Int.J.Curr.Microbiol.App.Sci (2023) *12*(09): 307-317

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 12 Number 9 (2023)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2023.1209.030

Relationship between Some Kinematic Parameters of Ram Semen and Reproductive Performance of Dairy Sheep after application of assisted Reproductive Technologies

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Keywords

Ram semen, CASA, kinematics, reproductive performance, estrus synchronization

Article Info

Received:
28 July 2023
Accepted:
30 August 2023
Available Online:
10 September 2023

A B S T R A C T

Aim of this study was to investigate the relationship between some kinematic parameters of ram semen and reproductive performance of dairy sheep after application of assisted reproductive technologies. The experiment was carried out with8 rams and 325 sheep from East Friesian breed and 7 rams and 162 sheep from Asaaf breed. Semen was collected by artificial vagina and subjected to primary assessment. Kinematic sperm parameters TM, PM, NPM, IS, VAP, VCL, VSL, LIN and STR were evaluated by computer-assisted semen analysis (CASA). All sheep were subjected to estrus synchronization by intravaginal sponges and artificially inseminated byfresh-diluted semen. Reproductive performance was recorded on the base of pregnancy rate (PR) and average litter size (ALS) calculated after lambing. Relationships between sperm kinematic parameters and these with the reproductive prformance were determined by correlation analysis. Significant differences between the mean values of age, body weight, ejaculate volume and sperm concentrationin the rams, and PR and ALS were not found. The sperm kinematic parametersshowed variability between individual rams. Total motility correlated positively with PM and LIN andnegativelywith NPM and IS ($P<0.05$). The correlation of PM with LIN and STR was positive, while this with NPM, IM and VCL was negative ($P<0.05$). A negative relationshipwas found of NPP and IS with STR and between LIN and VCL($P<0.05$). The relationship of PM, VAP, LIN, STR with PR, and of LIN and STR with ALS ($P<0.05$) was positive, whereas this between VCL with PR was negative ($P<0.05$). In conclusion, the evaluation of the kinematic parameters by CASA is useful diagnostic and prognostic tool in sheep reproduction. The parameters PM, VAP, LIN and STR have a strong positive relationship ($P<0.05$) with the functional activity of ram spermatozoa and the reproductive performance of the sheep. They could be included as indicators in evaluation of the fresh semen quality in a selection of East Friesian and Asaaf rams with high fertility.

Introduction

The use of semen from high fertility rams is a guarantee for success of the assisted reproductive technologies as estrus synchronization and artificial insemination in small ruminants (Salamon and Maxwell, 1995; Viñoles *et al.,* 2011; Gibbons *et al.,* 2019). The possibility of adequate movement of the spermatozoa through the various parts of the ovine reproductive tract after artificial insemination is very imortant(Kasimanickam *et al.,* 2007; David *et al.,* 2015). The cervix and the isthmus of the fallopian tube are the main barriers to spermatoza.

In these anatomical structures, the mucosal epithelium produces a viscous mucus through which the spermatozoa must penetrate to reach the fertilization site in the oviduct. Its association with local viscous secretions and the complex surface architecture of the mucosa of the cervix and fallopian tube may impede the passage of spermatozoa (Salamon and Maxwell, 1995; Yániz *et al.,* 2015).

Sperm kinematic parameters have a crusial rolethe necessary number of viable male gametes to reach oviduct and to provide successful fertilization of the egg, enshuring high conception rate and fecundity (Robayo *et al.,* 2008; Valverde *et al.,* 2020; Van de Hoek *et al.,* 2022). In routine practice, the degree of sperm motility has been evaluated mainly by a visual assessment of the mass motility (Gaffney *et al.,* 2011). A study of 711,562 sheep of 8 different breeds showed that mass motility can be a prognostic factor for fertility when conventional microscopic determination of the wave motion of the sperm mass from a specific ejaculate is used (David *et al.,* 2015).

