

Modern semen evaluation techniques in domestic animals: A review

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Semen quality evaluation is the most important part in predicting fertility of domestic animals. Due to the complexity of the fertilization process, a single semen evaluation test is not able to predict fertility. Instead, a set of semen tests has to be selected with high relevance for important sperm traits and low redundancy of assay results (Petrunkina *et al.* 2000). A sperm cell may still be infertile for other reasons, this implies that a fertile sperm has to have all its parts functional in the process. Other factors implicated in final fertility verdict of a semen sample also include the condition of semen storage and transport, physiological receptivity stage of the female besides the techniques of the inseminator. As the number of inseminated sperms increase, fertility also increase until it reach the maximum as reported by Dan Daas (1992), Therefore, estimates of fertility in controlled laboratory tests vary greatly from actual field fertility results.

Being one of the most significant causes of fertilization failure, sperm functional impairment keeps up-date as a subject on which research have been carried out for a long time. Various laboratory methods have been developed to assess the motility, morphology and viability of sperm cells. Problem with the microscopic method is that results are

subjective and variable, often resulting in erroneous or conflicting conclusions, as the assessment is also influenced by temperature and evaluator's skills. Accordingly, high variations in estimates of sperm motility parameters of the same ejaculate and the quality of frozen semen intended for use in artificial insemination greatly vary. Nevertheless a good indicator of sperm viability, the post thaw motility has not always proved to be a good predictor of fertility (Martinez, 2000).

Now a day, semen evaluation techniques to predict the fertilizing capacity of a semen sample using rapid and inexpensive procedures are in vogue for quality determination of semen. Yet, none of the presently available assays can perfectly evaluates the sperm's at collection, after extending and cooling to 5° c, or after freezing and thawing. This initiated a worldwide investigation looking for alternative, or at least complementary, in-vitro sperm assessment techniques.

Review of literature on correlation between various laboratory assays to fertility of the bull semen by Graham (2001), could not confirm these to be reliably correlated with fertility. Correlation between fertility and sperm motility ranged from 0.15 to 0.84; between fertility and morphology assays from 0.06 to 0.86 and for fertility in relation to cell viability

0.33 to 0.66.

For a laboratory assay to be useful in semen evaluation, it must be:

- . Objective – having little error due to human judgment
- . Repeatable- produce similar results when repeated
- . Accurate – precisely evaluates the sperm attributes
- . Rapid and
- . Inexpensive

Traditional semen Evaluation Techniques

Macroscopic Test

1. Volume
2. Colour
3. Consistency
4. Density
5. pH

Volume of semen, its colour and consistency, are observed visually, with diagnostic value to a certain extent, for functioning of accessory glands, possible sperm concentration and expected number of doses from semen sample. Colour can be an evidence of injury or pathology in the tract. The type of pH evaluates the quality of semen, good quality of semen always slightly acidic and poor quality semen is generally neutral or slightly alkaline in nature. Further analysis is done with a microscope having a warm stage and preferably with phase contrast for better visualization and assessment of sperm cells. Under field conditions, lower estimates of sperm cells motility are often reported, which may include the general lack of warm stage in microscopes at the field AI centers. As already indicated, such tests suffer from variations of subjective evaluation. Questionable sincerity in recording the field fertility results while struggling with unrealistically bloated targets, it will be disastrous to draw a correlation between field

fertility results and laboratory tests being done in most parts of the country.

Microscopic tests

Routine microscopic sperm motility assessment is a completely subjective evaluation and depends on the experience and ability of the person, yet it is the single most used parameter to determine the quality of semen intended for AI. Vital staining (Eosin-Nigrosin) of the spermatozoa allows quantifying the fraction of living cells, independent of their motility factors in the seminal plasma. This also provides a check on the accuracy of motility evaluation, since the percentage of dead cells not exceed the percentage of immotile spermatozoa.

6. Mass Motility

Is the collective movement of spermatozoa, mass motility should be examined as soon as possible; and is the most influenced parameter in the semen analysis.

7. Individual motility

Individual motility checks the progressive movement of sperm cells. A drop from undiluted semen is placed on a pre-warmed (37°C) glass slide. After placing a cover slip, the percentage motility of spermatozoa is assessed under a microscope at 40X.

8. Sperm concentration/Sperm count

Little more tedious is the Neubauer chamber for estimating sperm concentration, which may tend the technician to skip the test completely. Resultantly, the number of sperm per insemination dose not only varies greatly between batches, but may also be less than that required for optimum fertility. Initially, graphs were plotted for sperm concentration with spectrophotometer, which was then optimized with dedicated equipment for calculating the sperm concentration for providing the dilution rate based on given inputs. The drawback remains that it does not

differentiate between the dead and the live sperm cells while giving approximate count, hence to get the required population of progressively motile live sperm cells per dose, one has to consider other tests at the same time.

Spectrophotometer method

A spectrophotometer can be used to count stallion semen. Basically, the machine measures the amount of light that passed through a sample and calculates the concentration of cells that the density reflects. Using our machine:

- a) A blank tube is loaded with 3.42 ml of formal-buffered saline.
- b) The blank is inserted into the machine with the clear sides on the left and right.
- c) The machine passes a beam of light through the clear sample and determines that 100% of the light is passing through.
- d) Then, 180 μ l of semen is added to the tube, the tube mixed and reinserted into the machine.
- e) The sperm concentration is then calculated based on the percent of light that is transmitted through the sample.
- f) Any dirt, blood or other contamination in the sample may adversely affect the amount of light transmitted and result in an erroneous concentration reading.
- g) Total sperm numbers.

Multiply the concentration X the volume to give the total number of cells in the ejaculate.

Take that number and multiply by the percent progressively motile cells to get the total number of progressively motile cells.

