BULL SEMEN EVALUATION POST-THAW AND RELATION OF SEMEN CHARACTERISTICS TO BULL'S FERTILITY

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Abstract. Evaluation of the quality of cryopreserved bull spermatozoa is reviewed. The methods for the assessment of sperm quality have markedly improved over the last decades – starting with the assessment of morphological shapes and subjective motility analysis towards the more sophisticated analysis of the molecular changes in chromatin, membranes and catabolic activities of the sperm cell itself. Function of sperm plasma membrane under the hypo-osmotic conditions, distribution and concentration of ions, or function of different organelles seem to correlate with the degree of the viability of spermatozoa after freezing and thawing procedures. Many *in vitro* techniques that stimulate sperm function through female–derived factors, such as zona and oocyte, in zona pellucida binding assay, *in vitro* fertilization (IVF) and production of embryos, were employed to predict the outcome of artificial insemination in the field. Still, majority of methods used for semen analysis today are both - tedious and expensive, and, in many cases confined to human bias. In order to increase the predictive power of assessment, simultaneous analysis of multiple sperm attributes, or outcomes of several laboratory assessments must be combined to look for the overall effect of several independent sperm parameters.

Keywords: Sperm quality, sperm viability, prediction of sperm fertilizing potential, bull semen.

BULIŲ SPERMOS KOKYBĖS ĮVERTINIMAS PO ATŠILDYMO BEI SPERMOS KOKYBĖS SĄRYŠIS SU BULIAUS APVAISINAMĄJA GALIA

Santrauka. Šiame straipsnyje apžvelgiamas šaldytos bulių spermos kokybės įvertinimas. Per paskutiniuosius dešimtmečius spermos vertinimo metodikos pastebimai tobulėjo -pradedant spermatozoidų morfologijos tyrimais ir subjektyviai nustatomu judrumu, ir baigiant moderniais tyrimais, kuriais analizuojami chromatino, membranų pakitimai molekuliniame lygmenyje, bei įvertinamas spermatozoido metabolizmas. Nustatyta, kad spermatozoidų plazminės membranos funkcija hipoosmotiniuose tirpaluose, įvairių jonų pasiskirstymas bei jų koncentracija, o taip pat organelių funkcija koreliuoja su spermos gyvybingumu po atšildymo. *In vitro* tyrimo metodais, kuriuose skatinama spermatozoidų funkcija panaudojus tam tikrus moteriškojo organizmo faktorius, kaip antai kiaušialąstės skaidrusis dangalas ar pati kiaušialąstė, buvo siekiama numatyti būsimą apvaisinimo rezultatą remiantis gautais spermatozoidų prisitvirtinimo prie skaidriojo dangalo, *in vitro* apvaisinimo, embrionų gavimo rezultatais. Visgi, dauguma metodų, šiuo metu naudojamų spermos tyrimams yra varginantys, labai brangūs, ir, daugeliu atvejų, priklausomi nuo tyrimą atlikusio asmens įgūdžių ar meistriškumo. Norint tiksliau nustatyti spermos apvaisinamąją galią, tuo pat metu turi būti nustatomi keli spermatozoidų kokybiniai parametrai, arba kelių tyrimo metodų rezultatai turi būti kombinuojami tikslu nustatyti vieno nuo kito nepriklausomų parametrų įtaką apvaisinimui.

Raktažodžiai: Spermos kokybe, spermatozoidų gyvybingumas, spermatozoidų apvaisinamosios galios nustatymas, bulių sperma.