Regardless of the subjectivity of the method, the authors report optimization of the prognostic value of the study after a video-assisted determination of total motility for ejaculates with a score between 4 and 5, based on the speed of rotation of the waves, refined in 0.1 steps. According to Gallego *et al.,* (2018), however, the subjective assessment of sperm motility can be influenced by various factors and especially by the experience of the operator. An alternative method for evaluation of the kinematic parameters of sperm cells is Computer Assisted Semen Analysis (CASA), which provides an objective assessment of sperm motility and velosity characteristics (Rodriguez-Martinez and Vega, 2013; Palacín *et al.,* 2013; Yániz *et al.,* 2015; 2018; Van der Horst, 2020; Valverde *et al.,* 2020). Ram spermatozoa have fast movement and follow a strictly linear trajectory unlike those of other ruminants. The relationship between CASA determined kinematic parameters of the spermatozoa andsemen fertility rate has been studied primarily *in vitro* by various tests such as cervical mucus migration, zona pellucida penetration, adenosine triphosphate measurement, etc., and the results are very dabatable (Robayo *et al.,* 2008; Rodriguez-Martinez and Vega, 2013; Tanga *et al.,* 2021). In *vivo studies* are relatively few in rams, compared to other species, with variations from no correlations to significant positive relationships (O' Meara *et al.,* 2008; Del Olmo *et al.,* 2013; Vicente-Fiel *et al.,* 2014).

Robayo *et al.,* (2008) found through an *in vitro* test that specific kinematic parameters allow of the spermatozoa to migrate through cervical mucus with different rheological properties. Only the parameters VAP (average path velocity) and VCL (curvilinear velocity) positively correlated with the ability of sperm cells to migrate in sheep cervical mucus, while VSL (straight line velocity) and LIN (linearity) were associated with migration through goat cervical mucus. Del Olmo *et al.,* (2013) reported a strong positive correlation of fertility with VAP and VCL at 2 h after incubation of ram spermatozoa in semen freezing extender. However, O'Meara *et al.,* (2008) stated no relationship between kinematic parameters determined by CASA and *in vivo* registered fertility. Ejaculates of rams had better characteristics of sperm motility in breeding than non-breeding season, but no seasonal differences in morphological features were observed (Abadjieva *et al.,* 2014). According to Vicente-Fiel *et al.,* (2014) VAP, VCL, VSL (straight line

velocity), LIN and STR (straightness) values in fresh semen from low and high fertility rams differed significantly at hour 0 after ejaculate collection, while after the 6thhour the differences completely disappeared. Tang *et al.*, (2021) concluded that conventional semen analysis should be subjected to sperm quality control using of more modern methods, and the obtained values for motility characteristics should be investigated by clinical experiments before their inclusionin the clinical andrology. Aim of the current study was to investigate the relationship between some kinematic parameters of ram semen and reproductive performance of dairy sheep breeds after application of assisted reproductive technologies in breeding season.

Materials and Methods

Animals

The study was performed with8 rams and 325 sheep from East Friesian breed and 7 rams and 162 sheep from Asaaf breed, reared in intensive dairy sheep farms. The average age and body weight of the male and the female animals from the East Friesian breed were 40±6.9 months and 81.7±2.9 kg, respectively, and those from the Asaaf breed were 32.6±3.2 months and 73.9±4.6 kg. The rearing technology was free in group pens, the feeding was a total mixed ration according to the requirements of the sex and breed of the animals, and drinking water was *ad libitum*. Sheep were at the end of lactation (average daily milk yield <250 ml) with automated milking in a milking parlor. The experiment was carried out during the breeding season (September - October). The experiment was made according to all requirements for welfare and animal protection shown in Bulgarian legislation (Ordinance No. 20/1.11.2012).

Semen collection and evaluation, estrus synchronization and artificial insemination

The ejaculates were collected by artificial vagina and subjected initially to a macroscopic evaluation. Only ejaculates meeting the standard requirements for fresh ram semen (volume ≥ 0.5 ml; consistency dense, wave motion ≥ 3 on a scale of 1 to 5 and absence of agglutination and abnormalities in color and odor) were used. Age (months), body weight (kg) and ejaculate volume (ml) measurement using a graduated pipette were registered. Sperm concentration (x10^9/ml) and kinematic parameters total motility (TM, %), progressive motility (PM, %), non-progressive motility (NPM, %), immotile sperm (IS, %), VAP (μm/s), VCL(μm/s), VSL (μm/s), LIN (%) and STR (%) were determined by CASA analysis (Microptic S.L. Barcelona, Spain) and presented individually for each ram.