Take that number and multiply by the percent normal cells to get the total number of normal, motile cells.

9. Viability count

To determine the viability of spermatozoa, a drop of semen sample is mixed with a drop of the eosin and nigrosin stains on a pre-warmed slide using applicator stick and a thin smear is made using another slide (Khan and Ijaz 2008). After air-drying, the smear is observed under a phase contrast microscope (40X) for unstained heads of the spermatozoa (live) and stained or partial stained heads of the spermatozoa (dead). A total of 200 spermatozoa are counted to determine the percentage of live and dead spermatozoa. The mean of 3 observations are considered as a single data point.

10. Sperm morphology

Morphology is usually examined with an eosin-nigrosin stain (background stain) to highlight the cells. The final slide should have dark and light areas that allow you to view different coloured backgrounds as needed when examining the slide. Examine the cells under 1000X (oil) to fully assess the morphology. A phase contrast microscope should also be used with wet mounts, but is rare in a practice.

Sperm Abnormalities:

Abnormalities are classified as primary, secondary and tertiary. Primary abnormalities arise in the testes, whereas secondary abnormalities arise in the epididymis or ejaculate. Secondary abnormalities may be just as serious primary. Primary abnormalities decrease through transit and secondary abnormalities increase through transit. Tertiary abnormalities are those encountered during laboratory processing. However Major and minor abnormalities may be a better classification as described by Blom (1959).

Biochemical Tests: Biochemical analysis of secretion components from prostate, seminal vesicles and epididymis in semen give information about the functional state of these

organs. These markers include fructose as seminal vesicles marker, and other markers are measured using enzymatic assays.

11. Methylene blue reduction test: It is used to study the metabolic activity of spermatozoa. Reduction of methylene blue depends upon dehydrogenase enzyme activity of the spermatozoa. Hydrogen ions are liberated under anaerobic condition. These hydrogen ions are transferred to methylene blue and methylene blue reduces to leucomethylene blue which is colourless. More the no. of hydrogen ions liberated per unit of time less is the time taken by methylene blue to change its colour. Nakano (1930) was the first Scientist to study the metabolic activity of sperm cells by using methylene blue. Hankiewicz *et al.* (1964) observed strong correlation between dehydrogenase activity of semen, sperm motility, concentration of spermatozoa and live count, showed strong correlation between MBRT and fertility.

12. Resazurin Reduction test Resazurin, like methylene blue is an acceptor of hydrogen and is reduced in the process. The reduction of resazurin results in a series of colour changes; in solution it is blue. When oxidised, it changes through purple to pink resurufin irreversible reduction and finally white hydro-resurufin, during reduction. The reduction of hydro-resurufin is reversible. Erb and Ehlers (1950) have used the reduction of this dye as a measure of metabolic activity in place of methylene blue.

13. Fructolytic index

Fructolytic index is the amount of fructose used by 10^9 spermatozoa per hour at 37°C . This gives fairly good idea about the liveability of spermatozoa and sperm metabolism. The sample of semen should be well buffered otherwise the fructolysis will stop at certain stage and the results will be erroneous.

Bhosrekar (1975) recorded 1.4 ± 0.16 fructolytic index for buffalo semen.

14. Oxygen utilization test

Walton and Edwards (1938) were the first to use the oxygen consumption rate of semen samples as a measure indicative of potential fertilizing capacity of spermatozoa. This can be done by Warburg's apparatus. Oxygen uptake by spermatozoa is measured by manometric determination. The sperm cells diluted in various dilutors are kept in small flask, at body temperature in both the system is closed one, wherein the CO_2 produced is absorbed by an alkali. The O_2 consumed and CO_2 produced can be determined. The composition of the diluent, pH, as well as the temperature of the diluent influences the O_2 consumption.

15. Millovanov's resistance test

The test shows ability of the spermatozoa to withstand 1% sodium chloride solution.

16. Hyaluronidase content

Hyaluronidase enzyme is present in acrosomal system of spermatozoa. Since integrity of acrosome is directly involved in fertilizing capacity of spermatozoa. This enzyme has assumed a great importance in estimating fertilizing capacity of semen. If there is damage to the acrosome it is presumed that this enzyme will leak out in extracellular fluid and presence of hyaluronidase activity in extracellular fluid will indicate acrosomal damage.

Dhanda *et al.* (1981) studied sorbitol dehydrogenase and hyaluronidase activity in buffalo semen. They reported marked variation amongst individuals. According to them motility of sperm is inversely related to hyaluronidase activity. Danasoun (1988) observed loss of acrosomal enzymes acrosin and hyaluronidase from spermatozoa after thawing at different temperatures and time. The highest acrosome activity remaining in the spermatozoa and the

lowest hyaluronidase activity in the media were observed after thawing at 70° C.

17. Glutamic oxaloacetic transaminase activity (GOT):

This enzyme is purely cellular and is found in seminal plasma. The presence of this enzyme in plasma indicates the damage to the spermatozoa. Addition of glycerol increases the release of GOT in extra cellular fluid indicating damage to the spermatozoa.

18. Cold shock resistance test

This test is useful to test semen samples for varying degree of resistance of spermatozoa against cold shock. It has been concluded that greater the resistance towards cold shock, better is the fertility and may live longer when preferred. Cold shock causes irreversible loss of viability and there is rapid loss of ATP content of sperm. Various enzymes like alkaline protinase, acrosin and other vital proteins are lost from the acrosome. The cold shock resistance varies from bull to bull.

Microbial examination

19. Microbial load/Bacteriological culture

Direct measurement of infectious contamination is obtained from bacteriological cultures of both aerobic and anaerobic germs. In normal conditions semen is not sterile but rather colonized at low levels by a variety of germs. Recent studies have shown that bacterial colonization did not have negative impact on sperm-cervical mucus interaction.