Introduction. Artificial insemination (AI) is the first generation reproductive biotechnology that has made a profound contribution to the genetic improvement, particularly in dairy cattle. This impact would not have been possible without successful freezing of bull semen. The process of cryopreservation represents an artificial interruption of the progress of the spermatozoon towards post-ejaculation maturation and fertilization. The major disadvantage is that procedures involved in the cryopreservation process are harmful to spermatozoa and even the best preservation techniques to date result in about half of the sperm population that survive the freezing and thawing procedures. Change in temperature imposes changes on the composition and structure of various sperm plasma membrane domains [54], thereby modifying their function. As it has been demonstrated, the cryopreservation makes damage on sperm membranes, cytoskeleton, motile apparatus and nucleus, alter cell metabolism [for review see 39,85]. Moreover, freezing and subsequent thawing procedures render surviving spermatozoa different from spermatozoa before cryopreservation. They become very sensitive to any stresses by their environment *in vivo* as well as *in vitro* [39,85]. As a result, fertility from the AI with frozen-thawed semen is poorer than that obtained with fresh semen, which can be partially compensated by inseminating greater numbers of live spermatozoa. For this reason, proper assessment of the post-thaw quality of spermatozoa is of highest interest for AI industry, since it can provide insights upon the fertilising capacity of the cryopreserved spermatozoa. In order to be applied on a larger scale, the method has to be quick, relatively inexpensive and easily performed.

The aim of the present review is to summarize the current knowledge of the methods available to assess *in vitro* quality of frozen-thawed bovine spermatozoa and the relationship of the outcome of these tests to the fertility *in vivo*, after AI with that semen.

Definition of semen quality traits. It is generally accepted that there is a connection between the fertility of semen and its measurable properties. The ultimate goal of semen assessment is to find one or a few parameters to predict the fertilising ability of the semen. Many different methods have been evaluated throughout the last decades, but only few methods have been adopted for practical work. Most of these studies have used light microscopic evaluations of classical sperm parameters, including sperm concentration, motility, morphology and viability. Salisbury and VanDemark [70] proposed a model where both semen qualitative and quantitative parameters have to be considered in the equation for evaluation of male fertility potential. In fact, semen characteristics can be regarded as 'compensable' and 'uncompensable' in respect to the maximum likelihood of the conception attained by that semen. In some males, poor semen quality parameters can be compensated, in that fertility differences can be improved by adjusting the number of spermatozoa in the inseminate. There is also a category of subfertile males that cannot be brought to normal fertility levels by including additional sperm numbers in the insemination doses, thus rendering the semen traits of such males "uncompensable". Semen may contain both compensable and uncompensable sperm traits and each to a different degree [66]. In order to understand the nature of the impact that a given male has on reproduction, the problem should be approached by differentiating the essence of the deficiency at cellular and molecular levels. We must be able to identify sperm characteristics that prevent availability of sperm for fertilization (compensable traits) and identify sperm traits associated with incompetent fertilising sperm; that is, the sperm that can initiate, but not complete the fertilization process or sustain early embryogenesis (uncompensable traits). This subject, regarding compensable and uncompensable semen quality traits has been studied and reviewed by Saacke *et al.*, [66], den Daas [18].

Semen quality *in vivo* and *in vitro*. Sperm in the ejaculate are very heterogeneous in their attributes, including fertilizing capacity. Quality of spermatozoa that participate in the fertilization may not necessarily represent the whole ejaculate. Meaning that ejaculate which contains numerous subpopulations of sperm, individual numbers of which are prepared to fertilize an oocyte at different times but have very short life after initiation of their terminal attempt at fertilization. In the female, a very effective mechanism to control the number and the quality of spermatozoa that will finally compete in fertilization exists. The quantity of semen reaching the site of fertilization is reduced in relation to the number of spermatozoa inseminated. Also, compared to the inseminate, the quality of spermatozoa reaching the oviductal isthmus and ampullary isthmus is enriched in both - the viability and normal morphology [56,65]. Reduction of sperm numbers on their passage through the female genital tract is of major importance, since it represents the first mechanism to reduce or abolish the polyspermia. The final barrier against spermatozoa with certain traits participating in the fertilization is the vestments of the ovum – *zona pellucida*. Insemination with too low number of viable spermatozoa results in reduced pregnancy rates or no pregnancy at all. Sperm selection and reduction in numbers occurs no matter which sperm population is deposited – it applies both to the ejaculate and processed AI dose [57].

