Theriogenology 86 (2016) 1111–1131

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Development of a new fertility prediction model for stallion semen, including flow cytometry

I. Barrier Battut [a,*], A. Kempfer [a], J. Becker [a], L. Lebailly [a], S. Camugli [b], L. Chevrier [b]

[a] I.F.C.E, E.S.C.E., la Jumenterie du Pin, Exmes, France
[b] R&D Department, IMV Technologies, Saint Ouen sur Iton, France

ARTICLE INFO

Article history:
Received 17 December 2015
Received in revised form 4 March 2016
Accepted 1 April 2016

Keywords:
Stallion
Fertility
Spermatozoon
Flow cytometry

ABSTRACT

Several laboratories routinely use flow cytometry to evaluate stallion semen quality. However, objective and practical tools for the on-field interpretation of data concerning fertilizing potential are scarce. A panel of nine tests, evaluating a large number of compartments or functions of the spermatozoa: motility, morphology, viability, mitochondrial activity, oxidation level, acrosome integrity, DNA integrity, "organization" of the plasma membrane, and hypoosmotic resistance, was applied to a population of 43 stallions, 33 of which showing widely differing fertilities (19%–84% pregnancy rate per cycle [PRC]). Analyses were performed either within 2 hours after semen collection or after 24-hour storage at 4 °C in INRA96 extender, on three to six ejaculates for each stallion. The aim was to provide data on the distribution of values among said population, showing within-stallion and between-stallion variability, and to determine whether appropriate combinations of tests could evaluate the fertilizing potential of each stallion. Within-stallion repeatability, defined as intrastallion correlation (r = between-stallion variance/total variance) ranged between 0.29 and 0.84 for "conventional" variables (viability, morphology, and motility), and between 0.15 and 0.81 for "cytometric" variables. Those data suggested that analyzing six ejaculates would be adequate to characterize a stallion. For most variables, except those related to DNA integrity and some motility variables, results differed significantly between immediately performed analyses and analyses performed after 24 hours at 4 °C. Two "best-fit" combinations of variables were determined. Factorial discriminant analysis using a first combination of seven variables, including the polarization of mitochondria, acrosome integrity, DNA integrity, and hypoosmotic resistance, permitted exact determination of the fertility group for each stallion: fertile, that is, PRC higher than 55%; intermediate, that is, 45% < PRC less than 55%; or subfertile, that is, PRC less than 45%. Linear regression using another combination of 20 variables, including motility, viability, oxidation level, acrosome integrity, DNA integrity, and hypoosmotic resistance, accounted for 94.2% of the variability regarding fertility and was used to calculate a prediction of the PRC with a mean standard deviation of 3.1. The difference between the observed fertility and the calculated value ranged from −4.2 to 5.0. In conclusion, this study enabled to determine a new protocol for the evaluation of stallion semen, combining microscopical observation, computer-assisted motility analysis and flow cytometry, and providing a high level of fertility prediction.

* Corresponding author. Tel.: +33-2-33-12-12-04; fax +33-2-33-35-58-93.
E-mail address: isabelle.barrier@ifce.fr (I. Barrier Battut).

0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2016.04.001

1. Introduction

The equine species is one of the animal species for which selection is not primarily on the basis of fertility performance. This lack of selection on a reproductive index has resulted in widely variable fertility in the equine population. However, predicting fertility, or at least identifying subfertility in stallions before the breeding season, is extremely important for the equine industry. Conventional laboratory tests for the assessment of sperm quality have been developed for decades, including the microscopic evaluation of sperm morphology, motility (initially evaluated by a skilled technician under a microscope, though now more frequently using computer-assisted semen analysis) and the longevity of motility after storage [1–4]. The limitations of said standard "breeding soundness examination" in predicting fertility are well known, including the inability to identify approx. Twenty percent of all infertile stallions and the risk of rejecting approx. Twenty-four percent of all fertile stallions due to "low-semen quality" [3].

For that reason, additional tests have been developed in some laboratories, including the evaluation of DNA integrity, acrosome integrity, mitochondrial activity, hypoosmotic resistance, and so forth. (See review by Colenbrander et al. [5], Varner et al. [6]).

