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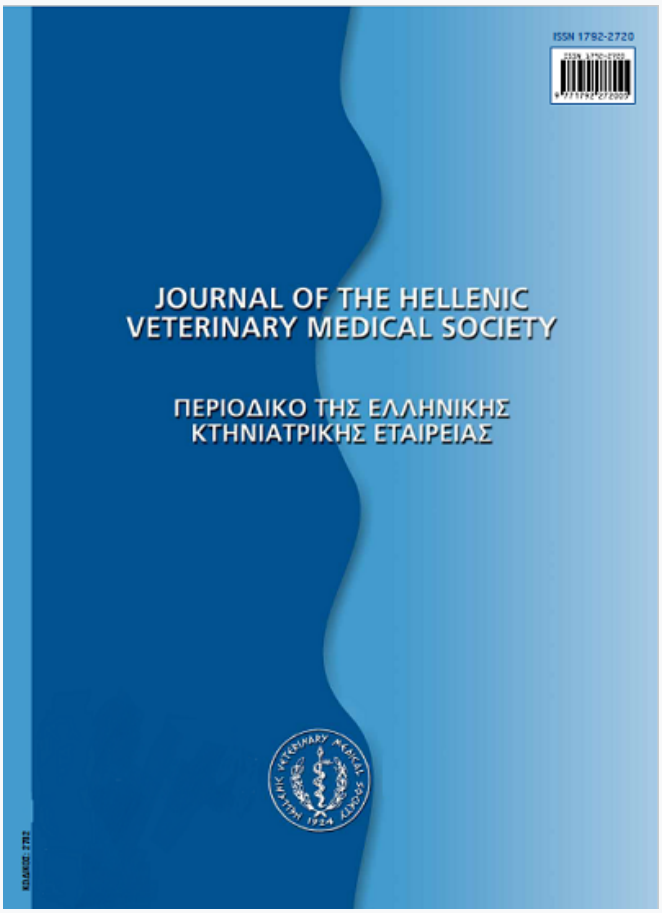

The application of in vitro fertilization techniques for the evaluation of ram fertility

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Review article
Ανασκόπηση

The application of in vitro fertilization techniques for the evaluation of ram fertility

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Η εφαρμογή της in vitro γονιμοποίησης ως τεχνικής για την αξιολόγηση της γονιμότητας του σπέρματος κριαριών

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ABSTRACT. The prediction of field fertility of a given ram by using *in vitro* tests would be of great importance for the reproductive management in sheep flocks. There are many *in vitro* procedures available for evaluating semen quality and fertilizing ability, and the method chosen depends on the objective of evaluating the sperm and the available resources. The in vitro evaluation of semen fertilizing ability was firstly developed for the artificial insemination (AI) purposes and secondly for the application of *in vitro* fertilization (IVF) technique. The IVF techniques allow the assessment of fertility in terms of ability to penetrate and fertilize *in vitro* mature oocytes and ultimately to yield component embryos following IVF and culture. In this review are briefly presented *in vitro* studies performed in an attempt to establish an accurate laboratory test for the evaluation and, even more, the prediction of field fertility in sheep.

Keywords: AI, IVF, *in vitro* embryo production, ram, semen.

