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Analysis of Awassi Sperm Motility in Two Media at Different Levels of Temperature, pH and Osmolality

Research Article

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ABSTRACT

The effects of five levels of osmolality and pH as well as three temperatures on Awassi ram sperm motility parameters assessed by computer-aided sperm analyzer (CASA) in tyrode albumin lactate pyruvate (TALP) and egg-yolk-tris (YET) media were studied. Semen obtained from five adult Awassi rams were pooled and diluted in the above two media at 100, 200, 300, 400 and 500 mOsm/kg at temperatures of 4˚, 20˚ and 37˚ degree celsius and also at pH levels of 5, 6, 7, 8 and 9 at the same temperatures. The osmolality, pH and temperature had a significant effect (P<0.05) on the percent motility (MOT %) and the percent of sperm showing progressive motility (PMOT %). With the exception of lateral head displacement, all the other CASA motility parameters were also significantly affected (P<0.05). Semen incubated in YET was able to tolerate osmolarities within 200 to 400 mOsm/kg range for MOT %. Alkaline condition at pH 9 had higher negative effect than acidic condition at pH 5. Compared to the 20 ˚C and 37 ˚C, the 4 ˚C had negatively affected MOT % and PMOT % and this was more obvious in TALP medium. In conclusion, our results suggest: 1) the significant effects of osmolality, pH and temperature on Awassi sperm incubated in both TALP and YET media and 2) the need for careful selection of temperature by which the semen may be manipulated during artificial insemination and *in vitro* fertilization experiments in these two media.

KEY WORDS computer-aided sperm analyzer, osmolality, ram, temperature.

INTRODUCTION

Sperm motility is one of the most commonly used parameters for the evaluation of sperm quality for both fresh and cryopreserved sperm (Comhaire *et al.* 1992; Holt, 2000). The measurement of motility provides methods of sperm assessment to develop media for artificial insemination (AI) and *in vitro* fertilization (IVF). Multiple methods have been developed to analyze physiological changes in sperm motility (Mortimer, 1997). In this respect, a major advance in the rapid and objective assessment of sperm motility in mammals has come from using the sophisticated image analysis methodology called computer-aided sperm analysis (CASA); (Amann and Waberski, 2014). Moreover, the availability of data recorded by CASA, and the comparison of sperm motility results facilitates the identification of subtle changes between males, subpopulations of spermatozoa and different media (Holt and Palomo, 1996; Alomar *et al.* 2006; Mortimer and Mortimer, 2013).

Sperm cells are totally dependent on their storage medium either *in vitro* or *in vivo*. Therefore, media and extenders have been designed, usually on an empirical basis, to protect and maintain spermatozoa during the processing and storage of the semen. For instance, media based on tris plus egg yolk has been widely used for AI in animal species including the ram (Salamon and Maxwell, 1995a; Salamon and Maxwell, 1995b). This AI medium is usually used for cervical and intrauterine inseminations (Eppleston *et al.*

1994) by using fresh or cryopreserved sperm. Most IVF labs use tryode albumin lactate pyruvate (TALP) medium for *in vitro* fertilization (Farrell *et al*. 1996). Lactate and pyruvate are crucial for sperm vitality, while albumin plays an important role in sperm capacitation (Langlais and Roberts, 1985; Visconti *et al*. 2002).

It is well known that several parameters of the dilution medium, such as osmolality, pH and temperature can affect semen quality of different species, such as rams, human and bulls (Curry *et al*. 1994; Varisli *et al*. 2009; Contri *et al*. 2013). In this respect, osmolality and pH of the medium markedly affected sperm survival and motility (Curry *et al*. 1994; Joshi *et al*. 2006). Although the impact of temperature on sperm motility is also acknowledged (Bag *et al*. 2002a; Nur *et al*. 2010), no study, has detailed the effect of osmolality, pH and temperature together on ram sperm motion characteristics assessed by the CASA system.