Modern semen evaluation Techniques

Sperm cell is a complex structure with almost all its parts contributing to successful fertilization of the ovum. Therefore, accurate assessment of semen quality would imply that the applied tests are able to discern deviations in the sperm cells, including their internal structures organelles, and their functional status so as to be correlated with ultimate fertilizing potential.

New automated machines, such as computer assisted sperm analysis (CASA) system and flowcytometry are now available to evaluate various characteristics of sperm cells. These allows us to evaluate different motility and morphology parameters, internal compartments and their functions in individual cells, which include the nucleus, plasma membrane and mitochondria as well as capacitation and acrosomal status. Major problem in using these computerized measuring devices include the extreme need for standardization, optimization and validation of the system before any practical use is possible, Indeed, the choice of internal image setting, which is important to identify and reconstruct the trajectory of different spermatozoa accuratel. Though, it is still a matter of conflict in many species. Standardization of technical setting is required to compare results between laboratories and may be of importance in view of the increasing international exchange of semen.

1. Sephadex filtration

Generally, semen ejaculates with low initial motility are discarded as they usually show reduced post thaw motility. In buffalo, it was recorded that 31 % of the total semen ejaculates produced per month had to be discarded due to poor initial quality. A good deal of heterogenesity in spermatozoa morphology is encountered in mammalian semen and significant reduction in the percentages of dead and abnormal spermatozoa has been reported following sephadex filtration in buffalo semen (Ahmad *et al.* 2003).

The mechanism by which sephadex retain dead, damaged or uncapacitated spermatozoa are still not well understood. The separation of spermatozoa was probably on the basis of complex and interacting properties of

sperm plasma membrane, the medium suspending the sperm and the sephadex particles. Landa *et al.* (1998) speculated that there was a physicochemical reaction between sperm plasma membrane bound proteins and sephadex particles.

Different types of sephadex G-25, G -50, G-100, G-200 and G50-200 were used. All types of sephadex filtration had a significant effect on sperm motility. Samples filtered through both Sephadex G-75 and G 50-200 revealed higher semen quality than sephadex 25, 50, 100 and 200. The mean percentage of live spermatozoa after filtration by different types of sephadex was significantly improved than control samples. More improvement was observed with sephadex 75 and sephadex 50-200 than other types of sephadex.

2. Immunological examination:

Since mucosal immunity and systemic immunity may occur independently in the female partner, thorough anti sperm antibody analysis requires assaying both the serum and cervical mucus (CM). The presence of anti-sperm antibodies in semen can alter the sperm fertilizing ability. Successful detection and quantization of antisperm antibodies is a necessary simple method for direct analysis of antibodies bound to sperm cells using the Sperm Mar assay or Immuno bead test (IBT). For preliminary screening, the Sperm Mar assay is rapid and allows the test to be performed on unprocessed semen, whereas, the IBT requires some sperm processing before assaying. Both assays permit semi quantitative assessment of the degree of antibody binding as well as the detection of IgA and IgG classes of antibodies. Positive samples are subsequently tested for anti-sperm IgA antibodies which are clinically more significant.

An indirect IBT assay utilizing serum or CM pre-treated donor sperm is an approach

that identifies both the presence and class of antisperm antibodies. Other methods for detection of antibodies against sperm include the tray agglutination test and immobilization test routinely used for screening serum samples and Solubilised CM because of assaying multiple samples in one run.

3. Cervical Mucus Penetration Test

Sperm – cervical mucus interactions

Cervical mucus is the major physical barrier that sperm cells have to cross to access to the female upper genital tract. Less than 1% of the sperm deposited in the vagina successfully penetrate the cervical mucus. Evaluation of sperm-cervical mucus interactions includes the post-coital test, the sperm cervical mucus contact test and the *in vitro* sperm-cervical mucus penetration assay.

Post-coital test

The *in vivo* evaluation of the spermatozoon-cervical mucus interaction is carried out with the post coital test (PCT). 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 in oestrous cycle, under the microscope and measuring the number and motilities of the spermatozoa. Showing a single advanced-fast motile spermatozoon in the cervix points out that the cervical factor is not responsible for infertility matter as found by Tejedor *et al.* (2000).

The number of motile sperm per high power microscopic field post coital are recorded and the test is considered positive when 10 or more motile sperm are found per field. Cervical mucus evaluation (including volume, consistency, ferning, cellularity and pH) is of utmost importance for the interpretation of post-coital test results with respect to sperm function. A decreased number of sperm in cervical mucus when the cervical mucus score is low reflects inadequate mucus rather than

impaired sperm function. Cervical mucus is colonized by sperm that are stored for several hours in cervical crypts. Sperm cells then gradually migrate through the cervix. Therefore sperm present in cervical mucus constantly for at least 12h following intercourse and the timing of post-coital test (6-12h after intercourse) allows testing the viability of sperm in this environment. Abnormal penetration of cervical mucus by sperm has been associated with the presence of immobilizing anti-sperm antibodies and repeatedly abnormal post-coital test with normal score cervical mucus.

The post-coital test however remains an inexpensive and non invasive procedure that gives information about the occurrence of ejaculation and the ability of the sperm cells to function within the cervical environment.

In vitro cervical mucus penetration test with bovine cervical mucus whose biochemical structures, glycoprotein structures, fluidity structures and their ferning appearances in the electron microscope scan are the same. 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 by Robayo *et al.* (2008).

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 as reported by Anilkumar *et al.* (2001). *In vitro* spermatozoon-cervical mucus penetration tests are performed in two ways, which are mucus contact test and capillary tube test.

