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NEW METHODS OF SEMEN ANALYSIS BY CASA

C. SOLER[1, 2], A. VALVERDE[1, 3], D. BOMPART[2], S. FEREIDOUNFAR[2], M. SANCHO[2], J.L. YÁNIZ[4], A. GARCÍA-MOLINA[1, 2], Yu.A. KORNEENKO-ZHILYAEV[5]

[1]*Departament de Biologia Cellular, Biologia Funcional i Antropologia Fisica, Universitat de València,* 46100 Burjassot, Spain;
[2]*Departament d'Investigaciy Biològica de Proiser R+D, S.L., Parc Científic de la Universitat de València,* 46980 Paterna, Spain;
[3]*Technological Institute of Costa Rica, San Carlos Campus, School of Agronomy,* 223-21001 Alajuela, Costa Rica;
[4]*Departamento de Producciyn Animal y Ciencia de los Alimentos, Escuela Politécnica Superior de Huesca, Universidad de Zaragoza, Ctra. Cuarte S/N 22071 Huesca, Spain,* e-mail: jyaniz@unizar.es (corresponding author);
[5]*OOO «Venera-Vet»,* 7A, ul. Kashtanovaya, pos. Bykovo, Podolsk Region, Moscow Province, 142143 Russia, e-mail contact@venera-vet.ru
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Abstract

Semen analysis constitutes the base for the establishment of fertility of a male. In addition, the number of insemination doses to produce from an ejaculate depends on the defined sperm characteristics. As soon as image analysis techniques were applied to biological problems the andrology was one of the first fields benefited both in the evaluation of genital track and sperm function. The improvement of image analysis expands possibilities of using developed systems in medical practice and animal husbandry. In ISAS®PBos («PROISER — Projectes i Serveis R+D S.L.», Spain) the semen analysis done includes concentration, motility and morphology. The systems with optimal rate of frame capture and transmission at images processing are developed for a two-dimensional analysis of spermatozoa motility in different species with regard to the spermatozoa sizes. ISAS®PBos calculates the percentage of morpho-abnormalities analysing the presence of cytoplasmic droplets and coiled tails base on the images used for motility analysis**,** and allows calculation of the optimal number of doses to produce from a particular ejaculate**.** Breed-dependent morphological diversity of spermatozoa found in different species, and a disclosure of structured subpolations of spermatozoa in the ejaculate, lead reproductive biology to the next level and open new prospects for the practice of animal breeding. In the study of spermatozoa at the subpopulation level the multivariate statistics which is based on an analysis of the principal components is applicable. In statistical estimation and mathematical modeling, it is proposed to use the Bayesian approach, on the basis of which a mathematical toolkit for estimating sperm quality will be developed in the near future. Essentially, the shortcomings of the early methods used for semen analysis are due to modifications of real motility of germ cells in counting chambers and the real shape and size of the spermatozoa under dehydration, fixation, staining and mounting. ISAS® 3DTrack and Trumorph® for estimation of sperm motility and morphology, respectively, avoid the limitations. ISAS® 3DTrack device, a lensless laser microscope, allows the analysis in a depth around 100 μm. Moreover, the analysis of the correspondent hologram allows the analysis of track in three dimensions what is also a big novelty. Trumorph® technique offers the maximum projection of the cells making it possible to obtain images of high resolution and definition of cells components in a wide range of species, including bull. ISAS® 3Fun with ISAS®3Fun kit and the correspondent software for automatic analysis is a new method enables a clear distinction of spermatozoa with intact plasmalemma and acrosome which are essential for sperm function**.**

Keywords: spermatozoa, motility, morphology, fertility, subpopulations, computer-assisted sperm analysis, statistical analysis

Semen analysis constitutes the base for the establishment of fertility ability of a male. In addition, the number of insemination doses to produce from an ejaculate depends on the defined sperm characteristics.

Traditionally, the semen evaluation was done by an experienced technician, analysing each sample under the microscope, basically for concentration and motility. When possible, concentration was estimated by using a good counting chamber (Neubauer, Bürker etc), when not it is just estimated

in the same time motility is done. For this the most common procedure is to make an approximation to 5 % value, not counting cell by cell but like a subjective approach. Regarding morphology it is not possible to do in a routine daily work and it is applied basically for control quality programs [1] but not for insemination seminal doses production.