Awassi sheep is the dominant breed in Middle East Area (Galal *et al*. 2008). It is well adapted to harsh environmental conditions and is raised primarily for triple-range of products; meat, milk and wool (Sleiman and Abi Saab, 1995). To improve the AI and IVF programs of this important breed, it is necessary to perform systematic studies aimed at improving the IVF and AI protocols by analyzing and developing the active media used in these protocols. Therefore, the main objectives of the current study were to compare the characteristics of sperm motility in Awassi ram semen diluted in TALP and in YET media in different osmolality, pH and temperature degrees using CASA system.

MATERIALS AND METHODS

Site description and experimental animals

The present study was carried out at the Division of Animal Production, Der Al-Hajar Research Centre, about 33 km South-East of Damascus. Five adult Awassi rams aged between 2 and 3 years were used. Animals were fed a diet based on concentrate, wheat straw and barley while water was available *ad libitum*.

Semen collection and semen analysis

Electrical stimulation was applied for semen collection with the aid of an electro-ejaculator (Minitube-GmbH-Electro Ejaculator, Germany) administrating a series of cycles pulses of short electrical stimuli with each cycle (two seconds impulse, then two seconds interval) delivering a slightly higher intensity (from 0 volt to 20 volt maximum) to obtain semen. The entire procedure was performed in approximately 2 min and repeated directly if the ram did not respond. It must be noted that the animals were handled according to the recommendations of the declaration of Helsinki and the internationally accepted principles for the care and use of experimental animals. The collected ejaculates were assessed for semen volume and sperm concentration. The ejaculates with less than 0.5 mL of volume and 1 $\times 10^9$ mL of concentration were removed from the analysis. Male variations were discarded by mixing the collected and accepted ejaculates of the five rams directly after semen collection.

Media preparation and experimental design

The first experiment was designed to examine the effects of five osmolality levels on ram sperm motility diluted in tyrode albumin lactate pyruvate (TALP) and egg-yolk-tris (YET) media at three different temperature degrees. The TALP solution prepared as a 300 mOsm/kg solution contained the following in g/L^{-1}: 6.6 g NaCl, 0.24 g KCl, 0.04 g NaH_2PO_4, 1.92 g $NaHCO_3$, 0.1 g $MgCl_2.6H_2O$, 0.3 g $CaCl_2.2H_2O$, 0.11 g $C_3H_3O_3Na$, 0.5 g $C_3H_5NaO_3$ and 6 g bovine serum albumin. The YET medium also prepared as a 300 mOsm/kg solution contained the following: 2.44 g tris (hydroxymethyl) aminomethane, 1.36 g citric acid monohydrate and 1 g glucose in 80 mL of distilled water, plus 20 mL of egg yolk, bringing the total volume to 100 mL. The two media components were held constant at pH 7 and only distilled water or NaCl solution of 700 mOsm/kg was varied to provide final solution of 100, 200, 400 and 500 mOsm/kg. The osmolarity was determined using an osmometer (Osmomat, 030-gonotec, Germany). The instrument was calibrated with 100, 300, and 1000 mOsm/kg standards before use and each test solution was assayed in duplicate or triplicate. In this experiment semen was diluted in the two media to give a concentration of 25 $\times 10^6$ sperm/mL. Semen was distributed into the experimental media at room temperature and incubated for 30 minutes before CASA analysis at 4 ˚C, 20 ˚C and 37 ˚C. This experiment was replicated for three times in each medium.

The second experiment was designed to examine the effects of five pH levels on Awassi ram sperm motility diluted in TALP and YET media at three different temperature degrees.

The two media were prepared as mentioned in the previous experiment and held constant at 300 mOsm/kg. Five different pH values of 5, 6, 7, 8 and 9 were prepared by the addition of 1 N of NaOH or 1 N of HCl just before the start of the trial. The pH of each semen dilution was determined using a pH meter (Bibby Scientific, Model 3505, United Kingdom). Semen was diluted in the two media to give a concentration of 25 $\times 10^6$ sperm/mL. Semen was distributed into the experimental media at room temperature and incubated for 30 minutes before CASA analysis at 4 ˚C, 20 ˚C and 37 ˚C. This experiment was replicated three times in each medium.