Semen evaluation techniques. From a biological point of view, only viable spermatozoa carrying intact genetic information are potentially fertile and therefore, most of the methods used so far focus on sperm viability and DNA integrity.

Motility and gross morphology estimated by light microscopy are by now most used parameters for semen quality assessment, especially in AI laboratories. Due to the simplicity of the evaluation technique motility is probably the most often used criterion for routine semen evaluation. Motility may be divided in quantitative motility (percentage of sperm cells with a progressive motility) and qualitative motility. The latter involves several different parameters, some of which are the speed of the moving sperm cells, altitude of head displacement and movement pattern (circular vs. linear movement, total distance vs. progression etc.). The accuracy in terms of repeatability of this test is, however, low, and very dependent on the ability of the operator (for review see [34]. Probably for this reason, reports on the relationship between subjectively-assessed sperm motility and fertility is inconsistent [48,52]. Computer assisted semen analysis (CASA) is objective method that gives extensive information about the kinetic property of the ejaculate based on measurements of the individual sperm cells. Using CASA, motility and movement characteristics of spermatozoa have been correlated to *in vivo* fertility [7,44,90]. Still, CASA-assessed motility is done on a rather limited number of spermatozoa and is predisposed to a certain degree of human bias.

Energetic exchange (ATP concentration). Energy for flagellar action is metabolized by the mitochondrial-dense mid-piece and these combine to propel the sperm head, carrying the male haplotype, to the ovum. Flagellar motion is the main energy-demanding process of viable spermatozoa [38], with most energy derived from the hydrolysis of adenine triphosphate (ATP) to adenine di- and monophosphates [58]. The concentration of ATP in semen is related to the number of motile spermatozoa [78], which means that measurement of ATP concentration may provide an objective method of estimating sperm viability. However results concerning correlations between sperm ATP concentrations and fertility have been contradictory [87,77,47].

Sperm morphology. The assessment of sperm concentration and morphology is based on the direct relation between the incidence of abnormal spermatozoa and the type of certain morphological defects with the *in vivo* fertility of the bull [75]. Accurate morphological screening of the ejaculates allows elimination of bulls with a potential low fertility, prior to the entrance of bulls a progeny testing program and the preservation of semen, thus contributing to a major savings for AI enterprises. There is undoubtedly a correlation between motility and

fertility as well as for morphology and fertility, provided there is a wide range of variation in the quality of the parameters assessed as well as fertility obtained with that semen. However, these correlations are reduced concomitantly with an increase in the lower limit set to accept an ejaculate for further processing. When the acceptable range for these parameters is narrow, motility and gross morphology only have limited value for separating ejaculates in respect to the expected fertility of the ejaculate.

Determination of sperm morphology by light microscopy by human eye suffers from subjectiveness, with different technicians often achieving different results on the same series of smears [2]. Increasingly, computer-aided sperm head morphometry analysis (ASMA) is being evaluated and applied in veterinary medicine [35,37,43 review by Boersma and Braun 5]. Computerized methods focus on evaluating the measurements that are able to quantify and classify sperm morphology correctly and offers repeatable and objective method of assessing bull sperm head morphometry within and between technicians. Measurements of sperm head variables such as area, length, width and perimeter have shown promise in the computerized analysis of bull sperm head morphology [36]. So far, information regarding sperm head morphometry and fertility of bull semen after AI is sparse. It has been observed, however, that certain parameters regarding texture of the digitized image of the sperm head correlates with *in vivo* bulls' fertility, expressed as non-return ranks [69].