In a traditional way, subjective motility and concentration were the most used parameters [2], while morphology assessment had a secondary place because it takes much more time to be performed, the definition of a universal pattern of classification is more difficult [3, 4], and all the process lacks convenient precision (5, 6 Nevertheless, Morphological characteristics of spermatozoa are genetically determined [7], relative to the spermatogenesis and epididymal maturation processes (8), and so being more informative than the motility that is much more affected by environmental factor [9].

As soon as image analysis techniques were applied to biological problems one of the first fields benefited was the Andrology, both into the evaluation of genital track and sperm function [10-13]. The entrance in the personal computerization era implies a fast appearance of computerized image analysis processes to evaluate concentration [14], motility [15, 16], and morphology [17, 18] including electron microscopy images [19]. Successful results of the predictive value of automated CASA [Computerized Assisted Semen Analysis] systems parameters [20, 21] derivates in the appearance of multiple commercial brands. The basic components of a CASa system included from the beginning, the microscope [with good phase contrast, if possible negative], the video-camera, the computer and the software [Fig. 1].

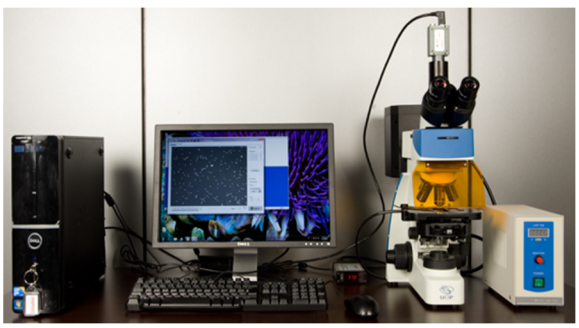

Fig. 1. Components of a CASA system.

The first generation of CASA systems were based on poor informatics tools, requiring a lot of computation time and resulting too much expensive. These facts limited their use to basic research and led to the widespread idea that its use is not recommended to routine human clinical or livestock production. One of the aims of PROISER (Projectes i Serveis R+D S.L., Spain) was the overcoming of this situation with the design of the ISAS®P series, originally devoted on boar seminal doses production but amplified to other species, including bull.

Centering our attention on ISAS®PBos, the semen analysis done includes concentration, motility and morphology. For concentration analysis the use of well-defined counting chambers is needed and, for this, we have developed specific chambers, both reusable and disposable [Fig. 2]. In the market it is possible to dispose of different designs but not all fit a good distribution of cells. In disposable chambers, this is due to rheologic and capillary forces and de design following parallel and thin way results in the best distribution [22-24]. When reusable chambers are used, to optimize the design and glass composition is also needed. Superficial tension of the glass due to surface ions implies a bad distribution related with the time of cover glass deposition [25].

These questions also affect the motility analysis, taking into account that

the cells are forced to move in a plane. For a cell having about 70 μm in length, movement in a space of only 10 μm of width produces a clear motility artifact. Depending on the species, the introduction of chambers of 20 μm reduces this effect, even it doesn't disappear (see later about for the analysis on high depth chambers.).

Fig. 2. Examples of reusable and disposable counting chambers.

Another significant aspect related with the quality of motility analysis refers to frame rate of sequence capture. Motility is defined by several kinematic parameters, some of which are very sensitive to the lapse between images capture. Particularly the VCL [curvilinear velocity] increases following an exponential curve [A. Valverde et al., unpublished results]. So only when the frame rate is close to the asymptotic value the kinematic parameters are really representative, being calculated for bull on 160 fps [26].

Now we are working in the establishment, species by species of the best conditions for the bidimensional motility analysis, it means the best chamber depth and characteristics, the optimal frame rate, the time of charge and analysis etc.