Motility analyses

The motility characteristics of the sperm were assessed by CASA technique, using the Hamilton-Thorne motility analyzer (HTM version 12.3, USA). Five microliters aliquots of diluted semen were placed in the system lame and loaded into the analyzer. At least three fields were counted for each sample. The motility characteristics included in the analysis were: the percent motility (MOT %), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), percent linearity (LIN, %), percent straightness (STR, %), lateral head displacement (ALH, µm) and the percent of sperm showing progressive motility (VAP≥75 µm/s and STR≥80%). More details about these parameters are given by Mortimer (2000). The HTM system settings of ovine spermatozoa are presented in Table 1.

Table 1 The settings for the Hamilton Thorne Biosciences system version 12.3 used to evaluate ram semen

Parameters	Settings
Frame rate (Hz)	60
Frames acquired (no)	30
Minimum contrast	60
Minimum cell size (pixels)	5
Low average path velocity (VAP) cut off	21.9
Low straight line velocity (VSL) cut off	6
Non-motile head size (pixels)	5
Non-motile head intensity	55
Static size limit (min/max)	0.60/8
Static intensity limit (min/max)	0.25/1.50

Statistical analysis

Statistical analyses were performed by using Minitab™ program (Minitab, Coventry, UK). Sperm motility parameters were investigated by repeated analysis of variance ANOVA using the general linear model procedure (GLM). The analysis was followed by multiple pairwise comparisons using a post-hoc (Tukey) test. The threshold of signification was set at $P < 0.05$.

RESULTS AND DISCUSSION

The effects of osmolality and temperature on CASA motion characteristics of Awassi sperm assessed in TALP and YET media are depicted in Figure 1. With the exception of ALH parameter, the osmolaltiy of the two media at each temperature degree had significant effect ($P<0.05$) on sperm motility percentage and other CASA velocities parameters. Also the temperature degree had significantly affected ($P<0.05$) sperm motility characteristics for the two media at each osmolality level. Sperm in YET medium were better able to tolerate the three temperature conditions and osmolarity rang of 200, 300, 400 mOsm/kg compared to TALP medium assessed by both MOT % and PMOT %. However, when the osmolarity of YET medium was low (100 mOsm/kg) or high (500 mOsm/kg) a clear and significant effect ($P<0.05$) of osmolarity on motility profile was noted as it was also the case for TALP medium. For the two media, the profile of VCL and VSL velocity parameters was exactly the same as VAP with the same significant indicators. Although both temperature and osmolality levels had a general significant effect on STR % and LIN %, no obvious differences were noted between the different endpoints of these two conditions.

The effects of pH and temperature on CASA motion characteristics of Awassi sperm assessed in TALP and YET media are shown in Figure 2. With the exception of ALH parameter, the pH of the two media at each temperature degree had significant ($P<0.05$) effect on CASA sperm motility parameters. Likewise, the temperature degree significantly affected ($P<0.05$) sperm motility characteristics for the two media at each pH level. The pH values 5 and 9 markedly altered the motility parameters in the two media and semen incubated at 4 ˚C were more negatively affected at pH 9 compared to the 20 ˚C and 37 ˚C at the same pH levels. As was the case in the osmolality experiment and for the two media, the profile of VCL and VSL velocity parameters was the same as VAP with the same significant indicators. Also both temperature and pH levels had a general significant effect on STR % and LIN % parameters**.** However, no obvious differences were noted between the different endpoints of these two conditions.

In the present study, we focused on sperm motility characteristics because it indicates active metabolism and integrity of sperm membrane, and is of a great importance for fertilizing capacity. As expected, sperm motility parameters were significantly influenced by osmolality, pH and temperature factors in the two media. However, the importance of the present results was in showing the effects of the interaction between the different osmolality-pH levels and the three temperature conditions. The temperatures employed in this study are the most likely sperm conditions encounter in any given medium. The 37 ˚C is the degree of body temperature and IVF incubators. The 20 ˚C is a normal room temperature of any semen laboratory for semen manipulation, while the 4 ˚C is a cooling temperature which is used to store semen. Generally, it would seem reasonable to process samples routinely at room temperature as it probably has a lower effect on semen quality compared to 30 ˚C (Farrell *et al.* 1996). The decrease of storage temperature to 4 ˚C may prove beneficial for prolonging the storage time of semen using fresh samples. In this study, we show that in comparison to both 20 ˚C and 37 ˚C and at different levels of osmolality and pH, the 4 ˚C tempereture had a negative effect on sperm motility characteristics especially in TALP medium.