All sheep were subjected to estrus synchronization by intravaginal sponges containing 30 mg flurogeston acetate (Syncro-part, Ceva Sante Animale, France) for 12 days and intramuscular injection of 500 UI equine chorionic gonadotropin (Folligon, MSD Animal Health, USA) on day of sponge withdrawal. The artificial insemination was with 0.2 ml fresh-diluted semen containing 80x10^6 motile spermatozoa from 56 to 58 h after the sponge removal.

Reproductive performance registration

After taking into account the lambing data, the parameters of the reproductive performance were recorded. Pregnancy rate (PR) was calculated as a number of sheep lambed, compared to number of inseminated sheep x 100. Average litter size (ALS) was calculated as a number of lambs born per sheep lambing. The values were given as mean±standard deviation according to the breed of sheep.

Statistical analysis

The results were processed using of computer program Statistica version 7.0 (Stat-Soft. - 1984-2000). The kinematic parameters were presented individually for each ram and age of animals, body weight, ejaculate volume and sperm concentration, conception rate and litter size were presented as mean±standard deviation. The mean values between

the different breeds werecompared by non-parametric method for comparison of means and proportions, using Student's *t*-criterion. The relationships between the different sperm kinematic parameters and these of kinematic parameters with pregnancy rate and litter size were determined by correlation analysis, calculating of Pearson's coefficient of correlation (R). Values were considered significant at P<0.05.

Results and Discussion

The comparative analysis showed no significant differences between the mean values of age, body weight, ejaculate volume and spermatozoa concentrationin the rams from the different breeds Table. 1.

However, data about the sperm kinematic parameters of semen in both breeds rams showed individual variability. In the East Friesian sheep, the total motility for rams No. 2, 4 and 8 was about 10% higher than those of the other males (Fig. 1). Similar increases (>15%) were recorded for PM in rams No.2, 3, 4, and 7, for NPM in No.1 and 3, and IS in No.1, 3, and 6. At the same time, variability in total motility and progressive motility in the Asaaf breed was less pronounced, with some differences in non-progressive sperm motility and immotile sperm for rams No.4 and 7 and No.2 and 6, respectively, (Fig. 2). More significant individual differences were recorded in the other kinematic parameters. In the East Friesian breed, the values of VAP for rams No. 4 and 7 (>150µm/s), VCL in No. 1, 5 and 7 (≥160µm/s), VSL in No. 7 and 8 (>60µm/ s) and LIN and STR in No.2 and 8 (>50%) were increased, in comparioson to remained males. In the Asaaf they were more clearly increased in VAP for rams No.5 and 7 (>120µm/s), VCL for No.1, 5 and 7 (≥180µm/s), VSL for No.5 and 6 (> 80µm/s), LIN in No.5 (>50%) and STR in No.5 and 6 (>60%).

The data for the reproductive performance in East Friesian and Asaaf sheep was indicative for close mean values of the pregnancy rate and average litter size (Table. 1). Due to the fact that there were no significant differences in the mean values of the biometric indicators of rams and the ejaculate parameters among both breeds, and with a view to eliminating individual effects ofram and breed, the relationships between the various kinematic parameters and the reproductive performance were investigated, common to both breeds.

The correlation analysis between motility characteristics and velocity indicators showed that TM correlated strongly positively with PM and LIN (P<0.05), while correlations with NPM and IS were negative (P<0.05) (Table 2). In turn, progressive sperm motility was positively correlated with LIN and STR, and the correlations with NPM, IM and VCL were negative (P<0.05). A similar negative relationship (P<0.05) was found between NPP and IS with STR and between LIN and VCL. The straight line velocity and linearity were strongly positively correlated with STR (P<0.05).