Regarding morphology analysis, the classical approach implied the use of different staining techniques (27). This process includes steps of dehydration, fixation and staining, all of them modifying the real structure and dimensions of the cell and this producing different level of artifacts when observing the cells (28). ISAS®PBos calculates the percentage of morpho-abnormalities analysing the presence of cytoplasmic droplets and coiled tails base on the images used for motility analysis, it means cells on suspension and using negative phase contrast. At his magnification (10×) it is not possible to obtain more detailed information about sperm morphology/morphometry. Fluorescence techniques could be also applied but, in this using this technique, only some head components could be measured (29). In the case of ISAS®PBos the offered report allows to the calculation of the optimal number of doses to produce from a particular ejaculate based, alternatively, on the quality of the analysed sample or on the historical results of the last five post-thawed analysis of samples from the considered animal.

Semen cryopreservation limits. The most important limit to the use of semen doses for AI (artificial insemination) is the time for the maintenance of the doses. In the beginning of the AI both males and females were placed in the same farm, in fact this is even now the common practice in some species like fox. But the evolution of the use of AI derivates to specific farms for each purpose, it means males for seminal doses production, females for calving and other for the growth of the animals. In this case, the possibility to preserve the doses results fundamental. The refrigeration of the samples was the first approach, and this continues being the most used in species like boar or rabbit. But the distance at which the samples can be transported and the time for its use is short, limiting the possible interchange of genetics in long distances. The introduction of freezing techniques allowed to solve this problem producing samples that, theoretically, could be transported around the world and for no limited time. This combination of techniques of freezing/thawing process is the most common now in human and bull species and a lot of different protocols were

developed in the last decades.

But this technique causes different alterations in the semen quality, like increase of oxidative stress conducing to changes in motility, DNA fragmentation, acrosome reaction etc [30]. The evaluation of these changes is very difficult if not impossible following manual evaluation of semen quality and thus, the use of CASA technology is particularly interesting for this determinations [31, 32].

Effect of genistein on bull sperm after freezing and thawing

Group	Viability	Sperm DNA fragmentation
Control	61.1±1.3[a]	6.8±0.7[a]
Genistein 30 min	51.6±1.3[b]	5.3±0.6[ab]
Genestein 60 min	50.2±1.4[b]	4.7±0.6[b]

In an attempt to improve the problem of oxidative stress on spermatozoa during cryopreservation the effect of addition of genistein was evaluated on bull semen [33]. We observed that the percentage of cells with high level of DNA fragmentation was lower after treatment with the antioxidant genistein (Table), but this reduction was in parallel to a decrease in motility and vitality, this treatment only seems to be indicated to samples with high DNA fragmentation levels or for ICSI procedure.

In other paper we asked recently, what we speak when we refer to dog semen (C. Soler et al., in press). In the case of dog we observed significant differences between the morphometric characteristics of spermatozoa from different breeds. It seems to be obvious that this differences must exist when we consider breeds so much different like British bulldog, chihuahua and German shepherd dod but until now must of the scientific literature has mixed different breed in the same work just like a dog. The same question could be also applicable to bull, where a lot of well-defined breeds, even inside *Bos taurus* were defined, but more if we included also the *Bos indicus*. In fact some studies have compared both species and morphometrical differences have been observed [34]. In the present we are performing one experiment comparing four different breeds trying to advance on this interesting topic that has two kinds of implications, just from the biological point of view and to define new approaches to how manage the ejaculates defining specific protocols for each breed

Looking to the future. Seminal subpopulations structure. In spite of the advances achieved in the last decades, the predictive capacity of the *in vitro* analysis on potential fertility of semen remains still limited [35, 36]. This limitation of the "classical" seminal parameters although may be improved using combined statistical analyses of various sperm quality parameters [37, 38]. As consequence, the complexity of the semen analysis has progressively been increased with the hope of improving fertility predictions [39, 40]. However, some of these quality parameters are highly correlated, with merely increasing the number of analytical tests not always improve the predictive ability of the spermiogram [28, 38, 41].

The application of high mathematics analysis tools to the study of semen characteristics has revealed the existence of sperm subpopulations in the ejaculate. This has been observed in the last decade in a great number of species and conditions, even its significance is not yet clear (42-47). The application of high mathematics analysis tools to the study of semen characteristics has revealed the existence of sperm subpopulations in the ejaculate. This has been observed in the last decade in a great number of species and conditions, even its significance is not yet clear (28). How this could happen is the most exiting research we can approach in the next decades until to achieve a good comprehension of the phenomena.