Sperm-cervical mucus contact test

Spermatozoon-cervical mucus contact test is done by mixing a drop of semen and a drop of cervical mucus on the slide. A cover slip placed on the slide and pressed slightly. Quantitative (number of sperm high power microscopic field, 400X) 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 sperm agglutinins stick to glycoprotein filaments as soon as they contact cervical mucus. Cervical mucus containing sperm agglutinins provides the penetrating spermatozoa with the sperm agglutinins causing the spermatozoa to stick to glycoprotein filaments.

In vitro cervical mucus penetration test /capillary tube test.

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, this is most widely used, whereas in another method, motile spermatozoon number is determined in 3 cm distance after 60 min of incubation.

4. Zona-free hamster egg penetration test

The zona-free hamster oocyte sperm penetration assay is a heterologous bioassay has originally been developed to test

capacitation, acrosome reaction, fusion and sperm chromatin decondensation.

Zona free hamster egg penetration test is one of the most impressive tests for evaluating spermatozoon functions. The zona removed hamster oocyte penetration test is defined as the penetration ability of spermatozoa in the zona removed hamster oocytes. It is 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 (Kathiravan *et al.* 2008). A great number of oocyte cumulus cells are cleaned off from the zona using hyaluronidase, trypsin and merceptal, 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 over 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 by Aitken (2006). 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).

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 difficulties 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. Since only the spermatozoa that have been through capacitated and completed the acrosome reaction will penetrate to the hamster egg whose zona is removed. Capacitation is acquired by incubating the spermatozoa in an appropriate capacitation fluid by swim-up process for 1 h. Glycosaminoglycans, Ca ionophores, follicle fluid, Test-Yolk buffer, platelet activation factor and progesterone, which induce *in vitro* capacitation and acrosome reaction are used.

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% CO₂ at 38.5-39°C. After the oocytes are washed with appropriate mediums and stained with various paint preparations following the incubation, examination is done under phase-contrast microscope at 400X. Swollen spermatozoon head or spermatozoon tail penetrated into the vitellus is evaluated as positive. 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).

5. Hemizona assay.

At the end of fertilizing process, sperm must contact and penetrate the oocyte. This requires intact receptor proteins on to the sperm to bind to the zona pellucida trigger the acrosome reaction and bind to the plasma membrane of the oocyte. Different techniques are used to evaluate the binding ability of the sperm cell. Homologous equine hemizona assay have also been developed. In the hemizona assay, sperm from the control and test stallion are labeled with the same fluorescent stain and incubated separately with the two hemizona. Two type of sperm – ZP binding assay have been used for bull spermatozoa, one using intact (not cleaved)

homologous oocytes by Zhang *et al.* (1998) and the other using bisected hemizona (hemizona binding assay) Fazeil *et al.* (1997) where each matching half is incubated with test vs control spermatozoa respectively. Significant correlation with AI – fertility has been found using both types of ZP- binding assays.

The hemizona assay (HZA) measures the binding of capacitated sperm to isolated zona pellucida. Oocytes are bisected by micromanipulation, thus allowing for an internally controlled comparison of sperm binding (from patient versus a fertile control) to matching hemizona surfaces. The two matched hemizona of the oocytes have the advantage of providing functionally equal surfaces allowing a controlled comparison of sperm binding and therefore limiting the amounts of oocytes used. Ethically this assay is acceptable since the microsurgical bisection of the oocyte prevents any inadvertent fertilization. The HZA has been found to be predictive of IVF outcome with positive and negative predictive values of 83% and 95% respectively. The major problem with this assay is the limited availability of zona sperm receptors mimic the natural functional hemizona used now.

6. ACROSOME REACTION

Acrosome is a cap structure covering the front side of spermatozoon nucleus, bounded to the membrane and due to its localization, available in different shapes among the golgi complex in the spermatid containing necessary enzymes for fertilization. Acrosome reaction is an irreversible event under physical conditions and after the spermatozoon reaches its full capacity, it springs into the action of binding to the zona pellucida. As a result of 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 acrosome ingredient is accepted as a substance that softens zona pellucida with the effects of the acrosomal enzymes or melts where available and thus, the penetration of the spermatozoa in this structure becomes easier by Neild *et al.* (2005). While the hyaluronidase enzyme, which the spermatozoa that have completed the acrosome reaction excrete, melts down the cumulus cell matrix, the acrosome on the surface of the spermatozoa plays a role in passing beyond the zona pellucida (Witte and Schafer-Somi 2007).

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 occurred. 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

Following spermatozoon-oocyte fusion, oocyte is activated as metabolic and after liberation 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.

Moce and Graham, 2008 ;narrated a procedure to study capacitation where spermatozoa acquire *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% CO₂. After capacitation, *in vitro* acrosome reaction is induced by agents such as, Ca ionophore A 23187, glycosaminoglycans and follicle fluid. In bulls, when stock solutions which are prepared especially by dimethyl sulfoxide and Ca A 23187 ionophore are to be used, 1 µl mixture is added to 50 µl sperm suspension to acquire 10 µM calcium after they are diluted 10 times with TALP.

Then, they are left for 1 h incubation at 39°C in a humid environment with 5% CO₂. 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 glutaraldehyde 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 Rosebengal in PBS with a 6 pH. Examine under a microscope for acrosomal reaction and are evaluated. 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, 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 post acrosomal areas) as reported by Ferrari *et al.* (2000).

7. Acrosomal integrity

Acrosomal integrity of spermatozoa can be measured by using fluorescently labelled plant lectins, proteins that recognize and bind

glucosidic residues in different parts of the acrosomal membrane. *Pisum sativum* agglutinin (PSA) derived from the pea plant and *Arachis hypogaea* agglutinin (PNA) derived from the peanut plant are the most commonly used for their specificity. However, PSA has a tendency to bind the egg yolk particles in the extenders and has slightly less specific binding by Gillan *et al.* (2005). PNA shows a high affinity and strong specificity for disaccharides with terminal galactose and binds to the outer acrosomal membrane, which becomes exposed during the acrosome reaction. Spermatozoa with reacted, damaged, or abnormally formed acrosome acquire green fluorescence after PNA labelling, while intact, normal acrosome have no fluorescence.