The results for the relationships between sperm kinematics and reproductive performance indicated a trend towards a positive correlation of TM with PR (P=0.052) and a high positive correlation between PM and PR (P<0.05). The relationships of VAP, LIN and STR with PR, and LIN and STR with ALS were also positive (P<0.05), whereas this between VCL with PR was negative (P<0.05).

An indicator of the effective application of estrus synchronization and artificial insemination in high-yielding dairy sheep is their reproductive staus, based on the consideration of basic parameters such as pregnancy rate, average litter size and percentage of single and multiple pregnancy (Bonev *et al.*, 2012; Fornazari *et al.*, 2018; Reinoso-Peláez *et al.*, 2023). The main elements determining the success of the assisted reproductive technologies are selection of rams with high fertility and choice of appropriate protocols for estrus synchronization (Abecia *et al.*, 2012; Gibbons *et al.*, 2019). One of the most important procedure before inclusion of the rams in the artificial insemination process is semen quality evaluation. In routine practice, it is most often performed on the basis of macroscopic

evaluation of ejaculate and microscopic examination to determine mass motility, sperm concentration and morphology (Evans and Maxwell, 1987; David *et al.,* 2015). However, these investigations are not a sufficient for detection of the males with the highest fertility, due to incomplete information regarding motility and subjectivity of the evaluation (Gaffney *et al.,* 2011; Gallego *et al.,* 2018).

In our study, the absence of significant differences in the biometric indicators of the rams from the two breeds was indicative of an equalized status of the used sires. The close mean values of ejaculate volume and sperm concentration confirmed this assertion and were in line with established ejaculate volume and sperm concentration for the East Friesian rams (>1.5ml and 3-6.7x10^9/ml) by Bara (2017) and for Asaaf (>1ml and 3x10^9/ml) by Fornazari *et al.,* (2018). Regardless of the fact, that the selection of the donors is carried out according to the standard requirements for fresh ram semen, the question of exact criteria for selection of the best breeders is still open.

The data for relationship between CASA-generated kinematic parameters and reproductive status provided additional information on the fertility of used rams. A similar experiment with Suffolk breed also found individual variations in VAP (from 115.8±5.7 to 126.5±5.7 µm/s), VCL (from 204.2±6.8 to 209.2±6.8 µm/s), VSL (from 93.2±5.9 to 108.2±5.9 µm/s) and LIN (from 44.0±1.2 to 51.5±1.2 %) (Robayo *et al.,* 2008). According to Abadjieva *et al.,* (2014), however, the percentages of fast, medium, low motile and immotile spermatozoa in rams from Synthetic Population Bulgarian Milk sheep obtained during the breeding season were 77.88±6.23%, 17.24±4.52%, 4.72±1.71% and 0.16±0.05%, respectively. The recorded VAP, VCL, VSL, LIN and STR were 58.1±3.46µm/s, 106.22±9.06 µm/s, 30.68±1.61 µm/s, 29.32±1.44% and 55.24±1.70%, respectively. Neila-Montero *et al.,* (2023) examined semen of Asaaf rams at the beginning and end of the breeding season by CASA and reported TM (>80%), PM (>75%) and LIN (>37%), close to the obtained in our study. The results of some above mentioned authors differed from those obtained for the current study, which supported our hypothesis for individual effect of ram and breed.

Explanations for the significant differences could be variations in the degree of maturity of the spermatozoa, the available energy reserves and the composition of the seminal plasma in different rams. The sperm maturation process induces motility changes that are responsible for the efficiency of their migration through the female reproductive tract and achieving successful fertilization (Van de Hoek *et al.,* 2022). Sperms acquire activated motility in the male reproductive tract during passage through the epididymal duct, involving acquisition of flagellation potential connected to 'sperm swimming' pattern (Yeung and Cooper, 2002). Furthermore, the male gamete motility also depends on energy reserves (Freitas *et al.,* 2016). The primary energy source is adenosine triphosphate, obtained by oxidative phosphorylation and the Krebs cycle in the mitochondria of the middle piece and glycolysis in the main piece of the flagellum. It is used by axonemal dynein adenosine triphosphatases to trigger flagellum beating. In addition, various authors (Maxwell *et al.,* 2007; Wang *et al.,* 2020) found an influence of components of the seminal plasma on sperm motility. The results of Ivanova *et al.,* (2021) showed breed dependence in the seminal plasma proteins of rams. According to Neila-Montero *et al.,* (2023), differences in seminal plasma protein expression are associated with energy metabolism, sperm-oocyte interactions, and flagellum structure, which lead to changes in the functional activity of sperm cells. Another reason for the individual and breed influence may be the different proportion of sperm subpopulations in the ejaculates of individual rams. In support of this, Yániz *et al.,* (2015) found that the distribution of sperm subpopulations in semen of high and low fertility rams differed significantly. A later study reported a higher proportion of motile sperm in the presence of an increased subpopulation of sperm cells with an intact plasma membrane and acrosome (Yániz *et al.,* 2018).