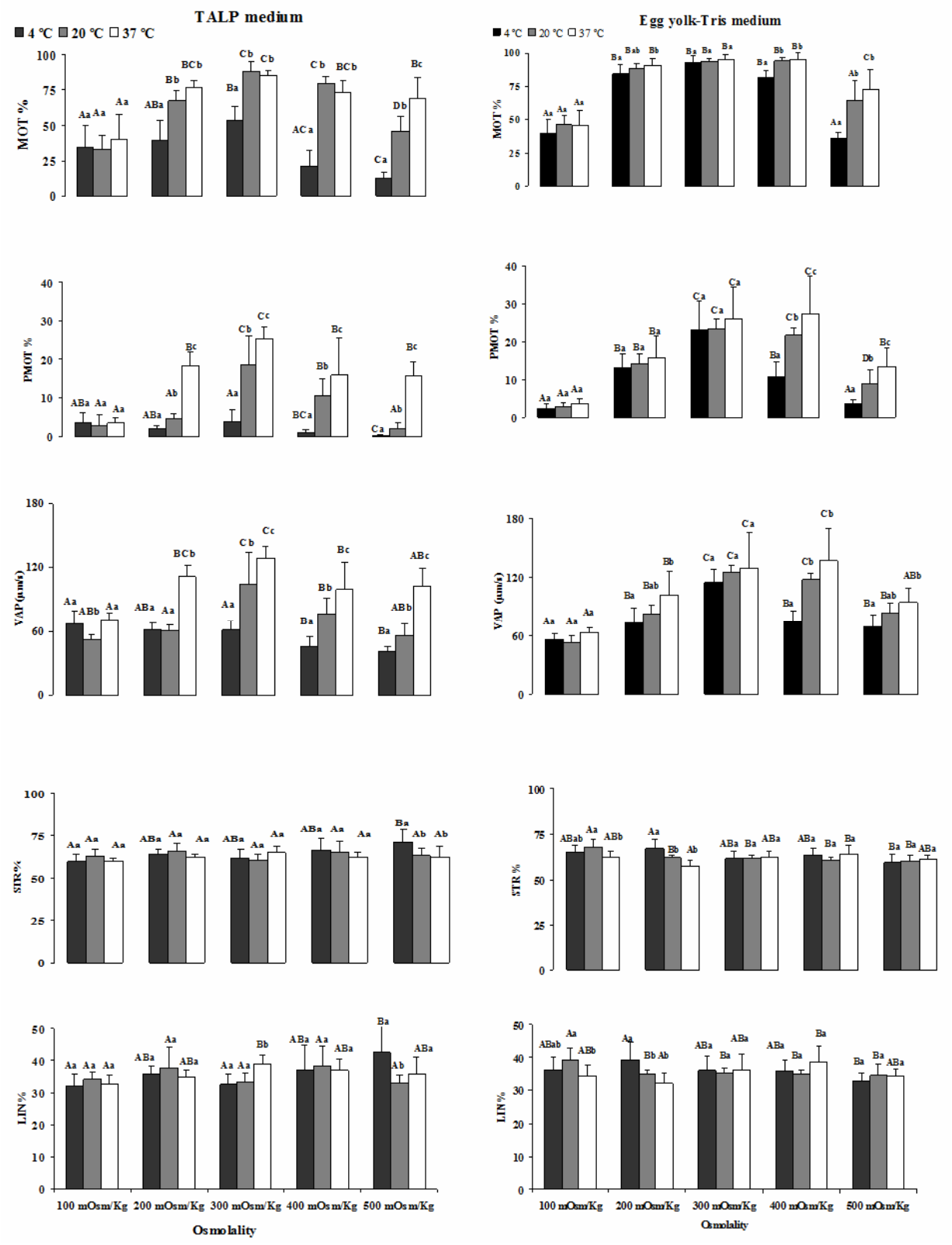

Figure 1 Effect of osmolality and temperature on CASA sperm motion characteristics of Awassi rams assessed in TALP and YET media
[A-D] Different letters within different osmolality levels in each temperature degree denote significant difference (P<0.05)
[a-d] Different letters within different temperature degrees in each osmolality level denote significant difference (P<0.05)