8. Sperm Quality Analyzer (SQA)

The SQA evaluates the following six parameters designed to estimate fertility of the semen sample viz Sperm motility index (**SMI**), Functional sperm concentration (**FSC**), Motile sperm concentration (**MSC**), Total Cell Concentration, Percent Normal Motility, and Percent Normal Morphology.

- a) **Sperm motility index (SMI)**: is value generated by computer on the basis of detected changes in a beam of light. This parameter is the basis for the other parameters. It is an item of numerical data internal to the device which determines the quality of ejaculate.
- b) **Functional sperm concentration (FSC)**: It defines the concentration of live, functional sperm in millions per ml (sperm which are motile and also morphological normal).
- c) **Motile sperm concentration (MSC)**: Defines the concentration of progressively motile sperm in millions per ml.

- d) **Total Cell Concentration:** one of the parameters, expressing the total number of cells, living or dead, per ml.
- e) **Percent Normal Motility:** another parameter, expressing the percentage of cells with normal motility.
- f) **Percent Normal Morphology:** parameter, the percentage of cells with a normal morphology

Prinosilova *et al.*, (2005) the semen was drawn into single-use plastic capillaries for measuring in the SQA and placed in the device so that the results could be read from the display. The device works on the principle of detecting changes in a beam of light after it has passed through the ejaculate in the capillary. The SQA measures changes in light impulses caused by sperm movement in time periods. SQA Analyses sample in 10-second periods. This test was repeated 4 times over a 45-second evaluation period and the values are processed to produce final result, which appears on display.

Overall ejaculate quality assessment of using routine sperm analysis and SQA

The sperm survival test is carried out at laboratory temperature (22°C) in ejaculates diluted with physiological saline buffered with a phosphate buffer at pH 7.2. The ejaculate is diluted to a concentration of 100×10^6 spermatozoa/ml. After 120 min the ejaculate is heated to 35°C and then evaluated by establishing functional indicators and the morphological assessment.

9. Sperm Chromatin Structure Assay (SCSA).

New studies suggest sperm Chromatin Structure Assay (SCSA) is a test to measure the level of DNA fragmentation in the sperm, to enhance the diagnosis of and treatment for male infertility. Research indicates that sperm with high-levels of DNA fragmentation have a lower probability of producing a successful pregnancy. It is observed that patients with a

DNA fragmentation level of greater than 30% are likely to have significantly-reduced fertility potential, including a significant reduction in term pregnancies. Sperm that appears to be normal by traditional semen analysis parameters (motile, morphologically normal sperm) may even have extensive DNA fragmentation. In an effort to achieve the most effective measurement of male fertility potential, the SCSA reports the percentage of the major populations of fragmented sperm present in a semen sample.

Sperm with immature chromatin due to less chromatin condensation, allowing for a higher degree of DNA stain ability. The SCSA is performed using an instrument called flowcytometer in which cells that have been stained with a fluorescent dye are sent through a glass channel in liquid suspension. The cells pass through a laser beam and the light from the beam causes the dye to emit fluorescent light of a certain colour. When performing an SCSA, the colours measured are red and green; green fluorescing sperm have very low level of fragmented DNA and red fluorescing sperm have moderate to high levels of fragmented DNA.

SCSA can measure 5000 individual sperm in just seconds and the data provides both a diagnostic and prognostic evaluation of the male's potential for sub fertility or infertility. Another advantage of note is the fact that the data are from objective, machine-defined criteria rather than from biased human eye measurements as with a standard semen analysis. In addition to having a higher level of repeatability than that of any other semen parameter, the SCSA randomly measures all cell types in the semen sample as opposed to evaluation of only washed samples.

While examining SCSA studies, length of sexual abstinence, age, exposure to high levels

of air pollution etc. cause significant variations in results. Sperm chromatin structure is also compromised in patients with testicular cancer. Significant exposure to prolonged heat in the testicles can also contribute to high levels of fragmentation. Drug use, exposure to chemicals or radiation and testicular trauma are other potential causes of abnormal results. Brahem *et al.*, (2011) reported the DNA fragmentation index (DFI) quantifies the susceptibility of sperm DNA to fragment and provide a result that settles into one of the following ranges:

| Fragmentation index | Result range |
|----------------------------|---------------------|
| < 15% DFI Integrity | Excellent DNA |
| 15 - 24% DFI Integrity | Good DNA |
| 25 - 29% DFI Integrity | Fair DNA |
| ≥ 30% DFI Integrity | Poor DNA |

10. Flowcytometric Assessment of sperm motility

Flow cytometry is a process in which fluorescently labelled cells (in these case, spermatozoa) travel individually at high speed through a flow cell, where they are illuminated by one or more lasers. This causes light scattering and fluorescence excitation of markers located on specific parts of the sperm, which is then picked up by photodetectors and sent to a computer program. The computer program presents the information in the form of relative fluorescent intensity units, which are typically displayed as either scatter plots or histograms by Martinez-Pastor *et al.* (2010). One of the main concerns with analyzing spermatozoa by flow cytometry is the presence of non-sperm events in the sample such as immature forms of spermatogenic cells, bacteria, blood cells, tissue, and in frozen-

thawed semen, extender contaminants such as egg yolk particles. During the data analysis, these non-sperm events can be taken into account and most of the time can be eliminated from evaluation by gating of scatter diagram/Histogram.