Sperm chromatin structure. There are many factors leading to production of abnormally shaped spermatozoa. Various toxic agents and deviations in scrotal temperature affect different endpoints in the formation of morphologically abnormal sperm. Abnormal chromatin structure may lead to problems in packaging of sperm nuclear material possibly related to morphologically abnormal spermatozoa [68]. Significant correlations between abnormal sperm chromatin structure and morphometric shapes of bovine sperm nuclei have been observed [69]. In contrast, morphological shapes of spermatozoa determined by visual examination of the sperm in light microscopy and visually classifying the cells as either normal or abnormal were low or inconsistent with abnormal sperm chromatin structure [3].

Abnormal sperm chromatin structure may be assessed by Sperm Chromatin Structure Assay (SCSA), which defines abnormal chromatin structure as susceptibility of DNA to denaturation *in situ* [22,24]. In the SCSA, whole spermatozoa, or sonication-released nuclei are either heated or treated with HCl to denature DNA *in situ* and then stained with the metachromatic dye acridine orange (AO). Suspension of stained cells is run through flow cytometer and excited with blue laser light, AO intercalated into native, double stranded DNA fluoresces green, whereas AO associated with single-stranded DNA fluoresces red. Thus, a shift from green to red fluorescence corresponds to the DNA denaturation. The extent of DNA denaturation quantified by the SCSA has been shown to be sensitive indicator of male fertility potential in various animal species and also in human (for review see [25]). Compared to visual estimation of sperm morphological abnormalities, SCSA is a very sensitive test. Karabinus *et al*. [51] showed that chromatin structural changes in spermatozoa after scrotal insulation of Holstein bulls could be detected 3 days after heat stress, whereas light microscopic observations did not detect abnormalities until 11 days.

Assays of plasma membrane integrity. The sperm plasma membrane is the primary site where lesions occur during freezing-thawing of semen [39,54]. An intact and functionally active membrane is essential for the spermatozoon to sustain metabolism, undergo capacitation and acrosome reaction (AR) and, further, attach to and penetrate the oocyte zona pellucida (ZP) [45,9]. It has been recognized during the last several decades that one of the major features discriminating dead from live cells is a loss of the transport function and physical integrity of the plasma membrane. Based on this phenomenon, a plethora of assays of cell viability has been developed. For example, since the intact membrane of live cells excludes a variety of charged dyes, such as trypan blue or propidium iodide (PI), incubation with these dyes results in selective labeling of dead cells, while live cells show no or minimal dye uptake. A combination of supravital staining dyes such as trypan blue/giemsa, eosin/aniline blue and some other classical dyes are widely used for differential live/dead staining of fresh ejaculated spermatozoa. For light microscopic evaluation a relatively high concentration of the dye (in mg/ml) is required. At these concentrations, eosin, and many other dyes are toxic, which can lead to underestimation of the proportion of live cells [86]. Moreover, these dyes are used nearly exclusively to stain fresh ejaculated spermatozoa, because glycerol, a most extensively used cryoprotectant, interferes with the staining. Today assays for exclusion of fluorescent dyes are the most popular for evaluation integrity of frozen-thawed sperm plasmalemma followed by examination with fluorescence microscopy or flow cytometry [30,86]. Microscopic evaluation enables direct observation of compartmentalization of dyes in target organelles or membrane domains of spermatozoa, however flow cytometric analysis is based on quantitative analysis of events (cells) that emit certain wavelength light after being excited by a laser beam. Still the latter technique has proven advantageous over microscopic assessment, allowing the examination of thousands of cells over a much shorter period than it is needed for microscopy [80].

Vital staining of spermatozoa. Once damaged, spermatozoa are not able to reseal the compromised plasmalemma [86], and therefore, cannot maintain those ion and co-factor concentrations essential to sperm survival. The development of staining technology using fluorophores for nucleic acid, intracytoplasmic enzymes, or membrane potential has provided with new tools for assessing the functionality of frozen-thawed spermatozoa. Single fluorophores or in combinations can be used determining sperm membrane integrity.