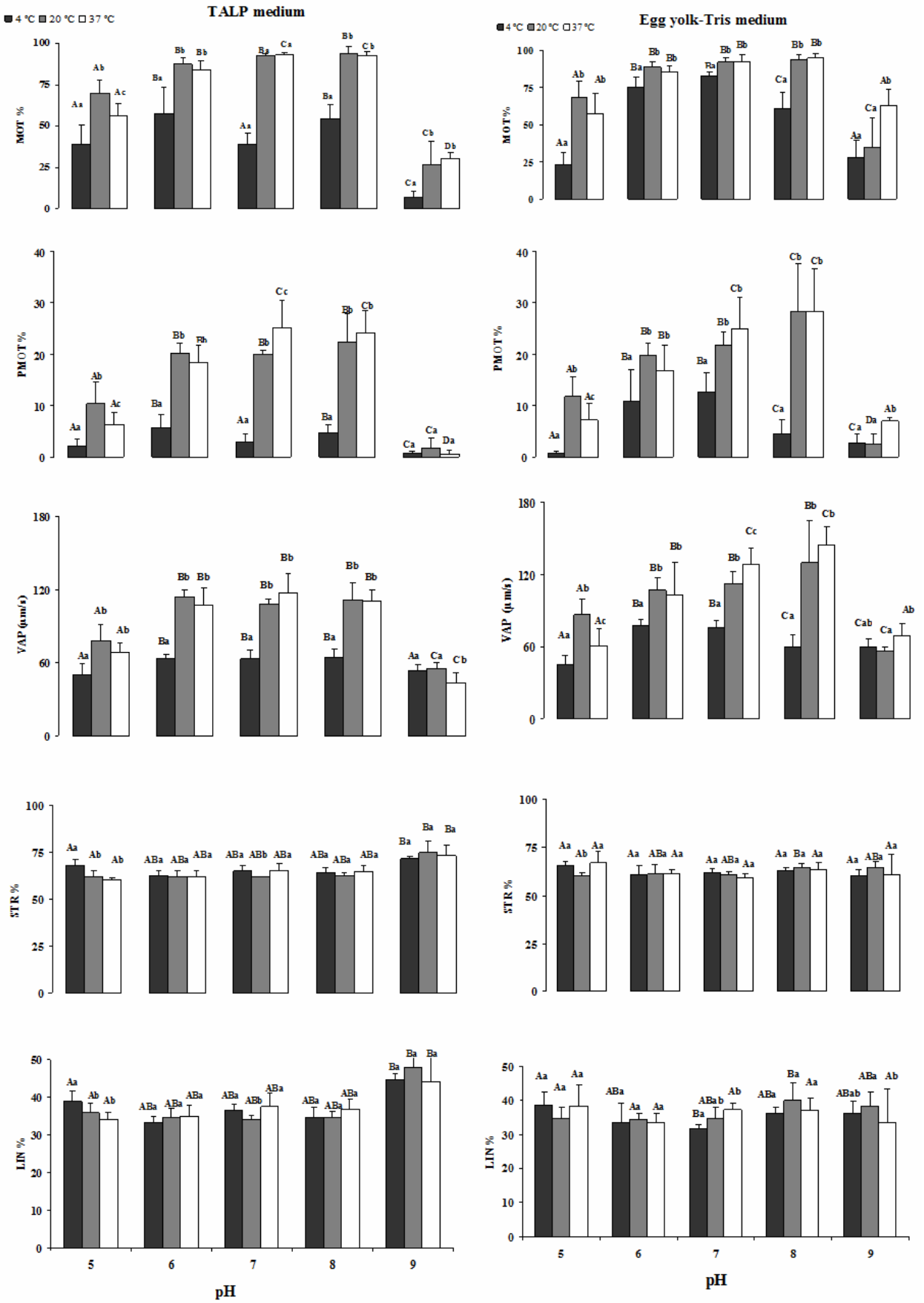

Figure 2 Effect of pH and temperature on CASA sperm motion characteristics of Awassi rams assessed in TALP and YET media
[A-D] Different letters within different pH levels in each temperature degree denote significant difference (P<0.05)
[a-d] Different letters within different temperature degrees in each pH level denote significant difference (P<0.05)

It is well known that cold shock (i.e. damage due to rapid cooling above 0 ˚C) could be caused by lipid phase transition effects and this might be involved in the manifestation of cold shock injury both on cooling and also rewarming of the sperm cells (Drobnis *et al.* 1993).