In a recent paper we have compared the "classical and advanced" mathematical approaches to the problem to evaluate the seminal characteristics. It was put on evidence that the Classical approaches, based on ANOVA or even

MANOVA, are no good enough to define the sperm population, being necessary the introduction of the use of multivariate statistics based on principal component analysis to define the subpopulation structure as a better definition of the real semen cell composition. In addition, the presence of differences between different ejaculates of the same animal indicates that the subpopulation structure, even having a genetic basis, could be influenced by environmental (external and internal) factors (48). Looking to this problem, the recent introduction of Bayesian approaches to the evaluation of semen quality is offering a new tool that will be developed in the next time (49-51).

New tools for new approaches. In any case, the analysis done until now, both for motility and morphology present an additional limitation, referred to the fact they must be considered like artifacts. The motility analysis had been requiring the observation using optical microscopy and this means that the depth of view is very low, and this implies that counting chambers depth cannot be higher than 20 μm. Taking into account that the length of sperm, depending on the species, could be considered higher than 50 μm it implies that the movement of the spermatozoa inside the chambers is non "natural" being constricted and so modified in its real pathway. In reference to morphology, all the techniques used until now implied the modification of the real shape and size of the spermatozoa, including processes of dehydration, fixation, staining and mounting (52).

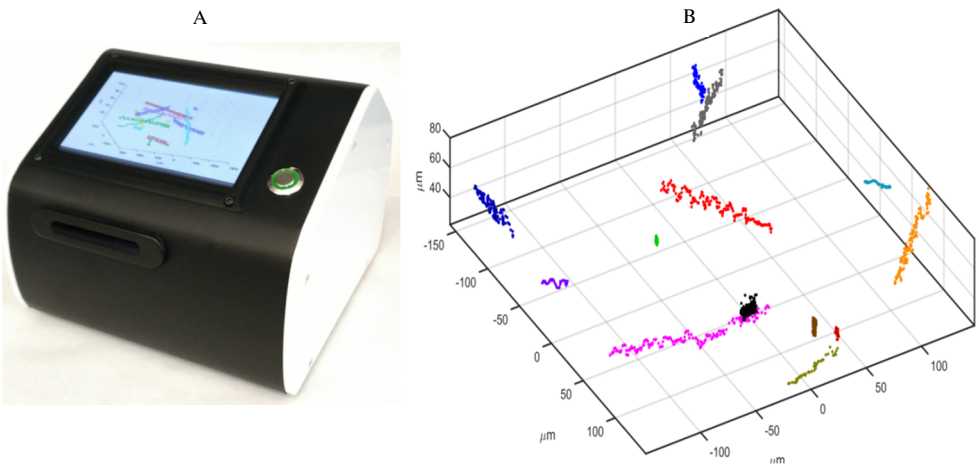

Fig. 3. ISAS®3DTrack device (A) **Tridimensional representation of boar sperm motility by using ISAS®3DTrack** (B).

To overcome this artifactual production, our team has developed two new facilities: the ISAS®3DTrack (Fig. 3, A, B) and Trumorph® (Fig. 4, A, B) for both motility and morphology analysis, respectively.

As we pointed out before, it is needed to significantly increment the depth in which cells must move during the analysis and for this we have developed the ISAS3DTrack, a lensless laser microscope that allows the analysis in a depth around 100 μm. Moreover, the analysis of the correspondent hologram allows the analysis of track in three dimensions what is also a big novelty. In essence, the proposed technology becomes in the basis architecture of a reduced cost, portable and compact system design of lensless holographic microscope with an illumination/detection scheme based on wavelength multiplexing, working with single hologram acquisition and using a fast convergence algorithm for image processing. Altogether, the proposed imaging platform allows high-resolution (μm range) phase-retrieved (twin image elimination) quantitative phase imaging of dynamic events (video rate recording speed) (M. Sanz et al.,

2015, 2017).