The most commonly used classic nucleic acid stains are bisbenzimidazoles Hoechst 33258 (H258) and Hoechst 33342 (H342) and phenanthridines, such as ethidium bromide (EtBr), propidium iodide (PI) and ethidium homodimer (EthD-1). Bisbenzimide dyes are water-soluble minor groove binding AT-selective DNA stains and are relatively non-toxic. Both bisbenzimide dyes are cell-permeant, however Hoechst 33258 has slightly lower membrane permeability than Hoechst 33342, which made it applicable for studies evaluating sperm cell viability. Hoechst 33258 has extensively been used for supravital nuclear staining to mark dead cells in human [15,16,62,78], caprine [4], ovine, [84], bovine [14] and porcine [86] species. Hoechst dyes are excited with UV light (~350 nm) and have a very large Stokes shift with maximal emission at blue 460 nm that makes them suitable for multicolor labeling experiments. Most commonly these are used in combination with the dye that can be detected at a longer wavelength emission, such as fluorescein (FITC, 515 nm). There are, however, several drawbacks that make bisbenzimides less attractive for semen analysis. The dyes are semi-permeant to intact membranes, and have extremely broad emission spectra that overlap that of other dyes, and therefore, require several filters for microscopical evaluation of combined staining. Moreover, UV light needed for the excitation of the cell might be harmful for the cells analyzed as well as the examiner. In multicolor analysis it is usually preferable to use a nucleic acid stain as the longest wavelength dye. Phenanthridines are usually the dyes of choice due to their large Stokes shift (ex/em ~530 nm/ ~620 nm) so that their emission can be readily separated from that of other dyes, e.g. fluorescein [41]. These dyes are impermeant to cells with intact membranes, which makes them useful as dead cell indicators and as counterstains in multicolor fluorescent analyses. Propidium iodide binds to DNA as well as to RNA by intercalating between the bases with little or no sequence preference at stoichiometry of one dye per 4-5 base pairs of DNA, while ethidium homodimer binds strongly to dsDNA, ssDNA, RNA and oligonucleotides. One molecule of EthD-1 binds per four base pairs in dsDNA and the dyes intercalation is not sequence selective. Once the dye is bound to nucleic acids, its fluorescence is enhanced ~ 20 to 30-times. These dyes can be excited with mercury or xenon-arc lamps or with the 488 line of argon-ion laser, making it suitable for fluorescence microscopy, confocal laser scanning microscopy, flow cytometry and fluorometry.

Sperm quality post-thaw can also be assessed with various dual fluorescent markers, such as non-specific esterase substrates, e.g. carboxyfluorescein diacetate (CFDA) [40] or calcein acetomethyl ester (CAM) [46] used in combination with membrane-impermeant nucleic acid stains. Thus, two-colour fluorescence cell viability assay is based on the simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability - intracellular esterase activity and plasma membrane integrity. The combination of dyes determining biochemical and physical properties has been used to assess sperm viability of several species including that of bull, ram, boar [31,40] as well as stallion [10]. The live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by enzymatic cleavage of non-fluorescent cell-permeant acetomethyl esters (AMs) to the intensely fluorescent form. The polyanionic calcein, for example, is retained within live cells producing an intense green fluorescence (ex/em ~495 nm/ ~511 nm). Ethidium homodimer enters cells with damaged plasma membrane and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence of dead cells (ex/em ~495 nm/ ~635 nm). The latter dye is excluded by the intact plasma membrane of the live cells.

Recently, a new membrane permeant nuclear stain for living cells (SYBR-14; [30]) has been tested in combination with propidium iodide to assess bovine sperm viability. This combination of dyes has several advantages over the above-mentioned dyes, among which is higher speed of labeling cells, higher stability while labeled. Both dyes label DNA circumventing ambiguity that may rise from targeting separate cellular components. In addition, both dyes fluoresce at excitation with visible light, thus avoiding harmful effects of UV exposure. Under illumination, SYBR-14 loaded viable sperm cells fluoresce bright green, whilst damaged cells take-up PI and fluoresce red. The relationship between SYBR-14/PI-stained and flow cytometrically-assessed semen viability post-thaw and *in vivo* fertility obtained with that semen is controversial. Some trials resulted in no significant correlations with field fertility [30], whereas promising results have been obtained in other studies [11].