Table.1 Biometric indicators and ejaculate parameters in rams and reproductive performance in sheep from different breeds (Mean±SD).

	Biometric indicators and ejaculate parameters			
Breed of rams	Age (months)	Body weight (kg)	Ejaculate volume (ml)	Sperm concentration (x 10^9/ml)
East Freisian (n=8)	40±6.9	81.7±2.9	1.68±0.63	2.58±0.96
Asaaf (n=7)	32.6±3.2	73.9±4.6	1.59±0.32	2.02±0.93
	Reproductive performance			
Breed of sheep	Conception rate (%)			Litter size (n)
East Friesian (n=325)	74.2±8.93			1.89±0.2
Asaaf (n=162)	66.7±7.8			1.75±0.1

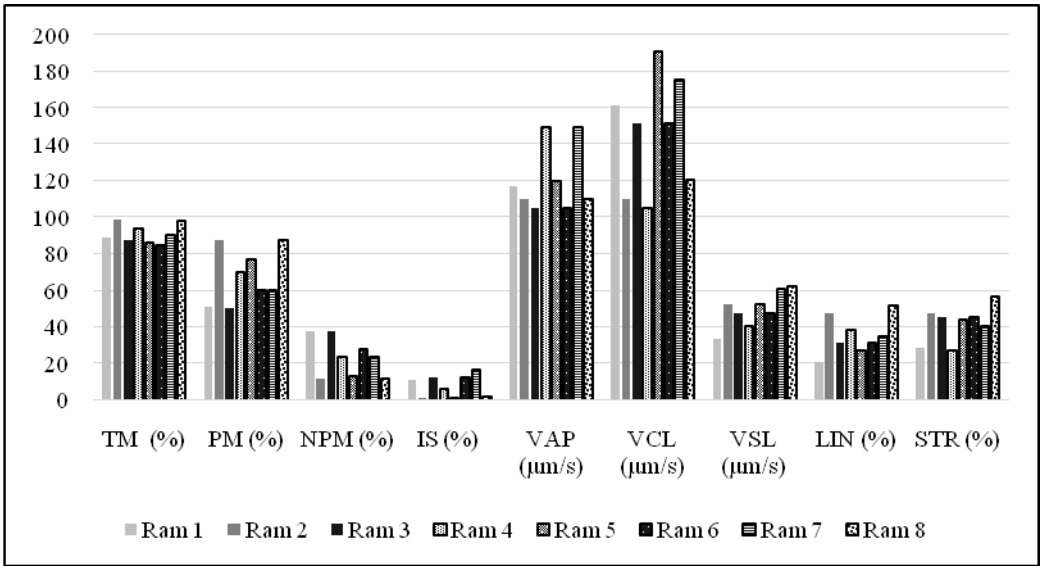
Table.2 Correlation coefficients between sperm kinematic parameters and reproductive performance characteristics.