Flow – Cytometry (FACS Analyzers), is a technology that examines thousands of spermatozoa within minutes, yet latter’s high cost further pose a hindrance in its wider applications in semen labs. Flowcytometry also utilizes fluorescence probes for sperm staining and increases the objectivity of analysis allowing large number of spermatozoa to be evaluated in a short time while providing reliable results. Flowcytometry allows simultaneous evaluation of multiple sperm characteristics, hence providing precise description of the sperm population.

Among the cell components, integrity of the plasma membrane has been one of the most studied parameters owing to its major role as the cell boundary and in cell to cell interactions. Presence of an intact plasma membrane is evaluated by staining a semen sample with propidium iodode (PI) a fluorescent probe that binds to DNA. Live cells having an intact plasma membrane will prevent PI from entering the cell, while cells with a damaged plasma membrane will permit PI to enter the cell and bind to DNA causing the cells to fluorescence red.

Vital Dye Based Sperm Flow Cytometry

Flow cytometry can be used to analyze a variety of structural and functional characteristics of spermatozoa, including plasma membrane integrity, mitochondrial activity/mitochondrial membrane potential, acrosome integrity, chromatin structure, changes in the sperm surface induced by sperm capacitation, and certain forms of morphological abnormalities present in a sperm

sample reported by Bochenek *et al.* (2001). Numerous biomarkers are available or in development for use in sperm evaluation with flow cytometry. Some, such as ubiquitin and PNA, are present only in poor quality sperm and are generally referred to as negative biomarker present in varying amounts depending on the quality of the spermatozoa. The stain forms aggregates which fluoresce orange. Therefore, the mitochondria with high membrane potential fluoresce orange and those with medium to low membrane potential fluoresce green.

Currently, one of the most commonly used viability stain combinations is SYBR-14 and PI dyes target the same cellular component, DNA, eliminating the ambiguity that can arise when different components are targeted. With these biomarkers, the nuclei of live spermatozoa display green fluorescence because of integration of SYBR-14 and dead/dying cells with compromised membrane integrity stain orange because of passive PI uptake through damaged plasma membrane. By combining the viability stains with other biomarkers additional sperm functions such as acrosomal integrity and mitochondrial function can be assessed. Sperm capacitation includes a process of plasma membrane destabilization which may lead to physiological acrosome reaction upon sperm-egg binding. Fluorescent markers such as SYBR-Green 1 have been adapted to identify bacteria in semen, and the use of flow cytometry for bacterial counts is becoming more routine as reported by Tripp (2008).

Protein Biomarkers of Sperm Quality

Potential sperm quality/fertility biomarkers include proteins that are exclusively associated with certain types of sperm defects and proteins more abundant in morphologically and functionally normal spermatozoa. One of

the protein biomarkers of bull sperm quality that have been studied in depth is ubiquitin. Abnormal spermatozoa are tagged by ubiquitination of the plasma membrane/sperm surface during epididymal passage by Baska *et al.* (2008). Many abnormal spermatozoa appear in the ejaculate, and their increased content is indicative of poor semen quality, or even infertility. Increased binding of fluorescently-labelled anti-ubiquitin antibodies to the sperm surface reflects the occurrence of sperm abnormalities, which is then detected by the flow cytometer. Ubiquitin as a sperm biomarker has been assessed in bulls by Sutovsky *et al.* (2002).

Heparin binding proteins (HBP) secreted by the seminal vesicles, prostate, and bulb urethral glands are present in the seminal fluid and bind to spermatozoa after ejaculation. The presence of a specific heparin binding protein, HBP-30, on the sperm plasma membrane has been correlated with increased fertility in bulls, inspiring a synonym the fertility-associated antigen (FAA). The presence or absence of FAA can only be determined via biochemical analysis it has no relation to breeding soundness or serving capacity.

Bull Sperm Proteomics: Searching for New Biomarkers

Sperm proteomics can be used to discover new biomarkers of fertility. The proteomics of seminal plasma and epididymal fluid could be arguably just as important as sperm proteomics because spermatozoa acquire numerous sperm surface proteins that convey fertilization potential in the epididymis by Sutovsky (2009). Comparison of bull sperm proteomes between fertile and sub/infertile bulls as well as proteomic characterization of bovine seminal plasma have given some insight into which proteins at what levels are indicative of fertility or infertility.

11. Hypo-osmotic swelling test

A simple test – HOST- has been developed for examining the sperm membrane integrity, based on the principle that all living cells with intact membrane should swell when exposed to a hypo-osmotic solution. 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 by Jayendra *et al.* (1992). HOS test investigates whether the spermatozoon membrane is functional or not. This test has been claimed to be relevant to fertilizing ability. The undamaged sperm's tail membrane would permit passage of fluid into the cytoplasmic space causing its swelling and the resulting pressure lead to characteristic curling of tail fibers. In contrast, the damaged or chemically inactivation membrane would allow fluid into freely move across the membrane without any accumulation inside it; hence no cytoplasmic swelling and curling of the tail should occur. Modification of the HOS-test has been presented for bull semen, using cell volumetris counting. The method indicated a significant correlation with fertility, however, the relationship between the degree of sperm damage after thawing and fertility is not always clear, but tends to increase when damage is extensive or when fertility values are widely spread. Therefore, HOS test is used as a method complementing the routine sperm analysis by Nie and Wenzel (2001).

Method of HOS test

Percentage of Hypo-Osmotic Solution Reactive Spermatozoa (HOST):

1. Plasma membrane integrity of sperm was assessed using hypo-osmotic swelling (HOS) assay. Sodium citrate (0.735g) and fructose

(1.351g) were dissolved in 100ml distilled water to prepare HOS solution and it was maintained at 37° C for 5 min before use.

2. 100 µl of each semen sample was mixed with 1000µl of HOS solution and incubated at 37° C for 60 min.