Osmotic resistance test (hypo-osmotic swelling test). Functional integrity of the plasma membrane can also be evaluated by measuring the resistance of sperm membranes to swelling in a hypo-osmotic medium. This much simpler method is based on the ability of the membranes to allow passage of water in order to establish equilibrium between the fluid compartment within the spermatozoon and the external surroundings [20,21]. It was suggested that the ability of spermatozoa to swell in the presence of hypo-osmotic medium reflects normal water transport across the sperm membrane, which is a sign of normal membrane integrity and functional activity [45]. The functionally active spermatozoa exposed to a hypo-osmotic stress swell due to the influx of water and subsequently increase in volume to establish the equilibrium between the cytosol and the extracellular milieu. Spermatozoa with compromised or inactive membranes are unable to regulate water influx and remain not swollen. Thus, hypo-osmotic swelling tests may be useful in assessing changes in the sperm membrane functional integrity during freezing thawing procedures [64]. Attempts have been made to correlate sperm plasma membrane integrity to fertility, but great variation is seen between studies and methods used [13,48,64].

Membrane destabilization. The destabilization of sperm membranes can be evaluated by tracking the distribution of Ca^{2+} in spermatozoa. There are two basic

classes of Ca^{2+} indicators. The main example of the first class is antibiotic chlortetracycline (CTC), which accumulates in organelles containing high concentrations of Ca^{2+}. The second class consists of molecules that reside in aqueous compartments such as cytosol and change their spectra when they bind to Ca^{2+}. Indicators of cytosolic free Ca^{2+} concentrations are quin-2, fura-2, indo-1 and fluo-3 [83]. Neutral uncomplexed CTC can easily cross the membranes where it ionizes to an anion and chelates Ca^{2+}. The latter complex binds preferentially to hydrophobic site, such as membrane and shows increased fluorescence as a result. The extent of the binding to membranes depends on the surface-to-volume ratio of the vesicle and the properties of the lipid. Due to the compartmentalization of the plasma membrane of spermatozoa, several distinct staining patterns can be evaluated which are associated with a functional status of spermatozoon [28]. A number of investigators found that human [17], mouse [29], bull [12], boar [60] and several other species exhibit similar patterns of CTC staining. The three patterns are evaluated, where Pattern F, with uniform fluorescence on the head, indicates uncapacitated, acrosome intact spermatozoa; Pattern B, with a fluorescence-free band on the post acrosomal region, indicates capacitated, acrosome intact spermatozoa; and pattern AR with uniformly fluorescence-free head and with a fluorescence band on the equatorial region, indicates acrosome reaction. Several studies [48,81] were performed aiming to find the key to the hypothesis that freezing/thawing destabilizes sperm membranes and the extent of destabilization is inversely related to the fertility values. It has been confirmed, that fertility of the bulls tested to be negatively related to the incidence of destabilized (pattern F) spermatozoa of frozen-thawed semen samples.

Function of different organelles. There are several assays of cell viability based on the functional tests of cell organelles. Thus, for example, the cationic dye rhodamine 123 (Rh123) accumulates in mitochondria of live cells due to mitochondrial transmembrane potential [50]. Dye uptake and equilibration of rhodamine 123 is rapid (few minutes), not temperature-dependent and not retained by cells when they are washed. This dye was historically applied to spermatozoa in combination with ethidium bromide [23]. Cell incubation with Rh123 results in green staining over the mitochondrial sheath in live cells while dead cells are identified with ethidium bromide, or other supravital fluorescent marker. Rh123 is not a suitable probe in the experiments in which cells must be treated with aldehyde fixatives or other agents that affect the energetic state of the mitochondria. This problem has been overcome by Molecular Probes, that developed MitoTracker probes - mitochondrion-selective stains that are concentrated by active mitochondria and well retained during cell fixation. While Rh123 and MitoTracker probes indicate the presence of membrane potential, another dye JC-1 has the unique property to differentiate between high and low membrane potentials. Mitochondria with high membrane potential fluoresce red-orange, while those with low to medium membrane potential fluoresce green. These dyes were used to study the effect of cryopreservation on bovine sperm organelle function and viability [79]. They showed that fluorometric measurement of mitochondrial function after thawing correlates with SYBR-14-assessed sperm viability and with microscopic assessment of motility.