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ΠΕΡΙΛΗΨΗ. Η πρόβλεψη της γονιμοποιητικής ικανότητας ενός κριού χρησιμοποιώντας *in vitro* δοκιμές, θα μπορούσε να έχει μεγάλη σημασία για την αναπαραγωγική διαχείριση σε ποίμνια προβάτων. Υπάρχουν πολλές διαδικασίες *in vitro* που διατίθενται για την αξιολόγηση της ποιότητας του σπέρματος και της γονιμοποιητικής ικανότητας, και η μέθοδος που θα επιλεγεί εξαρτάται από το στόχο της αξιολόγησης του σπέρματος και των διαθέσιμων πόρων. Η αξιολόγηση της γονιμοποιητικής ικανότητας του σπέρματος *in vitro*, κατά αρχάς αναπτύχθηκε για την εφαρμογή της τεχνητής σπερματέγχυσης (AI) και μετέπειτα για την εφαρμογή της τεχνικής της *in vitro* γονιμοποίησης (IVF). Οι τεχνικές της IVF επιτρέπουν την εκτίμηση της γονιμοποιητικής ικανότητας του σπέρματος με το να εκτιμούν την ικανότητα των σπερματοζωαρίων να διεισδύουν και να γονιμοποιήσουν ώριμα ωάρια *in vitro* και με το ποσοστό παραγωγής εμβρύων μετά από in vitro γονιμοποίηση και καλλιέργεια των γονιμοποιημένων ωαρίων. Στην παρούσα βιβλιογραφική ανασκόπηση παρουσιάζονται συνοπτικά μελέτες που πραγματοποιήθηκαν *in vitro*, σε μια προσπάθεια για τη δημιουργία μιας εργαστηριακής δοκιμής που με ακρίβεια να αξιολογεί, και ακόμη περισσότερο να προβλέπει την γονιμοποιητική ικανότητα σπέρματος κριών.

Λέξεις ερευτηρίασης: AI, IVF*, in vitro* παραγωγή εμβρύων, κριός, σπέρμα.

INTRODUCTION

The most valid test of sperm fertilizing ability is to obtain viable pregnancies and normal offspring following *in vivo* insemination. This represents a standard procedure for evaluating the males' fertility in farm animals. The prediction of sperm fertilizing ability is of great economic importance for breeding herds, especially when artificial insemination is applied, since it leads to the selection of males with good reproductive performance.

Studying the correlation between *in vitro* tests and *in vivo* fertility varies depending on how *in vivo* fertility is defined and how field fertility data are obtained and presented. For comparing *in vitro* and *in vivo* fertility data, the in *vivo* male fertility should be defined precisely. In most species, including sheep, the number of females served by a single male is too small, and thus the data are insufficient for evaluating male' s fertility. In that case, attention should be given in order to avoid the mating of a female by more than one male. On the contrary, the evaluation of ram's fertility is more accurate after artificial insemination. In that case, male's *in vivo* fertility is most often expressed as non-return (NRRs) or pregnancy rates after AI of a large number of females (Larsson et al., 2000). However, it should be taken into account that both maternal and environmental factors influence field fertility. One way to avoid the influence of these factors is to use NRRs that have been corrected for the influence of season, area, inseminator and parity (Zhang et al., 1999).

FERTILITY ASSESSMENT

Many *in vitro* assays have been developed to estimate the potential fertility of a male, in an attempt to eliminate males with subfertility. Also, by using *in vitro* methods, awaiting the results of hundreds of mating or AIs needed for evaluating the fertility under field conditions could be avoided (see review by Lonergan, 1994; Foote, 2003; Guillan et al., 2005; Rodriguez-Martinez, 2003; 2006; 2007; Rodriguez-Martinez and Barth, 2007). The latter procedures are highly reliable but also rather costly.

The evaluation of a semen sample can determine its degree of normality before being processed for AI or IVF. Most of these laboratory methods measure general characteristics of spermatozoa (morphology, motility, organelle integrity, etc.), all essential to fertility, if maintained until the spermatozoa are confronted with the oocyte. Other, more complicated methods (Zona Pellucida-assay, penetration-assays, Acrosome Reaction-assays, etc.) mimic *in vitro* the interactions between the spermatozoa and the female genital tract, the oocytes vestments and the process of fertilization *in vivo* (Rodriguez-Martinez, 2006). Also, there are other more elaborate tests including thermal resistance tests, ability of spermatozoa to travel in different media (e.g. cervical mucus, swim-up in media, etc.) hypo-osmotic swelling test and computer-based assessment of motility (Computer Assisted Semen Analysis, CASA) (Faigl et al., 2012). However, in

order to validate fertilization ability of semen, IVF tests are the most suitable for assessing the overall sperm function during fertilization, although these tests are expensive and time consuming, so they are far from satisfying on-farm commercial requirements.