The two media in the present study have been in use in our laboratory for several years and they were compared for their value in processing ram and buck sperm for CASA analysis. Human, rabbit and bull sperm can be diluted to concentrations suitable for CASA in TALP medium without substantial changes in CASA variables (Farrell *et al.* 1996). Moreover, the motility of human sperm changes little during several hours of storage at room temperature in TALP (Makler and Jakobi, 1981). Likewise and with the exception of pH 9 and 500 mOsm/kg levels, our results showed that the MOT % of the incubated ram sperm in TALP medium at room temperature did not significantly differ from that incubated at 37 ˚C for the different osmolality and pH levels.

The current work showed the effects of osmolality and pH in YET medium free from any cryoprotectant agent. The beneficial effect of cryoprotectant-supplemented media on post thaw mammalian semen and the osmolality of extenders containing different cryopreserve agents have been extensively reported in many studies (Salamon and Maxwell, 1995a; Salamon and Maxwell, 1995b; AK *et al.* 2010).

YET without cryoprotectant agent could serve by using fresh semen for both cervical and intrauterine inseminations. Moreover, AI with frozen semen dispensed through the cervix gives quite low fertility rates in ram. Although the use of laparoscopy with frozen-thawed sperm has improved the fertility rate significantly, this method is a costly and time-consuming technique especially in the developing countries.

Egg yolk in YET acts by coating the sperm plasma membrane and it has a reversible attachment which may be responsible for the protective activity during chilling (Watson, 1975). This could be responsible for the higher MOT % and PMOT % values in YET at 4 ˚C compared to TALP medium.

All semen samples were diluted to obtain a concentration of 25 × 10^6 sperm/mL which is within the recommended range for getting an accurate CASA measurement (Bag *et al.* 2002b). Subtle changes in CASA sperm motility and velocity patterns have been correlated with fertilizing ability *in vitro* and *in vivo* in several species including bull, dog and human (Vantman *et al.* 1989; Farrell *et al.* 1998; Rijsselaere *et al.* 2007). However, it is still not clear which characteristics of sperm movement assessed by CASA are of real diagnostic value for predicting fertility and *in vitro* fertilization rates in sheep.

In our present work, MOT %, PMOT %, VAP, VSL and VCL were the CASA measurements which showed a clear difference between the different endpoints of osmolality, pH and temperature. In contrast, ALH, STR % and LIN % parameters did not show an obvious difference between these endpoints. Herrara *et al.* (2005) reported that *in vitro* fertilization was significantly correlated to progressive motility, but not to VAP and LIN %. Moreover, Robayo *et al.* (2008) showed that VCL and VAP are the only sperm kinematics parameters positively correlating with the ability of sperms to migrate in ewe cervical mucus; and these could be important indicators of fertilization process.

Awassi semen incubated in YET medium was able to tolerate osmolarities between 200 to 400 mOsm/kg range at least for the MOT % parameter. Such result clearly showed the flexibility of Awassi ram sperm in adjusting to the different osmolality conditions of some media. Compared with other points, the 400 mOsm/kg point had the higher VAP, VSL and VCL values at 37 ˚C in YET medium which indicates the advantage of this level of osmolality and temperature degree on Awassi sperm velocity. It has been noted that extenders with high osmotic pressure (425 mOsm) are a better choice for ram semen freezing compared to extenders with low osmolarity (Ak *et al.* 2010).

The lower and the higher pH level (pH 5 and 9) were clearly able to significantly reduce sperm motility in TALP and YET media for all temperatures degrees. In this respect, the higher negative effect for both MOT % and PMOT % parameters was recorded in the alkaline condition at pH 9.