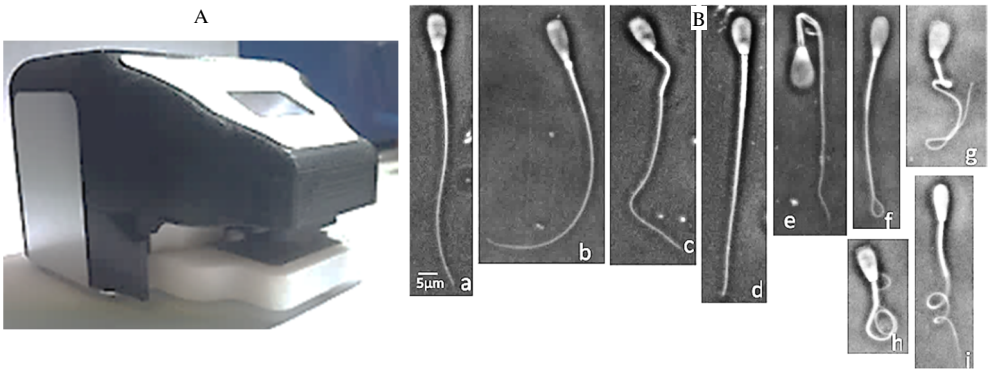

Fig. 4. Trumorph® device (A) **and different morphologies of bull sperm observed using Trumorph® technique** (B; a, b, c, d, e, f, g, h, i)**.** Image (a) represents what is considered a normal cell.

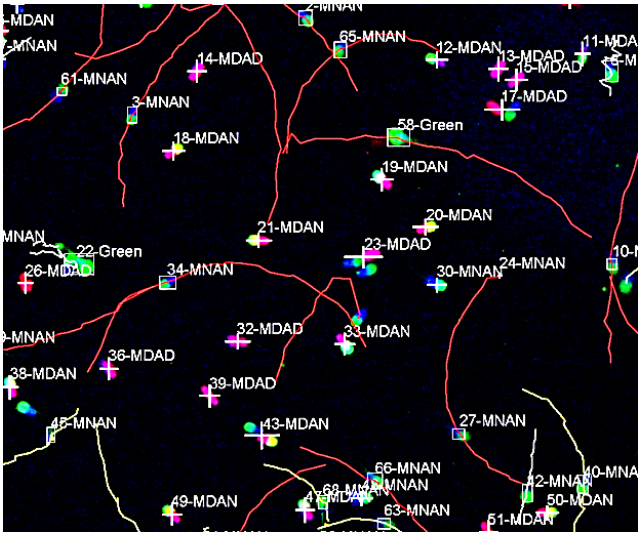

Fig. 5. Staining patterns of boar spermatozoa using the ISAS® 3Fun kit. Scale bar = 5 µm (left) **and computer-assisted sperm analysis of the sperm trajectories after staining with the ISAS® 3Fun kit (right).**

On the other hand, the Trumorph® technique (Fig. 5, A) is based on application of heating to immobilise the cells and a little pressure to spread the seminal plasma offering the maximum projection of the cells. With the use of objectives of 40×, negative phase contrast, it is possible to obtain images of high resolution and definition of cells components in a wide range of species, including bull (Fig. 5, B) (46, 53, 54).

Finally, fluorescent markers and flow cytometry allow the assessment of numerous structural and functional characteristics of spermatozoa in large populations (55, 56), but this technique is complex and too much expensive to be applied in a routine work for doses calculation. With the aim to avoid these limitations we have developed the ISAS®3Fun kit and the correspondent software for automatic analysis. This new method enables a clear distinction of spermatozoa with intact plasmalemma and acrosome, which are essential for sperm function (57), no altering the motility and so offering the possibility to analyse these three parameters simultaneously and cell by cell (see Fig. 5).

So, our proposal for the close future must to combine, using advanced mathematical analysis, as much as possible seminal parameters, including both the “classical” and the new ones to define a new mathematical pathway for predicting fertility. This effort must to start by the definition of the optimal conditions for the use of any of the tools considered. Some work on this was done but it must be completed. The analysis of the best possible data will rend a new way

to approach both the best comprehension of the subpopulations structure meaning from the evolutive and physiological points of view and the improvement in the seminal evaluation and seminal doses production for assisted reproduction techniques.

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