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Functional Tests in Semen Quality Determination

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Abstract: Being one of the most significant causes of fertilization failure, sperm functional impairment keeps up-to-date as a subject on which researches have been carried out for a long time. The semen quality evaluations are the most important factor in estimation of fertility in domestic animals. Nowadays, acrosome reaction, hypo-osmotic swelling test, zona free hamster egg penetration test, bovine cervical mucus penetration test and computer-assisted sperm analysis tests have been used for quality determination of semen. By this way, opportunity to evaluate the fertilization capacities of the spermatozoa is given and thanks to its applicability, the economical contribution it provides to the country's livestock farming is considerably high. The information enlightened with the contemporary sources regarding the sperm function tests mentioned in this study have been compiled separately.

Key words: Fertilization, *in vitro* evaluation, semen quality, spermatozoa, hypo-osmotic, Turkey

INTRODUCTION

Traditionally, male fertility is defined by the evaluation of the number, motility, concentration and morphologies of the spermatozoa in the ejaculate. As the essential principle, the spermatozoa motility, concentration and the number of morphologically normal spermatozoa must be above the limit values for pregnancy to develop and to determine fertility functionalities of the spermatozoa play a rather determinant role than the number of spermatozoa do. Depending on today's technological developments, sperm function tests are composed of *in vitro* tests including acrosome reaction, hypo-osmotic swelling test, zona free hamster egg penetration test, bovine cervical mucus penetration test and computer-assisted sperm analysis tests and these tests provide a different point of view on reproduction.

ACROSOME REACTION

Acrosome is a structure covering the frontal side of the spermatozoon nucleus, bounded to the membrane and cap-like shaped due to it's localization, available in different shapes among the golgi complex originated species in the spermatid and containing necessary enzymes for fertilization (Baviste, 1980; Abou-Haila and Tulsiani, 2000).

Acrosome reaction is an irreversible event under physical conditions and after the spermatozoon reaches the full capacity, it springs into the action of bound to the zona pellucida. As a result of the acrosome reaction, vesicles are formed in the spermatozoa by the conjunction of the plasma membrane and the external acrosome membrane and the acrosome ingredient is ejaculated on the spermatozoon surface. The release of the acrosome ingredient is accepted as a kind of exositosis; zona pellucida softens with the effects of the acrosomal enzymes or melts where available and thus, the penetration of the spermatozoa in this structure becomes easier (Neild *et al*., 2005; Flesch and Gadella, 2000). Except oocytes, all mammals have zona pellucida consisting of glycoproteins and primary spermatozoon receptors on it and besides all these, there are cumulus oophorus cells in many species. While the hyaluronidase enzyme, which the spermatozoa that have completed the acrosome reaction excrete, melts down the cumulus cell matrix, the acrosine on the surface of the spermatozoa plays a role in passing beyond the zona pellucida (Witte and Schäfer-Somi, 2007; Shetty *et al*., 2003).

The most significant characteristic of acrosome is the acrosomal enzymes that it nestles in the segment called the equatorial segment. These enzymes are protease, glycosidase, proacrosin, acrosin and the most important of all, hyaluronidase and they play a great role in the spermatozoo passing cumulus oophorus. The spermatozoa which passes the cumulus bounds to zona pellucida firmly (Abou-Haila and Tulsiani, 2000; Witte and Schäfer-Somi, 2007; Shetty *et al*., 2003; Bhattacharyya and Kanjilal, 2003). At this stage, spermatozoon is important in terms of the interactions between the plasma membrane

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receptors and zona pellucida. Meanwhile, the spermatozoon bounded to zona pellucida starts to lose acrosomal cap and consequently starts to perform acrosome reaction. Thus, thanks to the spermatozoon activity which is hyperactivated by the other acrosomal enzymes and capacitation that intensify in the part below the acrosomal region called the equatorial segment, spermatozoon passes beyond the zona pellucida (Flesch and Gadella, 2000; Witte and Schäfer-Somi, 2007; Zeginiadou *et al*., 2000). Acrosome reaction is directed to two important functions which are passing beyond the zona pellucida and spermatozoon and oocyte combining at cell membranes level. The real acrosome reaction can be observed under light microscope in species with large acrosomal cap because in living spermatozoa which have completed acrosome reaction, the acrosomal cap is not observed as a distinctive structure. Various methods have been developed in order to determine that acrosome reaction has definitely occured. These methods are:

- Naphtyl-yellow/eritrosin B
- Acridine orange-UV
- Triple staining (Trypan Blue, Bismarc brown and Rose bengal)
- Chlortetracyclin-UV
- Pisum sativum (Pea-lectin) methods (Neild *et al*., 2005; Flesch and Gadella, 2000; Bhattacharyya and Kanjilal, 2003; Glazier *et al*., 2000)

The spermatozoon which passes beyond zona pellucida holds ooplasm after passing through the perivitellin cavity. This first adherence takes place in the part that is just over the equatorial segment. After this hold, fusion between spermatozoon and oolemma occurs and after the plasma membrane of spermatozoon melts, spermatozoon becomes a part of oocyte plasma membrane. Following spermatozoon-oocyte fusion, oocyte is activated as metabolic and after the exostosis of cortical granules, the meiosis is completed. Due to cortical granule activation, the permeability of zona proteins change and new spermatozoon access is prevented. This is called the oocyte zona reaction (Flesch and Gadella, 2000; Moce and Graham, 2008) (Fig. 1).

Spermatozoa become *in vitro* capacity after they are left to incubation in mediums such as TALP and Krebs Ringer at 37°C in an atmosphere with 5% CO_2. After capacitation, *in vitro* acrosome reaction is induced by agents such as, Ca ionophore A 23187, glycosaminoglycans and follicule fluid. In bulls, when stock solutions which are prepared especially by dimethyl sulfoxide and Ca A 23187 ionphore are to be used, 1 µL mixture is added to 50 µL sperm suspension to acquire

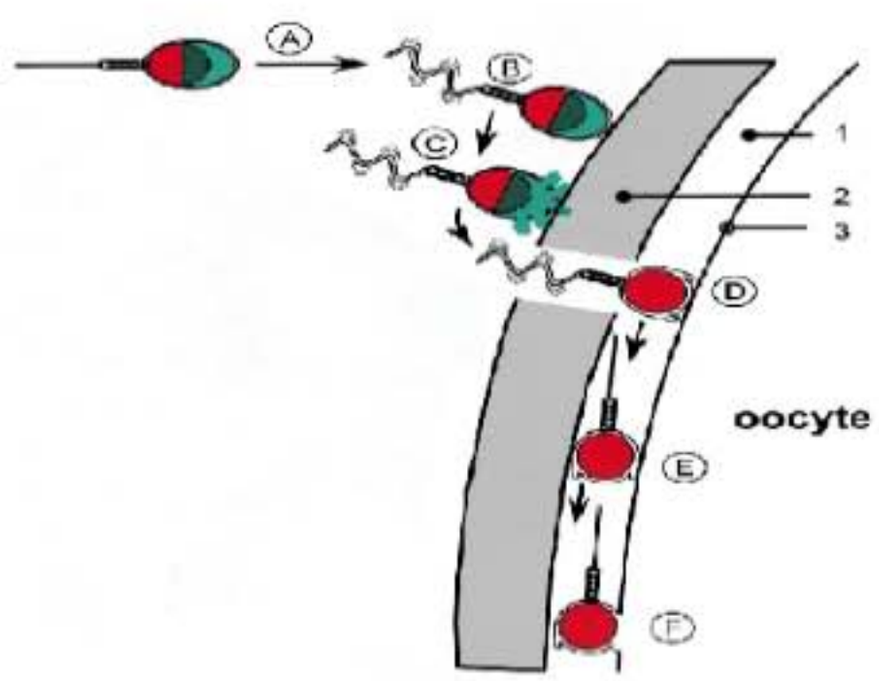

Fig. 1: Sequence of mammalian fertilization. (a) Freshly ejaculated sperm cells are activated in the female genital tract during a process called capacitation. (b) Capacitated sperm cells are hypermotile and are able to bind to the egg extracellular matrix (ZP). (c) Binding of sperm cells to the ZP triggers the acrosome reaction and acrosomal enzymes are secreted. (d) Hydrolytic enzymes secreted from the acrosome degrade the ZP and subsequent sperm cells penetrate the ZP, enter the perivitelline space and bind to the oolemma with the apical tip. (e) Subsequent to apical tip binding, oolemma binding changes to the hairpin structure of the acrosome reacted sperm cell. (f) After hairpin structure binding to the oolemma, the sperm cell fuses with the oocyte and the sperm cell is subsequently incorporated in the oocyte. 1: perivitelline space; 2: ZP; 3: oolemma (egg plasma membrane) (Flesch and Gadella, 2000)