Throughout the world, a lot of *in vitro* studies evaluating semen quality and fertility have produced inconsistent results. There are likely many reasons for these inconsistencies, some of which are, definition of fertility used (non-return rate or pregnancy diagnosis), non-sperm effects (management and environment), failure to accurately estimate fertility after AI of few females, the use of high sperm numbers and the sperm heterogeneity of ejaculation (Saacke et al., 1980; Rodriquez-Martinez, 2006; Mocé and Graham, 2008). Amann and Katz, (2004) stated that the malfunction of any one of many essential and independent sperm attributes (known and unknown) can render a given sperm incapable of fertilizing an oocyte. Furthermore, attributes necessary for fertilization will certainly depend on whether AI or natural mating is used (including, but not limited to, the effect of the site of semen deposition), whether ovulation synchronization and timed AI is used, and female factors (Deligiannis et al., 2005; Dalton, 2011; Valasi, 2011).

AI AND FERTILITY ASSESSMENT

The main goal of AI industry is to obtain a simple, accurate method for predicting the fertilization capacity of semen in different species. Many factors influencing fertility after AI have been comprehensively described in the last decades (Evans and Maxwell, 1987; Chemineau et al., 1991; Valasi, 2011; Faigl et al., 2012; Valasi et al., 2012; Khalifa et al., 2013) e.g. number of spermatozoa, preservation technique (frozen, chilled, fresh), method of technique of insemination, time of insemination, type of oestrus (natural or induced/synchronized), hormonal treatment of female, age and breed of females, individual, season of treatment, stress factors and health status.

The AI success depends mainly on semen preservation, site of deposition and breed. The viability of spermatozoa in the female tract is affected by semen preservation and there is also an increased incidence of embryonic mortality after insemination with preserved semen in sheep (Gillan et al., 2004). Several researchers (reviewed by Salamon and Maxwell, 1995a; 1995b) have also reported inadequate transport of preserved spermatozoa through the reproductive tract of small ruminants after cervical AI (cervix: Lopyrin and Loginova, 1958; utero-tubal junction or both structures: Mattner et al., 1969; Platov, 1983).

However, fertility of preserved semen can be increased if the number of spermatozoa in the insemination dose and the depth of cervical insemination are increased (Faigl et al., 2012). Achieving sufficient penetration of the cervix is problematic and the conception rates are variable (Gillan et al., 2004). The narrow lumen and complex anatomical structure of the uterine cervix are major limiting factors precluding the widespread application of non-surgical intrauterine AI in ewes. This inherent obstacle causes the reproductive technologies in sheep to be more difficult to perform and less cost effective. The attempts to improve transcervical AI in ewes have focused primarily on using different types of insemination catheters and pharmacological cervical dilators to facilitate intrauterine deposition of semen or embryos (Candappa and Bartlewski, 2011).

Cervical AI of frozen-thawed semen generally yields an unacceptably low (10-30%) pregnancy rate in sheep (Salamon and Maxwell, 2000). Although, intra-uterine insemination using laparoscopic procedures, gives very good pregnancy rate (60-70%) the use of this technique is likely to be limited because of the costs involved, restrictions on the conditions under which the surgical procedures required may be carried out, as well as welfare concerns. In contrast to previous studies, Richardson et al. (2012) indicated that pregnancy rate following insemination of frozen-thawed semen into vaginal fornix is comparable to that obtained following cervical insemination and thus may represent a more feasible option for AI on a large scale in sheep.

There are differences due to the breed of ewe in the success of cervical AI using fresh (Papadopoulos et al., 2012) or frozen-thawed semen (Donovan et

al., 1998; 1999; 2000). Breed effects on the timing of ovulation, and hence inappropriate timing of AI, were not the explanation for the major breed differences because there were no significant differences among breeds for the intervals from progestagen withdrawal to either the preovulatory surge or to ovulation (Donovan et al., 2000). The large differences among ewe breeds provide a challenge for evaluating the importance of variation in transport through the cervical barrier, survival in the uterus and physiological mechanisms governing the milieu at the site of fertilization. Also, the relatively large variation among individuals rams in the fertilizing ability of semen has implications for research on protocols for cryopreservation, *in vitro* evaluation of semen after thawing and the possibilities for developing effective screening methods for selecting rams for use in AI (Hanrahan, 2003).