In agreement with our results, Contri *et al.* (2013) noted the increase pH degree to 8.5 resulted in the immobilization of bull spermatozoa through a significant reduction in their mitochondrial activity. In contrast, human sperm had exhibited a greater reduction in motility in response to acidic conditions than to alkaline conditions (Makler *et al.* 1981). The relationship between intracellular pH and motility was previously studied, and the link between these parameters was suggested to be protein phosphorylation (Carr and Acott, 1989).

CONCLUSION

The results of the current study suggested that osmolality and pH significantly affect Awassi ram sperm motility patterns assessed by CASA and this was partially related to the medium employed, but most importantly to temperature. This finding implies the need for careful selection of temperature by which the semen may be manipulated during AI and IVF techniques. Our study is also useful for indicating which CASA motility measurements are worth further investigation to develop a set of prognostic sperm motility factors for AI and IVF media.

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REFERENCES

Ak K., Cirit U., Nur Z., Bacinoglu S., Pabuccuoglu S., Ozdas O.B. and Birler S. (2010). Effects of extender osmolarity, cooling rate, dilution rate and glycerol addition time on post-thaw ram semen characteristics and fertilization. *J. Fac. Vet. Med. Istanbul Univ.* **36,** 33-46.

Alomar M., Mahieu J., Verhaeghe B., Defoin L. and Donnay I. (2006). Assessment of sperm quality parameters of six bulls showing different abilities to promote embryo development. *Reprod. Fertil. Dev.* **18,** 395-402.

Amann R.P. and Waberski D. (2014). Computer-assisted sperm analysis (CASA): Capabilities and potential developments. *Theriogenology.* **81,** 5-17.

Bag S., Joshi A., Rawat P.S. and Mittal J.P. (2002a). Effect of initial freezing temperature on semen characteristics of frozen-thawed ram spermatozoa in a semi-arid tropical environment. *Small Rumin. Res.* **43,** 23-29.

Bag S., Joshi A., Naqvi S.M.K., Rawat P.S. and Mittal J.P. (2002b). Effect of freezing temperature, at which straws were plunged into liquid nitrogen, on the post-thaw motility and acrosomal status of ram spermatozoa. *Anim. Reprod. Sci.* **72,** 175-183.

Carr D.W. and Acott T.S. (1989). Intracellular pH regulates bovine sperm motility and protein phosphorylation. *Biol. Reprod.* **41,** 907-920.

Comhaire F.H., Huysse S., Hinting A., Vermeulen L. and Schoonjans F. (1992). Objective semen analysis: Has the target been reached? *Hum. Reprod.* **7,** 237-241.

Contri A., Gloria A., Robbe D., Valorz, C., Wegher L. and Carluccio A. (2013). Kinematic study on the effect of pH on bull sperm function. *Anim. Reprod. Sci.* **136,** 252-259.

Curry M.R., Millar J.D. and Watson P.F. (1994). Calculated optimal cooling rates for ram and human sperm cryopreservation fail to conform with empirical observations. *Biol. Reprod.* **51,** 1014-1021.

Drobnis E.Z., Crowe L.M., Berger T., Anchordoguy T.J., Overstreet J.W. and Crowe J.H. (1993). Cold shock damage is due to lipid phase-transitions in cell-membranes a demonstration using sperm as a model. *J. Exp. Zool.* **265,** 432-437.

Eppleston J., Salamon S., Moore N.W. and Evans G. (1994). The depth of cervical insemination and site of intrauterine insemination and their relationship to the fertility of frozen-thawed ram semen. *Anim. Reprod. Sci.* **36,** 211-225.

Farrell P.B., Foote R.H., Mcardle M.M., Trouern-Trend V.L. and Tardif A.L. (1996). Media and dilution procedures tested to minimize handling effects on human, rabbit and bull sperm for computer-assisted sperm analysis (CASA). *J. Androl.* **17,** 293-300.

Farrell P.B., Presicce G.A., Brockett C.C. and Foote R.H. (1998). Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. *Theriogenology.* **49,** 871-879.

Galal S., Gursoy O. and Shaat I. (2008). Awassi sheep as a genetic resource and efforts for their genetic improvement: A review. *Small Rumin. Res.* **79,** 99-108.