10 µM calcium after they are diluted 10 times with TALP. Then, they are left to incubation for 1 h at 39°C in a humid environment with 5% CO_2. Soon after sperm samples are incubated in a 2% of trypan blue in PBS with a pH of 7.3 for 15 min at 37°C and fixed for 40-60 min in glutaraldehide of 4%, they are stained for 5 min in 0.8% of bismarck brown with 1.8 pH and then stained for 30 min at 24°C in 0.8% of rose bengal in PBS with a 6 pH. Next, they are taken under a microscope for acrosomal examination and are evaluated (Moce and Graham, 2008). They are subjected to staining regarding acrosome reaction test after the serial processes in appropriate mediums. In the evaluation, 200 spermatozoa are evaluated under phase contrast microscope with immersion objective. The spermatozoa are classified as live spermatozoa which have been through acrosome reaction (light rose-coloured postacrosomal areas and white acrosomal areas), dead spermatozoa with abnormal acrosome that is to say,

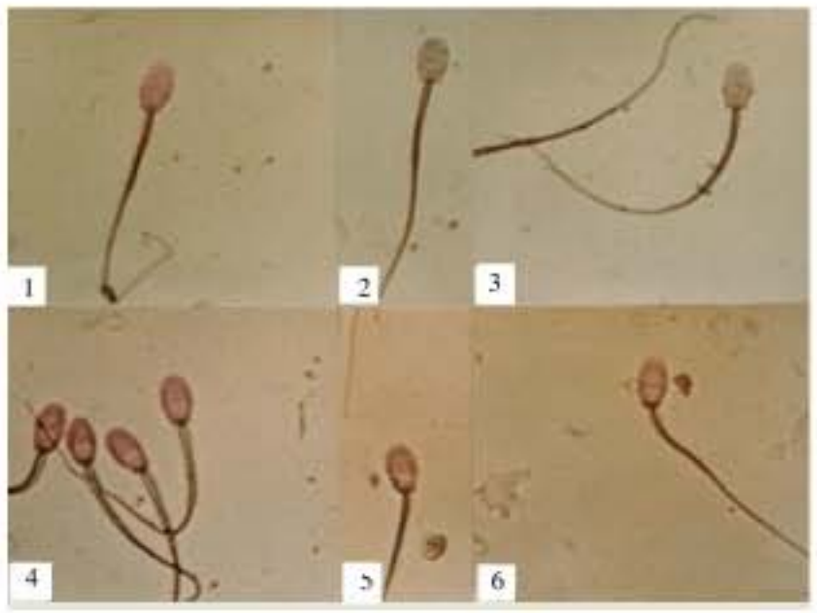

Fig. 2: Triple staining technique was painted with spermatozoonlar. Spermatozoa stained by a simplified triple stain technique. 1) a live spermatozoon acrosome reacted; 2) a dead spermatozoon with an intact acrosome; 3) a dead spermatozoon with degenerative acrosome reaction; 4) live spermatozoa with intact acrosomes; 5, 6) live spermatozoa with acrosome partially intact (Ferrari *et al*., 2000)

degenerative acrosomal reaction (white acrosomal area and blue postacrosomal areas), live spermatozoa with healthy acrosome (light rose-coloured postacrosomal area and pink acrosomal areas) and dead spermatozoa with healthy acrosome (blue postacrosomal areas) (Ferrari *et al*., 2000) (Fig. 2).

Acrosome reaction is a necessary process before fertilization and infertility cases and abnormal acrosome reaction are related. The complex membrane structure of spermatozoon and the acrosome reaction it undergoes provide useful information to determine infertility.