	TM	**PM**	**NPM**	**IS**	**VAP**	**VCL**	**VSL**	**LIN**	**STR**	**PR**	**ALS**
TM	1										
PM	0.78*	1									
NPM	-0.56	-0.96*	1								
IM	-1.00*	-0.78*	0.56	1							
VAP	0.45	0.55*	-0.28	-0.28	1.00						
VCL	-0.74*	-0.59*	-0.36	-0.39	0.16	1.00					
VSL	0.35	0.40	0.45	0.53	0.10	0.15	1.00				
LIN	0.74*	0.80*	0.42	0.49	0.25	-0.60*	0.29	1.00			
STR	0.47	0.56*	-0.68*	-0.76*	-0.39	-0.10	0.92*	0.88*	1.00		
PR	0.51	0.62*	-0.11	-0.11	0.62*	-0.65*	0.35	0.86*	0.55*	1.00	
ALS	0.30	0.21	-0.23	-0.20	0.31	-0.47	0.52	0.87*	0.77*	0.55	1.00

Total motility - TM, Progressive motility - PM, Non-progressive motility - NPM, Immotile sperm - IS, Average path velocity - VAP, Curve linear velocity - VCL, Straight line velocity, VSL, Linearity - LIN, Straightness - STR, Pregnancy rate - PR, Average litter size – ALS

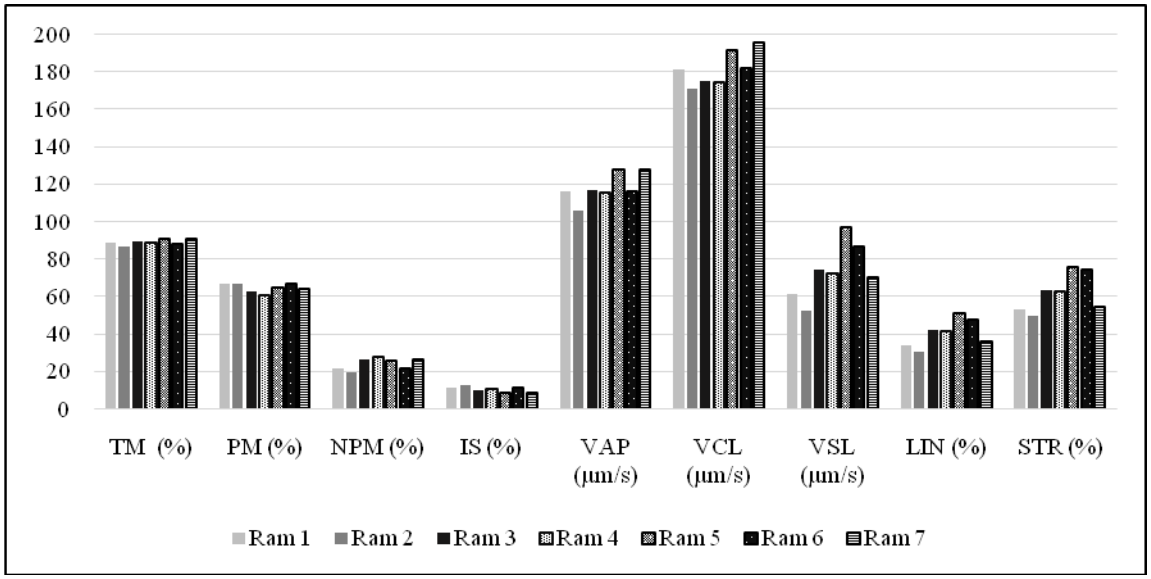
*- *Significant* correlations are marked with *asterisks* at *significance* level P<0.05

Fig.1 Semen kinematic parameters in rams of the East Friesian breed.



Total motility - TM, Progressive motility - PM, Non-progressive motility - NPM, Immotile sperm - IS, Average path velocity - VAP, Curve linear velocity - VCL, Straight line velocity, VSL, Linearity - LIN, Straightness - STR

Fig.2 Semen kinematic parameters in rams of the Asaaf breed.