Acrosomal status. There are two basic classes of fluorescent probes of acrosomal status: those that can be used on living, non-permeabilized cells, and those that detect intracellular acrosome-associated material and consequently require the cell to be permeabilized before labeling. In the first group there are chlortetracycline and antibodies that bind to externally exposed antigens, and in the second group - lectins and antibodies that bind to intracellular, acrosomal antigens. Lectins are the most accessible agents of this group. A variety of lectins that have specific affinities for particular saccharide molecules of the acrosomal matrix or the outer acrosomal membrane have been used to assess sperm acrosomal status. There is a large variety of lectins available on the market. Some of them are toxic (*Ricinus communis* agglutinin) and should be handled with care. The most popular ones are fluorescein isothyocyanate (FITC)-conjugated *Pisum sativum* agglutinin from the edible pea and *Arachis hypogaea* agglutinin from peanut (PNA). These lectins were used to assess acrosomal status of spermatozoa from humans [16], horses [10], pigs [61], bulls [14], rams [74] and some other species. A fluorescent nuclear stain as H258, PI or EthD-1 is usually included as a supravital stain in the lectin labeling procedure [10,16,61]. The two fluorophores can be visualized either simultaneously with a single, or using separate filters for each of the fluorophores. Intact acrosome post thawing is crucial for fertilization. The acrosome status in frozen-thawed bull spermatozoa has also been related to fertility [67]. The effect of cryopreservation on various sperm organelle function has been studied [32,79]. However, simultaneous evaluation of various organelle functions post-thaw seems most promising in order to assess post-thaw sperm quality [33].

Sperm-oocyte interaction. For experimental purposes, various sophisticated methods for semen assessment may be used. Various steps of fertilization procedure and rate of embryo development have been used in evaluation of semen quality, and may give valuable information on one part of the fertilizing ability of the semen from the sire in question. Successful fertilization is known to involve several sequential steps. These are 1) sperm capacitation in the female genital tract, 2) binding of the capacitated sperm to the zona pellucida (ZP), 3) induction of sperm acrosome reaction, 4) penetration of ZP, and 5) fusion of the spermatozoon with the vitelline membrane of the oocyte. An essential step in the process of fertilization is the recognition and binding between spermatozoa and the oocyte's extracellular coat, the zona pellucida. Since the cumulus cell layer surrounding zona pellucida in bovine is lost at the ovulation and during the fimbral and ampullar transit in the oviduct [59] ZP is the only mechanical obstacle for spermatozoa on their way to fertilize the oocyte.