Herrara C., Brogliatti G., Cavia R., Conde P., Revora M. and Pasqualini R.S. (2005). CASA sperm parameters and their relation with *in vitro* fertilization. Pp. 411-415 in Proc. 15th Int. Congr. Anim. Reprod., Porto Seguro, Brazil.

Holt W.V. (2000). Basic aspects of frozen storage of semen. *Anim. Reprod. Sci.* **62,** 3-22.

Holt W.V. and Palomo M.J. (1996). Optimization of a continuous real-time computerized semen analysis for ram sperm motility assessment, and evaluation of four methods of semen preparation. *Reprod. Fertil. Dev.* **8,** 219-230.

Joshi A., Mathur A.K., Naqvi S.M.K. and Mittal J.P. (2006). Influence of osmolality of complete semen extender on motion characteristics of frozen-thawed ram spermatozoa. *Asian-Australasian J. Anim. Sci.* **19,** 716-1721.

Langlais J. and Roberts K.D. (1985). A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. *Gamete Res.* **12,** 183-224.

Makler A. and Jakobi P. (1981). Factors affecting sperm motility. V. Washing and resuspension of human spermatozoa in various artificial media. *Fertil. Steril.* **35,** 442-446.

Makler A., David R., Blumenfeld Z. and Better O.S. (1981). Factors affecting sperm motility. VII sperm viability as affected by changes of pH and osmolarity of semen and urine specimens. *Fertil. Steril.* **36,** 507-511.

Mortimer S.T. (1997). A critical review of the physiological importance and analysis of sperm movement in mammals. *Hum. Reprod. Update.* **3,** 403-439.

Mortimer S.T. (2000). CASA-Practical aspects. *J. Androl.* **21,** 515-524.

Mortimer D. and Mortimer S.T. (2013). Computer-aided sperm analysis (CASA) of sperm motility and hyperactivation. *Methods Mol. Biol.* **927,** 77-87.

Nur Z., Zik B., Ustuner B., Sagirkaya H. and Ozguden C.G. (2010). Effects of different cryoprotective agents on ram sperm morphology and DNA integrity. *Theriogenology.* **73,** 1267-1275.

Rijsselaere T., Mase D., Hoflack G., de Kruif A. and Van Soom A. (2007). Effect of body weight, age and breeding history on canine sperm quality parameters measured by the Hamilton-Thorne Analyser. *Reprod. Domest. Anim.* **42,** 143-148.

Robayo I., Montenegro V., Valdes C. and Cox J.F. (2008). CASA assessment of kinematic parameters of ram spermatozoa and their relationship to migration efficiency in ruminant cervical mucus. *Reprod. Domest. Anim.* **43,** 393-399.

Salamon S. and Maxwell W.M.C. (1995a). Frozen storage of ram semen. I. Processing, freezing, thawing and fertility after cervical insemination (Review). *Anim. Reprod. Sci.* **37,** 185-249.

Salamon S. and Maxwell W.M.C. (1995b). Frozen storage of ram semen. II. Causes of low fertility after cervical insemination

and methods of improvement. *Anim. Reprod. Sci.* **38,** 1-36.

Sleiman F.T. and AbiSaab S. (1995). Influence of environment on respiration, heart rate and body temperature of filial crosses compared to local Awassi sheep. *Small Rumin. Res.* **16,** 49-53.

Vantman D., Bands S.M., Koukoulis G., Dennison L. and Sherins R.J. (1989). Assessment of sperm motion characteristics from fertile and infertile men using a fully automated computer-assisted semen analyzer. *Fertil. Steril.* **51,** 156-161.

Varisli O., Uguz C., Agca C. and Agca Y. (2009). Motility and acrosomal integrity comparisons between electro-ejaculated and epididymal ram sperm after exposure to a range of anisosmotic solutions, cryoprotective agents and low temperatures. *Anim. Reprod. Sci.* **110,** 256-268.

Visconti P.E., Westbrook V.A., Chertihin O., Demarco I., Sleight S. and Diekman A.B. (2002). Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *J. Reprod. Immunol.* **53,** 133-150.

Watson P.F. (1975). The interaction of egg yolk and ram spermatozoa studied with a fluorescent probe. *J. Reprod. Fertil.* **42,** 105-111.