Total motility - TM, Progressive motility - PM, Non-progressive motility - NPM, Immotile sperm - IS, Average path velocity - VAP, Curve linear velocity - VCL, Straight line velocity, VSL, Linearity - LIN, Straightness - STR

Regardless of all aforementioned, most of the experiments have been carried out in laboratory by obtaining indirect evidence for the fertilizing capacity of spermatozoa without considering the fertility of rams in field trials. This once again argued the need ofadditional investigation of the relationship between sperm kinematics and reproductive abilities of males, based on information for the reproductive performnace of the inseminated sheep. The reproductive status of sheep has been used by various authors (O' Meara *et al.*, 2008; Vicente-Fiel *et al.*, 2014; Yotov *et al.*, 2022) for *in*

vivo determination of the reproductive potential of rams, but most attempts are limited until the determining relationship between reproductive findings and mass motility, individual motility of sperm cells and immotile spermatozoa determined by conventional methods or CASA, without consideration the sperm kinematics (Gaffney *et al.,* 2011; David *et al.,* 2015).

We accepted that high total and progressive sperm motility is the first necessary circumstance for higher sperms fertility, but the final result may also depend on other kinematic parameters, which play an important role for spermatozoa transport through the reproductive tract of female animals (Yániz *et al.,* 2015; Van de Hoek *et al.,* 2022). It was the basis for determining the relationships between different CASA parameters on the one hand, and their dependencies with reproductive performance on the other one, eliminating the effects of ram and breed.

The correlation analysis confirmed our hypothesis about an influence of sperm velocity characteristics on their total and progressive motility and the reproductive performance of sheep. The increase in VAP, LIN and STR had a strong positive influence ($P<0.05$) on TM and PM, and increasing PM had a positive effect ($P<0.05$) on pregnancy rate. At the same time, the increase in VCL had a negative effect on both types of motility and LIN, associated with a decrease in PR. The higher LIN and STR correlated positively ($P<0.05$) with the pregnancy rate and average litter size.

The above was in agreement with the results of other authors (Del Olmo *et al.,* 2013; Vicente-Fiel *et al.,* 2014) who conducted similar experiments with rams of other breeds. This result provided more information about the possibilities of using different sperm kinematic parameters in selection East Friesian and Asaaf rams with high reproductive abilities. Irrespective of that**,** the determination of accurate reference values requires additional research on a larger number of ejaculates collected during the breeding season, considered to age and breed of the animals.

To obtain more correct information regarding *in vivo* determination of reproductive capacity of breeders and efficiency of the used assisted reproduction technologies, it is recommended to investigate the relationships between the individual characteristics of the reproductive status (Tanga *et al.,* 2021). The additional analysis in this direction showed a tendency ($R=0.55$; $P=0.051$) increase in pregnancy rate to lead a higher average litter size, also reported by other researchers (Petrović *et al.,* 2018; Madrigali *et al.,* 2021). However, in this study a strong positive relationship between PR and ALS was not found. An explanation for the lack of a strong positive correlation between these parameters may be the fact that the pregnancy rate is calculated as a ratio between the number of inseminated and the number of ewes lambed, without taking into account the type of pregnancy.

In many cases PR is very high, but a significant percentage of pregnancies can be singletons. Based on this result and the information for direct dependence of number of the lambs born from number of the ovulations (Drouilhet *et al.,* 2013), we can recommend the interpretation of ALS as indicator for *in vivo* determination of the reproductive capacity of rams to be analysed in line with genetically determined prolificacy of sheep.

In conclusion, the evaluation of the kinematic parameters by computer-assisted semen analysis is an extremely useful diagnostic and prognostic tool in sheep reproduction. The parameters PM, VAP, LIN and STR have a strong positive relationship ($P<0.05$) with the functional activity of ram spermatozoa and the reproductive performance of the sheep. They could be included as indicators in evaluation of the fresh semen quality and preliminary selection of East Friesian and Asaaframs with high fertility.

Acknowledgements

The authors gratefully acknowledge to all members of the Department of Obstetrics, Reproduction and Reproductive Disorders, Faculty of Veterinary

Medicine Trakia University and Agrimlk Farm to their assistance during the investigations performance.

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How to cite this article:

Branimir Sinapov and Stanimir Yotov. 2023. Relationship between Some Kinematic Parameters of Ram Semen and Reproductive Performance of Dairy Sheep after application of assisted Reproductive Technologies. *Int.J.Curr.Microbiol.App.Sci.* 12(09): 307-317.
doi: https://doi.org/10.20546/ijcmas.2023.1209.030