HYPO-OSMOTIC SWELLING TEST

Rather than the number of spermatozoa, their functionality play an important role in determining infertility in males. Therefore, various spermatological parameters are used to determine fertility (Jeyendran *et al*., 1984a; Aitken, 2006). However, only a limited number of methods can be used to determine the functional integrity of the membrane of the sperm. The biochemical integrity of the plasma membrane has a great importance not only for spermatozoon metabolism but also for capacitation, acrosome reaction and the event of sperm being connected to the oocyte surface. For this reason, determining the membrane function can be the indicator of spermatozoa fertilization capacity (Jeyendran *et al*., 1984b).

Hypo-osmotic swelling test is one of the new procedures used in the evaluation of the reaction that the functional integrity of plasma membrane of spermatozoa reveals under hypo-osmotic conditions (Jeyendran *et al*., 1984b, 1992; Van der Ven *et al*., 1988). HOS test investigates whether the spermatozoon membrane is functional or not. Therefore, HOS test is used as a method complementing the routine sperm analysis (Nie and Wenzel, 2001).

This method is based on the principal of carrying the water from an active membrane whose functional integrity is not spoiled, until osmotic pressure is balanced between two environments having different osmotic pressure (Jeyendran *et al*., 1992).

In the event that cell plasma membrane is left to hypo-osmotic environment, the fluid penetrates into the cell until osmotic pressures are equalized. Due to this fluid penetration, the volume of the cell and the cell membrane grow and as a result, swellings are formed in the plasma membrane. The cell membrane covering the fibrils in the tail of spermatozoon is looser than the membrane in the head part and thus, swelling in the tail is more apparently shaped compared to the other regions. Curling is caused by the swelling of plasma membrane. While swelling in the tail is observed with the spermatozoon which is physically and chemically healthy in environments with hypo-osmotic pressure, these reactions are not seen in cells which are not healthy (Jeyendran *et al*., 1984b, 1992; Nie and Wenzel, 2001; Lagares *et al*., 2000) (Fig. 3).

In the studies performed on humans, the HOS test results are evaluated as; ejaculates above 60% are normal, ejaculates below 50% are abnormal, ejaculates between 50 and 59% are suspect (neither normal nor abnormal) (Jeyendran *et al*., 1992).

Although, HOS test is used to determine the spermatozoa membrane integrity in various animal species and humans, the suggested osmotic pressure level for test application vary depending on species. The appropriate osmotic pressure to form maximum tail curls are declared as 150 mOsm for human (Jeyendran *et al*., 1984a), bull (Kathiravan *et al*., 2008) and buffalo (Shukla and Misra, 2007) sperm, 100 mOsm for ram (Aisen *et al*., 2005) sperm, 25-100 mOsm for stallion (Neild *et al*., 1999) sperm and 60 mOsm for dog (Kumi-Diakam, 1993) sperm.

For example, for 100 mOsm HOST solution, first sodium citrate-fructose hypotonic solution is prepared. For this, distilled water is added to 9 g fructose plus and 4.9 g sodium citrate mixture until it reaches 1 lt. to prepare the HOST solution. This prepared solution and sperm are put into an eppendorf tube and left for incubation for 1 h at 37°C. In the examination performed after incubation by

Fig. 3: Different types of swelling and curling in sperm (400x)

taking one drop from the solution and putting it under lamella with an enlargement of 400 times under a phase contrast microscope, the results should be evaluated by counting 400 spermatozoa (Jeyendran *et al*., 1984a, b).

ZONA-FREE HAMSTER EGG PENETRATION TEST

Zona free hamster egg penetration test is one of the most impressive tests for evaluating spermatozoon functions (Graham *et al*., 1987). The zona removed hamster oocyte penetration test which is used for the first time on humans to solve the fertilization problems of males is defined as the penetration ability of the human spermatozoa in the zona removed hamster oocytes (Yanagimachi *et al*., 1976; Jeyendran *et al*., 1984a; Das, 1985; Maruyama *et al*., 1985; Henkel *et al*., 1996). Later, it became a sensitive test giving highly valuable information in determining the functions and fertilization potential of the fresh and frozen-thawed spermatozoa obtained from various animal species and humans (Ferrari *et al*., 2000; Aitken, 2006; Kathiravan *et al*., 2008; Graham *et al*., 1987; Berger, 1989).