3. After incubation, the spermatozoa were fixed with formaldehyde (10% formalin 0.1 ml) for subsequent observation of swollen sperm. Such fixation retained the shape of spermatozoa, which could be observed even at a later stage.

4. After placing a drop of incubated well mixed semen sample on a glass slide and covering it with a cover slip, a total of 200 sperm were counted in at least 5 different fields of view and the percentage of sperm that reacted with different swelling patterns of tail were observed.

Interpretation

Spermatozoa were classified in 4 different classes according to the presence of following tail swelling pattern:

Pattern A: No swelling, no membrane reaction

Pattern B: Swelling of the tip of the tail

Pattern C: Different types of hair -pin like swelling of the mid piece

Pattern D: Complete tail swelling

Selvaraju *et al.* (2008) found the mean hypo-osmotic swelling responsive spermatozoa in Murrah buffalo to be 50.25 per cent neat semen diluted with egg yolk-tris buffer.

Tiwari *et al.* (2009) reported in vitro fertility based on hypo-osmotic swelling test (HOST) of semen of Tarai Buffalo bulls. The

average values of semen volume and hypo-osmotic reacted spermatozoa were 2.31 ± 0.46 ml and 71.30 ± 3.42 per cent, respectively. There was highly significant ($P < 0.01$) variation among bulls for hypo-osmotic swelling test. There was none significant correlation between semen volumes with HOST.

12. Computer Aided Sperm Analyzer (CASA)

Computer aided sperm analysis (CASA) attracted increasing interest in human and live stock sector. The track semen analysis system, based on individual sperm assessment, offers an accurate and rapid calculation of various semen parameters such as total motile, progressive motile, linearity and velocity parameters. The CASA can assess various semen characteristics simultaneously and objectively. It has been proposed for semen evaluation of several mammalian species and is valuable in detecting changes in sperm motion, which cannot be identified by conventional semen analysis methods. Such sperm motion has been correlation with sperm penetration of cervical mucus, penetration of oocytes, and the results of in-vitro fertilization.

CASA helps in determining the sperm viability by using a vital stain- VIADENT'. The dye stains only the cells with non-intact membranes thus identifying non-viable cells. Using IDENT stain, sperm are easily identified from debris under fluorescent illumination. IDENT (DNA specific dye based on Hoechst bisbenzimidazole) stains all DNA containing objects, which fluoresce under appropriate light. Cytoplasmic detritus, which is devoid of DNA material, will not fluoresce and lower degree of fluorescent intensity than the haploid sperm. Interestingly, although sperm cells have only half the DNA complement of a somatic cell, it is highly condensed, resulting in a higher degree of fluorescent intensity.

CASA consists of optics, light source and the computer hardware and motile, morphology and vitality that can do analysis module that contain software is a combination. 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 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 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. 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 reported by 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 larger 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 are recommended whereas in the stallion from 300-500 cells seems to be optimal by Verstegen *et al.* (2002).

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.

To reduce variability and misinterpretation of results, a larger number of cells have 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 haematoxylin are most used for morphologic assessment in CASA by Davis and Katz (1993). Magnification also affects operation. Goat sperm heads did not present significant differences when analyzed at 20, 40, or 60X but bull sperm heads must be analyzed at 60 X. 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.

Computer Aided Sperm Analysis System (CASA).

- MAIN SOFTWARE FEATURES:
- Storage of textural data images and clips.
- MultiMedia Catalog database. We can take advantage of all the powerful features of MultiMedia Catalog in application to semen analysis. The database is a perfect tool for archiving patient data, analysis results, video clips and images, fast and flexible search and spectacular reporting with images, graphs and other features adjusted to our needs. Automated analysis of sperm concentration and motility on samples.

- Automated morphology analysis on stained samples.
- Manual assessment of concentration of WBCs, immature germ & round cells.
- Manual measurements for special research or other individual tasks.
- Using MMC Sperm, you can record all the clips to your hard disk to repeat the motility analysis at any time while without it you are restricted to the live time of spermatozoa.
- Morphology analysis becomes more accurate. The software will assess every cell that you have acquired.
- The use of the automated software for semen analysis you receive objective quantitative data: head length, width, area, and form factor. The same applies to motility estimation of sperm movement becomes more objective if you know its speed and exact track.
- Using Computer Aided Sperm Analyzer, it is much easier to train new staff in your laboratory and control the results of their work.
- The MultiMedia Catalog database provides the ability to create unlimited number of records with infinite number of fields to archive all the required patient information accurately and search for it fast. Selecting the proper database field types helps you prevent misprints in your database. Drop-down lists will aid in fast and accurate filling the records with repeatable information.

MOTILITY ROUTINE

Sperm concentration assessment (M/ml) and motility analysis are performed. The analysis strictly follows the semen and sperm-cervical mucus interaction.

The following parameters are calculated:

- VCL = curvilinear velocity (micron/s)
Time-average velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope.
- VSL = straight line velocity (micron/s)
Time-average velocity of a sperm head along the straight line between its first detected position and its last.
- VAP = average path velocity (micron/s)
Time-average velocity of a sperm head along its average path. This path is computed by smoothing the actual path.
- BCF = beat cross frequency (beats/s)
the average rate at which the sperm's curvilinear path crosses its average path.
- LIN = linearity the linearity of a curvilinear path.
- STR= straightness the linearity of the average path.
- Elongation of sperm head
- Based on the parameters mentioned above, the motility of each spermatozoon is graded:

A = rapid progressive motility

B = slow or sluggish progressive motility

C = non progressive motility

D = immotility

Additionally, the software enables the user to assess the concentration of:

White blood cells

Immature germ cells

Round cells

- We can see the exact values of the parameters for the selected spermatozoon and classification result in a pop-up window. The clip can be played again, rewind, played frame by frame for detailed analysis. You can save clips to your

hard disc uncompressed without loss of quality.