Capacitated spermatozoon interacts with the ZP in a highly precise manner. Extensive studies suggest that binding to the ZP is a two step process. First, spermatozoa loosely and reversibly adhere to the oocyte's extracellular coat, and the second stage is strong and irreversible binding. Many sperm can bind to the surface of ZP, and enter the ZP, but only one will penetrate the ZP and fuse with the oocyte's plasma membrane. The intrinsic feature of spermatozoa to bind to the homologous ZP has been used in order to predict the fertilising potential of semen samples in a zona-binding assay (ZBA; [8]). In fact zona binding assay can be approached either by using intact oocytes [26] or bisected zonae pellucidae (hemizona binding assay HZA); [27] accessory spermatozoa, i.e. those entrapped in the ZP of the oocyte/embryo [66], or solubilized bovine ZP [82]. In a zona-binding assay, large numbers of oocytes are required due to the considering variation in binding sites among oocytes. Therefore a large number of oocytes is crucial in the ZBA trying to find a relationship between the outcome of this assay and the fertility of bulls [27,90]. In HZA, oocytes are surgically bisected and used to assess sperm binding to the homologous halves. In this respect, it enables comparison of sperm binding between fertile controls and test samples [27]. It has been shown that HZA indices of the semen samples and 56-day NR rates were significantly correlated [27]. Relationship between accessory sperm and fertilization status/embryo quality has also been confirmed [19,66]. These studies, however, are based on relatively low numbers of ova/embryos evaluated that are usually not sufficient for statistical significance [63]. Considering the fact that both ZBA and HZA are laborious procedures, highly dependent on skills of the operator and are based on relatively low numbers of oocytes/zonae evaluated, incubation of spermatozoa with solubilized biotinylated ZP proteins appears to be most promising. It surpasses the variability in the ZP-binding sites among the oocytes and makes it possible to examine large numbers of spermatozoa in a flow cytometer.

The outcome of the *in vitro* fertilization system differs among the bulls [73,89]. In some studies, significant correlations between embryo cleavage and field fertility have been demonstrated [72], but not in others [71]. Relations between the proportions of fertilized oocytes and field fertility are also variable [6,55,88]. The individual bull has been shown to constitute a considerable source of variation in the proportion of oocytes fertilized and their subsequent embryonic development [72,88]. The donor-specific effect on *in vitro* fertilization could be minimized by adjusting the number of spermatozoa in the insemination dose [42]. Still, cleavage rate of bovine oocytes *in vitro* has been shown to be affected by bull effect, but not by sperm concentration [53]. In vitro fertilization assays, including sperm-ZP binding, sperm penetration and various steps of IVF are promising, however they are very expensive, highly dependent on reproducibility of the laboratory and skills of the operator (H. Rodriguez-Martinez, personal communication).

Analysis of multiple sperm attributes. As mentioned above, spermatozoa need to possess certain attributes in order to achieve fertilization [1]. It is unlikely that a process so complex as fertilization could be predetermined by a single parameter of sperm quality. It is agreed that spermatozoon is multifunctional cell that must possess a large number of attributes that make it potentially fertile. Any spermatozoon lacking any of these attributes or not possessing enough of an attribute will be infertile. Because sperm require many attributes, an assay, measuring a single attribute will fail to detect defective spermatozoa, and will have low power to predict which samples are likely to possess low, or high fertility. So far, the analysis of a single sperm viability parameter cannot predict the outcome of a process as complex as fertilization. It has been shown that combination of several post-thaw sperm quality attributes, as compared to any single sperm quality trait, can explain more variation in fertility between the bulls [49]. For this reason, in order to increase the predictive power of the test, simultaneous analysis of multiple sperm attributes [33], or outcomes of several laboratory assessments can be combined statistically to look for the overall effect of several independent sperm parameters [48,87,90].

Summary Although many efforts have been made, so far there are no currently available methods, or sperm assessment techniques that could accurately predict sperm fertilizing potential. If successful, such a method could contribute to improved herd fertility. It could also result in major savings for AI enterprises, for the bulls with inferior fertility could be selected and culled prior entering the fertility and progeny testing programmes. There are still many unanswered questions regarding the likely, not yet established links between sperm quality and fertility, i.e. the unknown traits of sperm quality that might be essential for fertilization. Most promising tests available are sperm chromatin structure assay (SCSA) and sperm viability assessed by flow cytometry, however the present review has shown that today we still do not have single *in vitro* sperm quality assessment method that can accurately predict sperm fertilizing potential. In order to increase the predictive power of the test, assessment of several sperm attributes must be combined in a simultaneous analysis, or outcomes of several laboratory assessments must be combined statistically to look for the overall effect of several independent sperm parameters.

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