A great number of oocyte cumulus cells are cleaned off from the zona using hyaluronidase, trypsin and mercaptal, respectively. Since only the spermatozoon which completed the acrosome reaction could be connected to oolemma, spermatozoa are left in various capacitation mediums for a short time or for one night to induce this reaction. Later, they are left together with zona removed oocytes. The results are evaluated according to the number of spermatozoa which are connected to oolemma and those which access in the oocyte (Aitken, 2006; Graham *et al*., 1987; Hoshi *et al*., 1982; Kumar and Sharma, 2005).

With this test, the potential of spermatozoon, which completed its acrosome reaction to connect to the plasma membrane of the oocyte is tested. It gives information about the final step of the spermatozoon-oocyte interaction (Kumar and Sharma, 2005; Shibahara *et al*., 1998).

The usage of the zona free hamster egg penetration test is limited due to requiring heavy work and labour, its complexity, high price and the difficulties in its standardization. The difficulty in its standardization derive from the differences in providing the conditions to enable the spermatozoon capacitation and to start the acrosome reaction. *In vitro* zona free hamster egg penetration test consists of the stages of preparing the spermatozoa, preparing the oocytes and incubation of spermatozoon-oocyte (Aitken, 2006).

Since only the spermatozoa that have been through capacitation and completed the acrosome reaction will penetrate to the hamster egg whose zona is removed, the spermatozoa taken must primarily be capacitized *in vitro*. Capacitation is acquired by incubating the spermatozoa in an appropriate capacitation fluid by swim-up process for 1 h. Glycosaminoglycans (Kumar and Sharma, 2005), Ca ionophores ((A 23187) Kathiravan *et al*., 2008; Kumar and Sharma, 2005) follicle fluid (Landim-Alvarenga *et al*., 2001). Test-Yolk buffer (Romano *et al*., 1998) platelet activation factor (Kumar and Sharma, 2005) and progesterone (Katila, 2001), which induce *in vitro* capacitation and acrosome reaction are used.

Superovulation was applied on 6-12 weeks old Golden Syrian female hamsters (*Mesocricetus auratus*) and then the animals were killed by cervical dislocation or in a different way and both oviducts were dissected and taken to appropriate mediums with 0.1% of hyoluronidase and 0.1% of trypsin and left to incubation for a length of time so that cumulus cells and zona pellucidas were moved away (Kumar and Sharma, 2005; Shibahara *et al*., 1998; Rutllant *et al*., 2005; De Jonge, 2005; Yogev *et al*., 1999). In order to mature the oocytes, Ham F-10, Ham F-12, Brister BMOC-3, Waymouth MB 752/1, Dulbecco B2, KRB (Krebs Medium) and MEM (Minimum Essential Medium) together with TCM-199 (Tissue Culture Medium) and TALP (Tyrode's Albumine Lactate Pyruvade Medium) are the most commonly used mediums (Berger, 1989; Shibahara *et al*., 1998; Gundogan, 1998; Yang *et al*., 1989; Kuplulu and Un, 2001).

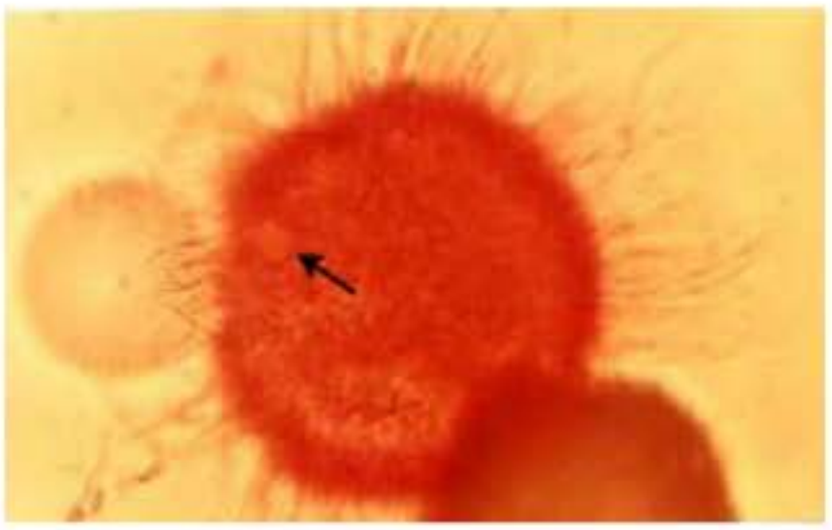

Fig. 4: Murrah buffalo spermatozoa interacting with zona-free hamster egg (400x) with a visibly swollen sperm head within an egg (Shukla and Misra, 2007)