- The number of sperms detected and tracked is displayed on the screen and we can check if you have tracked enough cells. When the analysis is finished, the results of automated classification and manual counting are automatically transferred to the database.

MORPHOLOGY ROUTINE

Morphology of the sperm head is an important criterion for the correct diagnosis. The software is set up to analyze still images of smears stained with the Diff-Quik stain. We have selected Diff-Quik as the head is stained pale blue in the acrosomal region and dark blue in the post-acrosomal region which is a good basis for precise image analysis. The following parameters are assessed for every spermatozoon.

- Area of the head
- FFC = form factor circle the degree of similarity of the sperm head to a circle.
- Perimeter of the head
- Brightness
- ELL_B = Big axis of ellipse outlining the sperm head, the length of the sperm head
- ELL_S = Small axis of ellipse outlining the sperm head, the width of the sperm head
- Elng = elongation of the sperm head
- FFE = form factor ellipse the degree of similarity of the sperm head to an ellipse.

The software classifies spermatozoa into Norm and Head Pathology classes automatically based on head parameters. You can easily correct the results manually and also specify other abnormalities (Tail Pathology, Neck Pathology). It is now customary to record the number of

morphological sperm defects divided by the number of defective spermatozoa, a measure called the teratozoospermia index (TZI). After the analysis is finished, the TZI is calculated automatically. The teratozoospermic index values should read between 1.00 (each abnormal spermatozoon has only one defect) to 3.00 (each abnormal spermatozoon has head, neck and tail defects).

STATISTICS on all the parameters which have been measured in Motility and Morphology is displayed in the database (mean values of parameters). Statistics provides additional information on sperm quality. Corresponding database fields are filled in automatically during analysis like those for motility and morphology results. Statistic can be printed or to represent it to if required. Extended report template should be selected for this purpose. Should the statistical data not be required for the patient, a brief report without statistics can be selected.

REPORTING The report is generated according to the instructions the user provides in the database and is stored to corresponding database record. Following features are available grouping of data, changing the order of data appearance, displaying graphics etc. Virtually any requirement can be implemented.

Motility Sperm concentration is estimated using special counting chambers with fixed depth to provide standard volume and distribute spermatozoa in one focal plane to let them move free and stay in focus all the time they are being tracked. Sperm counting chambers provide the ability to work with undiluted semen. The preparation consists usually in mixing the sperm sample carefully while avoiding bubbles.

Morphology To achieve good quality images of stained spermatozoa for morphology assessment, seminal plasma should be diluted and removed after centrifugation. The sperm pellet is resuspended in an appropriate volume to obtain the highest sperm concentration possible, but not exceeding $80 \times 10^6/\text{ml}$. An aliquot of 0.2 to 0.5 ml of semen, depending on sperm concentration, is diluted to 10 ml with normal saline at room temperature. The tube is centrifuged at 800g for 10 minutes and most of the supernatant tipped off. The pellet is resuspended in the remaining saline, typically 20-40 μl , by gently tapping the tube. Then 5-10 μl of this suspension is placed on a glass microscope slide and the drop is spread across the slide with a pipette to make a smear as described below.

Preparation of smears

At least two smears should be made from the fresh semen sample for duplicate assessment and in case of problems with staining. The slides should first be thoroughly cleaned, washed in 70% ethanol and dried, before a small drop of semen (5 to 20 μl) is applied to the slide. If the sperm concentration is over $20 \times 10^6/\text{ml}$, then 5 μl of semen can be used: if the sperm concentration is less than $20 \times 10^6/\text{ml}$, then 10 to 20 μl of semen should be used. The 'feathering' technique whereby the edge of a second slide is used to drag a drop of semen along the surface of the cleaned slide may be used to make smears of spermatozoa, but care must be taken not to make the smears too thick. Feathering works well when viscosity is low but is often unsuitable for viscous semen. Alternatively, a drop of semen can be placed in the middle of a slide and then a second slide, face down, placed

on top so that the semen spreads between them: the two slides are then gently pulled apart to make two smears simultaneously.

CONCLUSION

In this study, it is obvious that sperm function tests extend the profiles of sperm analysis and moreover, with regard to its more effective use in pre-determining the fertility potential. The complex membrane structure of spermatozoon and the acrosome reaction it provides useful information to determine infertility. The sperm motility index provides a reliable and objective reflection of semen motility parameters and quality. The advent of sperm quality biomarkers and the implementation of flowcytometry should benefit the cattle industry greatly, being able to identify markers of good fertility as well as poor fertility of a semen sample in a fast and objective manner that could reduce the need for multiple inseminations and prevent expenses covering offspring testing of sub-fertile producing poor pregnancy rates in AI service. While the idea of using flow cytometry is new and some of the biomarkers are still being developed, an increase in speed, accuracy, and precision in the assessment of fertility in bulls should be welcomed by the cattle industry. Even if flowcytometry were only used to determine sperm viability and concentration, it could potentially still lead to more straws of semen being produced, and better quality control overall, which leads to an increase in profits. Finding the sub-fertile bulls that fly under the radar could potentially save producers considerable amounts of money, especially if sub-fertility could be determined early on before the bulls reach

breeding age. Furthermore, flowcytometry could be used to aid in the development of new cryopreservation techniques, as some scientists are testing the effect of cryopreservation on various sperm characteristics, such as organelle function and viability acrosomal integrity and viability, and mitochondrial function and viability. Hypo-osmotic swelling test examining the membrane integrity. Zona free hamster test, Hamezona assay and cervical mucus penetration test is very useful to evaluate the fertilizing capacity of sperm.

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