IVF AND FERTILITY ASSESSMENT

In vitro fertilization (IVF) is the *in vitro* method that mostly mimics gamete-to gamete interaction during *in vivo* fertilization. It has been used repeatedly to determine the relative fertility of semen samples in farm animals by evaluating different endpoints in early embryo developmental stages (Rodriguez-Martinez, 2007). Among the sperm assays that have been developed, IVF tests are the most suitable for assessing the overall sperm function during fertilization. The binding and penetration of the zona pellucida is one of the most important barriers the spermatozoa must overcome in the fertilization process. However, there are still pronounced differences between IVF and *in vivo* fertilization, including sperm transport, conditions of sperm capacitation, and sperm:egg rations at the site of fertilization (Lonergan, 1994).

The effect of individual rams on the fertilization rate after IVF, as well as on early embryonic development was demonstrated by Fukui et al. (1988). Smith and Murray (1996) found differences in fertilizing ability not only between individual rams, but also between ejaculates from the same ram. Attempts to correlate *in vitro* tests and *in vivo* fertility using ram semen have given contradictory results. Choudhry et al. (1995) demonstrated a correlation between the number of penetrated sperms per zona-free hamster oocyte and *in vivo* fertility. Codde and Berger (1995) were unable to found a correlation between *in vivo* fertility and the ability of spermatozoa to bind or penetrate zona pellucida, although there were significant differences between rams among these *in vitro* parameters.

In Dublin, Byrne et al. (2000) examined how freezing rate through the critical temperature zone (-10°C and -25°C) affected the fertility of spermatozoa as assessed *in vivo* and *in vitro*. The overall aim was to provide a method for evaluating fertilizing ability of ram spermatozoa without the need to resort to *in vivo* methods. The authors concluded that IVF evaluation of frozen/thawed semen provides a valid assessment of the ability of the semen to achieve fertilization *in vivo*.

Based on the encouraging results of the previous work that would enable the identification of individual rams whose semen is able to maintain high percentage of motility after freezing and thawing and yield high pregnancy rates, Papadopoulos et al. (2005) tried to improve pregnancy rates by evaluating the quality of frozen–thawed ram semen through *in vitro* fertilization procedures prior to insemination. A series of studies took place to examine directly for the first time the relationship between IVF results and fertility following cervical insemination with a fixed dose of frozen-thawed ram spermatozoa. In conclusion, there was little evidence that evaluation of the quality of frozen-thawed ram semen through IVF provided a useful predictor of pregnancy rate following cervical insemination. This may be attributed to the IVF procedures used, which were designed to maximize blastocyst yield rather than for detecting differences in fertilizing ability between batches of sperm. It may be that selection of motile sperm using a separation technique may eliminate possible differences between rams (Morris et al., 2003).

Also, O' Meara et al. (2005) based on the findings of Byrne et al. (2000) and Papadopoulos et al. (2005), studied to assess how lowering sperm concentration

during IVF would affect the ability to discriminate between rams classified as having high or low fertility *in vivo* based on cervical AI of frozen-thawed semen. The effects of lowering sperm concentrations in IVF below 0.25 x 10^6 spermatozoa/ml for frozen-thawed ram semen have not been explored. It may be possible that the use of a high sperm concentration masks intrinsic differences between individuals in an IVF system. There was a need to identify the lowest sperm concentration that was compatible with getting sufficient fertilized oocytes to the blastocyst stage in order to reliably differentiate between rams. The above study suggested that using a low concentration of spermatozoa (0.0625 x 10^6/ml) for IVF may be a useful method for predicting field fertility of frozen-thawed ram semen. At higher concentrations the magnitude of the differences between rams is diminished. Papadopoulos et al. (2005) could not accurately detect differences between rams at field level (NRR ranges from 45.7% to 73.8%) via IVF evaluation (proportion of oocytes cleaved) using a concentration of 1 x 10^6 spermatozoa/ml. Thus, it was proposed that a lower concentration might enhance individual ram differences *in vitro*. Fewer spermatozoa added to oocytes might reflect an *in vivo* situation, where large numbers of sperm are not present at the site of fertilization (Hunter, 1993).