The spermatozoa which are capacitized and whose acrosome reaction is shaped and the oocytes which are cleared of cumulus cells and zona pellucida, are taken to the appropriate *in vitro* Fertilization (IVF) medium and incubated for 3 h in an atmosphere including 5% CO_2 at 38.5-39°C. After the oocytes are washed with appropriate mediums and stained with various paint preparates following the incubation, examination is done under phase-contrast microscope at an enlargement of 400. Swollen spermatozoon head or spermatozoon tail penetrated into the vitellus is evaluated as positive (Das, 1985; Kumar and Sharma, 2005; Shibahara *et al*., 1998; Takemoto *et al*., 1985). The penetrated oocytes and by how many spermatozoa each oocyte is penetrated are determined. The event that 10% of the oocytes are penetrated and each oocyte has 5 spermatozoa are considered to be normal values (Shukla and Misra, 2007) (Fig. 4).

BOVINE CERVICAL MUCUS PENETRATION TEST

Cervical mucus is a hydrogel structure and contains 94% of water and the water rate may increase up to 98% during ovulation. The basic content of cervical mucus is a mucin type glycoprotein which is rich in carbohydrate. The other elements are carbohydrates, serum proteins, lipids, enzymes, amino acids and inorganic salt. Seven percent of the inorganic salt is composed of sodium chloride and contains potassium, calcium, magnesium, copper, zinc, iron phosphates, sulphates and bicarbonate (Rutllant *et al*., 2005; De Jonge, 2005; Tsiligianni *et al*., 2001).

The cervical mucus and its content undergo changes depending on cycle. In the middle of the cycle, close to ovulation period, the cervical mucus is rather wet, alkali structured and poor in terms of cell. In this period, the

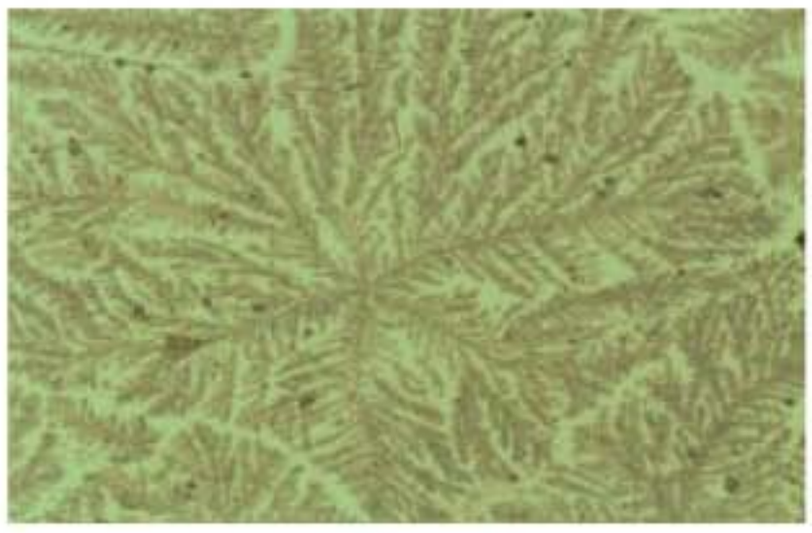

Fig. 5: Ferning

yielding characteristic of the cervical mucus (Spinnbarkeit) increases (Rutllant *et al*., 2005). The mucus which is collected in a time close to the ovulation period and dried on a lamina, reveals a typical crystallisation order or ferning from Fig. 5 (Tsiligianni *et al*., 2001).