The sperm chromatin structure assay was introduced by Evenson in 1980 [7] as a method to determine the susceptibility of sperm DNA to denaturation. This assay has been shown to provide additional information, independent from motility and morphology (see review by Love [8], Varner et al. [6]).

Acrosome integrity can be evaluated in the stallion using appropriate fluorescently labeled lectins, such as fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) or *Arachis hypogea* agglutinin (FITC-PNA; see review by Neild et al. [9]). Furthermore, the response to an induction of acrosome reaction via treatment with progesterone [10] or calcium ionophore A23187 [11,12] has been shown to be related to fertility in the stallion. The presence of seminal plasma was reported to enhance the response to an induction of acrosome reaction using ionophore A23187 [13].

The phospholipid disorder ("scrambling") in the plasma membrane that occurs in the early stage of capacitation allows intercalation of the fluorescent stain, merocyanine 540, which can be detected by flow cytometry in stallion spermatozoa [14]. This test could be used to evaluate premature capacitation, or at least membrane damage, which could occur during sperm storage.

The hypoosmotic swelling test (HOS test) has been used to evaluate the functional integrity of the sperm plasma membrane [15] and has been applied to equine semen, thereby showing a relation to fertility [9,16]. Spermatozoa exposed to hypoosmotic conditions, usually 50 mOsm/kg, exhibit a typical tail curling, known as "swelling", in response to the influx of water and the expansion of the plasma membrane. Damaged sperm membranes do not allow such water movements, and therefore do not show tail "swelling". Spermatozoal assessment can be performed microscopically. More recently, a new approach of that test has been developed, using multihypotonic steps and the evaluation of membrane integrity with propidium iodide (PI), followed by flow cytometry [17,18]. The hypoosmotic resistance of spermatozoa takes the form a curve that can be modelized, thereby enabling to calculate the critical osmolality for each individual, defined as the osmolality at which 50% of the spermatozoa remain alive. Such critical osmolality has been shown to be related to fertility in the boar [19].

The mitochondrial energy metabolism is a key factor in providing energy for motility and fertilization. The mitochondrial function can be assessed using stains, which preferentially link onto the depolarized membrane, or which fluoresce differently according to the mitochondrial membrane potential (high or low). Such stains have been successfully used in the stallion [20–22].

High levels of reactive oxygen species (ROS) may be detrimental to stallion sperm survival during storage, and the percentage of dead hydrogen peroxide-positive sperm was shown to be negatively related to the foaling rate [23]. However, the response to an exposure to oxidative agents, such as H_2O_2, could also provide valuable information because mild peroxidation seems to be involved in the capacitation process (see review by Aurich et al. [24]). For bull spermatozoa, the oxidation level in live cells is negatively correlated with fertility, whereas oxidation after exposure to H_2O_2 is positively correlated to fertility [25].

The improvement in flow cytometric technology, now being more user-friendly, has allowed the development of a large number of tests for the on-field evaluation of sperm quality parameters. It is now obvious that no single test would be able to predict fertility or explain infertility [21]. Recently, combinations of seven appropriately selected tests, including 12 variables, were reported to be very efficient in predicting bull fertility [25]. Several studies previously evaluated the relation between fertility and a panel of laboratory analyses performed on stallion spermatozoa, on both fresh semen [22] and frozen semen [21], but their investigations were limited to a maximum of four sperm compartments, for example, viability, motility, chromatin structure stability, and mitochondrial function. The predictive value remained limited. To our knowledge, no study has analyzed the results of a larger number of tests, including, for the same sample, motility, morphology, mitochondrial activity, oxidation level, acrosome integrity, DNA integrity, and plasma membrane integrity on a large number of stallions with widely differing fertility. It is therefore not known whether increasing the number of tests would increase the level of fertility prediction.

The present experiment was designed to define a new protocol for the assessment of stallion semen, thereby improving the prediction of fertility. The response to this objective entails determining the appropriate combination of tests, and the minimal number of ejaculates needed to characterize a stallion. For that purpose, a panel of nine tests, evaluating a large number of spermatozoal compartments—motility, morphology, viability, mitochondrial activity, oxidation level, acrosome integrity, DNA integrity, "organization" of the plasma membrane, and hypoosmotic