Factors such as date and a date-by-ram interaction caused significant variation in blastocyst rate achieved throughout the experiments took place during the study of O' Meara et al. (2005). This is in agreement with Zhang et al. (1997) who found that testing date was the greatest source of variation in the blastocyst development rate. Embryo development *in vitro* is largely dependent on the conditions under which *in vitro* fertilization and *in vitro* culture is carried out (Bavister, 1995). Thus, it could be suggested that several other factors are necessary for the cleaved oocyte to develop to the blastocyst stage thus reducing its reliability as an indicator of fertility *in vivo* (O' Meara et al., 2005). Recently, studies have demonstrated that heterologous IVF with bovine oocytes, can be useful to predict the field fertility of ram spermatozoa (García-Alvarez et al., 2009; Valente et al., 2010).

SPERM QUALITY CHARACTERISTICS AND FIELD FERTILITY ASSESSMENT

O' Meara et al. (2008) further investigated the *in vivo* fertility of rams with a) the use of visual and flow cytometric assays in evaluating the viability, acrosomal status and membrane integrity of spermatozoa, b) the use of visual and computer-assisted sperm analysis (CASA) sperm motility analysis and c) the level of platelet-activating factor (PAF) present in frozen-thawed ram spermatozoa with *in vivo* ram fertility. The main findings from this study were that neither visual nor computer-assisted assessments of sperm motility characteristics were capable of predicting the potential fertility of frozen-thawed ram semen. The percentage of viable, acrosomal reacted, capacitated or dead cells and live cells following the osmotic resistance test failed to predict *in vivo* fertility (O' Meara et al., 2008). Although, differences are apparent among rams for variables such as acrosomal status and capacitation status, the results of the above study showed large variation between replicate straws of semen within rams for motility parameters and clusters. Hence, these variations were added to the already complicated process of attempting to predict potential fertility *in vitro*. It is possible that tests based on more straws per ram (more than 3) would improve the predictive power of the *in vitro* process (O' Meara et al., 2008).

It is still not clear which characteristics of sperm movement assessed by CASA are of real diagnostic value for predicting fertility and fertilization rates in sheep. However, motility is widely believed to be one of the most important characteristics associated with the fertilizing ability of sperm (Tsakmakidis, 2010). Regarding to *in vitro* fertilization results, Herrara et al. (2005) reported that *in vitro* fertilization results was significantly correlated to progressive motility, but not to average path velocity (VAP), linearity (LIN) and beat cross frequency (BCF). Moreover, sperm velocity patterns of VAP and straight line velocity (VSL) were significantly related to the number of sperm penetrating oocyte (Farrell et al., 1998). The VSL is an indication of forward progression and VAP is an indication of capacitation of spermatozoa (Farrell et al., 1998).

CONCLUDING REMARKS

Any attempt to predict ram's fertility based on *in vitro* tests is a rather complicated process. IVF is often not highly correlated with in vivo fertility but is a good approach for evaluating fertility for IVF purposes. However, IVF is largely dependent on laboratory stability, thus making the accounting of variation within tests and comparisons among studies rather difficult. The variability among replicate straws is another factor that must be considered in any future studies on *in vitro* test. It is suggested that a technique that mimics the physiology of fertilization within the female reproductive tract and the ability of spermatozoa to bind to and penetrate an oocyte may yield a more useful measure of fertility than analysis of a single sperm parameter.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest to declare.

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