In vitro cervical mucus penetration test was first performed on people in order to investigate infertility problems and to gain information regarding the functions and quality of spermatozoa (Stumpf and Lloyd, 1985; Murase *et al*., 2001; Anilkumar *et al*., 2001). In the first studies (Takemoto *et al*., 1985; Borghi and Asch, 1983; Ola *et al*., 2003) human cervical mucus were used. However, because of the disadvantages of cervical mucus female menstrual cycle, such as the difficulty of obtaining it along the preovulator phase and obtaining in insufficient amounts, bovine cervical mucus whose biochemical structures, glycoprotein structures, fluidity structures (rheologically) and their ferning appearances in the electron microscope scan are the same, started to be used instead (Takemoto *et al*., 1985; Ola *et al*., 2003; Galli *et al*., 1991; Engel and Petzoldt, 1999). After progesterone treatment, *in vivo* penetration of the spermatozoa in the cervical mucus may not be formed. When the cervical mucus is too wet, the penetration of spermatozoa is higher (Tsiligianni *et al*., 2001).

It was shown that Cervical Mucus (CM) acted as a barrier which eliminates the spermatozoa with abnormal morphology and allows only the spermatozoa with normal morphology to pass and that the spermatozoa which cannot penetrate into the mucus lack the ability to fertilize the ovum (Ola *et al*., 2003; Keel and Schalue, 2000; Robayo *et al*., 2008).

In the *in vitro* cervical mucus penetration test, it is stated that the incubation temperature affect the speed of penetration of the spermatozoa to the cervical mucus, meaning that the high temperature and short-time incubations increase the penetration speed of the spermatozoa to the cervical mucus (Yogev *et al*., 1999; Stumpf and Lloyd, 1985; Tas *et al*., 2006).

The most important factors in evaluating the success of the penetration of the spermatozoa to the cervical mucus are stated as the change in the lateral head of the spermatozoon, the proceeding speed of the spermatozoon that is to say its motility, morphology, acrosome integrity and concentration (Yogev *et al*., 1999; Anilkumar *et al*., 2001).

The *in vivo* evaluation of the spermatozoon-cervical mucus interaction is carried out with the postcoital test (PKT). The principle of the test is based on the examination of a drop of liquid taken from the cervical mucus, after 9-24 h after mating when the female is on eustrous cycle, under the microscope and measuring the number and motilities of the spermatozoa. Showing a single advanced-fast motil spermatozoon in the cervix points out that the cervical factor is not responsible for infertility matter (Tejedor *et al*., 2000).

A more controlled evaluation of the spermatozoon-cervical mucus interaction can only be acquired with the use of *in vitro* conditions (Keel and Schalue, 2000). The different cervical mucus penetration test performed with the use of different scoring principles has been defined. If the details of the evaluation criteria are not considered, the cervical mucus penetration is highly dependant on the moving characteristics of the spermatozoa. The particularly important points for the cervical mucus penetration are the average speed and the order of the movement, which are the moving characteristics of the spermatozoon (Tang *et al*., 1999).

In vitro spermatozoon-cervical mucus penetration tests are performed in two ways, which are mucus contact test and capillary tube test.

Spermatozoon-cervical mucus contact test is done by mixing a drop of sperma and a drop of cervical mucus on the slide. A coverslip placed on the slide and pressed slightly. Quantitative (number of sperm high power microscopic field, x400) assessment of sperm penetration in cervical mucus. Change in motility pattern of spermatozoa as result of sperm agglutination in sperm-cervical mucus contact test. Spermatozoa loaded with spermagglutinins stick to glycoprotein filaments as soon as they contact cervical mucus. Cervical mucus containing spermagglutinins provides the penetrating spermatozoa with the spermagglutinins causing the spermatozoa to stick to glycoprotein filaments (Hafez, 1987).

In the capillary tube test, spermatozoa's ability to penetrate into the cervical mucus colon, which is in a capillary tube is tested. In the test, 5 cm long, 3 mm wide and 0.3 mm deep flat tubes are used. First, cervical mucus is aspirated in these tubes. One end is closed with a plastic tap and the sperm samples are plunged vertically from the open end before it is left for incubation. This is based on the principal of placing the tubes directly under a microscope after incubation to evaluate the spermatozoa penetrated into the mucus. Various evaluation methods were suggested about the Capillary Tube Method. The longest distance covered by the spermatozoon in the capillary tube after 90 min of incubation is measured in the Penetrak test, which is most widely used, whereas in another method, motil spermatozoon number is determined in 3 cm distance after 60 min of incubation (Engel and Petzoldt, 1999; Keel and Schalue, 2000; Mahony, 2001).

