environmental temperature during autumn and winter might have negative influences on metabolism that could affect fatty acid composition of spermatozoa as well as had clear results on body temperature.

Considerable attention has been paid to the effect of season of collection on sperm morphology (22), because the percentage of spermatozoa with normal morphology positively correlates with the rate of fertility (13). Our result showed that ejaculates collected in breeding season showed higher percentage of normal sperm than

that in ejaculates collected in non-breeding season. Correlation between sperm morphology and fertility was detected for the proportion of normal sperm in the breeding season only (47). As in the breeding season the proportion of morphologically normal sperm was significantly higher in the fertile group (47). Furthermore, our data showed that the semen characteristics of first and second ejaculates were nearly similar, which is consistent with a previous report found that the difference between total sperm number was significantly less pronounced among the two ejaculates (48).

Conclusion

From the presented results, older stallions (15-22 years) produce ejaculates of larger volume but the motility, viability and abnormalities percentages were better in ejaculates collected from stallions aged 4-11 years. Ejaculates collected during spring and summer showed better characteristic than that collected during autumn and winter. It is recommended to perform semen collections during spring and summer from middle age Arabian stallions selected for natural and AI programs under subtropical conditions.

Acknowledgment

The authors thank Adel-Rahman El-Rewashedh, the Technical director of Arabian Equine Farm for their Help to accomplish this work.

The authors declare no conflicts of interest.

GS, TMB, and AAA conceived and designed the experiments, AAA, and AMS performed the experiments, AM and GS analyzed the data, GS and AAA wrote the first draft of the manuscript, GS and AM revised and edited the manuscript.

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