COMPUTER-ASSISTED SPERM ANALYSIS (CASA)

CASA consists of optics, light source and the computer a hardware (the computer part of the machine) and motilite, morphology and vitality that can do analysis module that contains a software (computer program) is a combination. CASA was born to objective, accurate, reliable, need for repeated analysis of the spermatozoon (Guctas, 2006).

The more recent track semen analysis system, based on individual spermatozoon evaluation, offers an accurate calculation of different semen parameters. This method was first developed using multiple time-exposure photomicrographies to follow spermatozoid movements. The images obtained allow the analysis of several parameters, including semen concentration, semen motion and some morphology, particularly sperm head morphology (Verstegen *et al*., 2002).

Because of the post acquisition processing of digitalized data, the CASA are able to objectively determine morphological parameters or distinguish subpopulations in sperm head motion, which are not measurable or observable manually. The disadvantages of CASA are related to the cost of the equipment, the extreme need of validation, quality control and standardization of the measures realized (Davis and Katz, 1993).

It is vital to use appropriate optics and the best illumination to enhance the contrast of the spermatozoa heads, which in turn facilitates the manual selection of thresholds. The acquisition frequency of images is important, as reconstructed trajectory is influenced by the frame rate and several kinetic parameters are frame rate-dependent. However, the choice of frame rate is still conflicting, as it is not only equipment-dependent but related to species and experimental condition as well (Kraemer *et al*., 1998).

In general, more variations are observed for the analysis of different fields than for the repeated analysis of the same field, so the largest the number of cells analyzed, the more reduced the coefficient of variation.

Consequently, the precision of the results increases as the number of fields and cell analyses increase. In bull, the measure of 30 fields and approximately 300 cells (Budworth *et al*., 1988) is recommended whereas in the stallion from 300-500 cells seems to be optimal (Verstegen *et al*., 2002).

Semen must often be diluted before analysis because the sperm cell concentrations of raw semen are too high for the successful analysis of individual spermatozoa tracks. The extender used to dilute semen must not contain particles of a size similar to sperm cells' heads because they will not be differentiated from non-motile sperm (Mortimer and Swan, 1995).

Computer-assisted sperm analysis provides the means for an objective classification of a given population of spermatozoa. Using digital images of each sperm cell's track, CASA machines are able to analyze, by processing algorithms, the motion properties of spermatozoa. Many factors are may affect the quality of sperm cell movements. They include temperature of measurement, semen processing (washing and capacitation, freezing/thawing processes), semen concentration and pathological process in the donor (Verstegen *et al*., 2002).

Manual assessment of sperm morphology has always been demonstrated as problematic. Large variations between technicians and laboratories in the subjective evaluation of semen characteristics are known to exist making accurate interpretation of results difficult (Ombelet *et al*., 1997). In 1983, 26 professional observers were asked to assess the morphology of one semen sample and they rated the abnormal forms between 5 and 85% (Verstegen *et al*., 2002). Simultaneously, the same sample was assessed by three very skilled technicians and the percentage of abnormalities ranged between 25 and 40%.

Many factors affecting morphological studies responsible for variations include differences in smears preparation techniques, interpretation, classification systems and technicians' experience (Gravance *et al*., 1998).

To reduce variability and misinterpretation of results, a larger number of cells has to be counted. Staining methods are certainly species-dependent. A number of stains have been suggested for sperm morphology assessment; however, previous research indicates these stains do not necessarily provide the appropriate gray-level contrast for accurate computer-assisted morphometric analysis. Papanicolaou's staining and hematoxilin are most used for morphologic assessment in CASA (Davis and Katz, 1993). Magnification also affects operation. Human samples did not present differences when analyzed at 40 or 100x magnifications; goat sperm heads did not present significant differences when analyzed at 20,40, or 60x but bull sperm heads must be analyzed at 60x. The advantage of lower magnification is the decrease in time needed to analyze the sample. Correct illumination and focus are essential to a consistent reading.

CONCLUSION

In this study, it is obvious that sperma function tests extend the profiles of the sperma analysis and moreover, it was regarded that with its more effective use in pre- determining the fertility potential, it provides great benefits for artificial insemination, *in vitro* Fertilization (IVF), embriyo transfer and infertility problems.

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