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Trypan Blue/Giemsa Staining to Assess Sperm Membrane Integrity in Salernitano Stallions and its Relationship to Pregnancy Rates

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Contents
Aim of this study was to test the reliability of Trypan blue/Giemsa staining to evaluate sperm membrane integrity, acrosomal intactness and morphology in stallion to verify whether it could be applied *in vitro* as useful tool for sperm fertilizing ability. Fertility data on inseminated mares were collected to evaluate the relationship of sperm quality to pregnancy rates. Forty-one ejaculates were collected from 3 stallions of Salernitano Horse Breed and evaluated for gross appearance, volume, visual motility and membrane integrity with Trypan blue/Giemsa staining and thirty-five mares were inseminated during the breeding season from April to July. Differences among stallions were found in volume, sperm concentration ($p < 0.05$) and visual motility ($p < 0.01$). A decrease in sperm motility, concentration ($p < 0.05$) and total sperm number was found in June–July ($p < 0.01$). Live sperm with intact acrosome (LSIA) and proximal droplets (PD) were lower ($p < 0.01$) in June–July, while acrosome reacted sperm (ARS) percentage increased ($p < 0.05$). No fertility differences were found among stallions with an average fertility per cycle of 44.6% and a pregnancy rate of 68.6%. Higher percentages of LSIA were found in the ejaculates used to inseminate mares that became pregnant vs those used in mares not pregnant ($p < 0.05$). The significance of LSIA as test variable to verify the reliability of Trypan blue/Giemsa staining was confirmed by Receiver operating characteristic ROC analysis and the sensitivity of the test was 85% at a cut-off value of 48% LSIA. Trypan blue-Giemsa showed to be an accurate method that can be applied on field to evaluate sperm membrane integrity and to identify poor-quality ejaculates.

Introduction

Several assays have been developed to estimate semen quality, often expensive and time-consuming, and many studies have correlated *in vitro* assays with fertility in bull (Ostermeier et al. 2001; Al-Makhzoomi et al. 2008; Kastelic and Thundathil 2008), boar (Gadea 2005; Didion et al. 2013), stallion (Jasko et al. 1992; Love and Kenney 1998; Heckenbichler et al. 2011;), man (Bonde et al. 1998; Lewis 2007). A sperm cell must possess many attributes to fertilize an oocyte (Graham and Mocé 2005), as intact and functional plasma and acrosomal membranes. Each assay evaluates different sperm features and conventional laboratory tests include concentration, motility, membrane integrity or viability and morphology. No single assay reliably predicts fertility (Mocé and Graham 2008) for the complex nature of the spermatozoon itself and for the female effect. The possibility of using a simple procedure to evaluate sperm morphology, as well as membrane integrity or viability and the acrosome status at low cost, without the employment of expensive equipment, could be useful to assess simultaneously different sperm compartments, giving a great support to define stallions' reproductive capability to increase the efficiency of assisted reproduction.

Several staining techniques performed by light microscopy have been used, such as eosin-based stains (Blom 1950a,b; Casserett 1953), Giemsa (Kútvölgyi et al. 2006), India ink and Wright's stains (Kenney et al. 1983), among others. Eosin/nigrosin staining, the most commonly employed, has shown a good agreement with flow cytometry and automatic cell counter technique when used on semen with high levels of sperm membrane intactness (SMI), while for low levels of SMI, it tended to overestimate them (Love 2012). A simple dual staining procedure with Trypan blue/Giemsa has been used in different species with good results (Didion et al. 1989; Nagy et al. 1999; Boccia et al. 2005). Kovács and Foote (1992) stated that the procedure had not given satisfactory results for stallion sperm, because of the minor size of the head that needed a higher magnification compared to bull and boar sperm, and because the lower semen concentration of the stallion resulted in more disturbing background caused by seminal plasma and extender proteins (Kútvölgyi 2012). Later Kovács et al. (2000) adapted this method to stallion spermatozoa, using more diluted fresh and frozen semen and higher magnification (1000×). The integrity of plasma membrane is determined by the ability of viable cells to exclude Trypan blue dye, whereas the stain will cross the membrane of dead cells with compromised membrane. Giemsa staining is associated to detect the presence or absence of the acrosome that appears light purple when intact. Damaged acrosomes are pale lavender and loose acrosomes are dark lavender; sperm cells with no acrosome are grey in the acrosomal region. The membrane integrity of the sperm tail can be also evaluated. Live cells have sky-bluish postacrosomal region, while dead cells are dark blue. Evaluation of morphological abnormalities including cytoplasmic droplets is also possible (Kovács et al. 2000).

The aim of this study is to assess sperm quality and membrane integrity of stallion semen using the Trypan blue/Giemsa staining in order to verify whether this technique could be applied routinely during the breeding season to evaluate simultaneously plasma membrane integrity, acrosomal intactness and morphology. Field fertility data on inseminated mares were collected to evaluate the relationship of assessed sperm quality and membrane integrity to pregnancy rates.

Material and Methods

Semen collection

During the breeding season, from April to the end of July, semen was collected from 3 stallions of Salernitano horse breed belonging to the Regional Center of Equine Improvement (Centro Regionale di Incremento Ippico - S. Maria Capua Vetere), a warmblood type of horse native of southern Italy in Campania region, a very ancient breed currently considered as endangered. Semen was collected from the stallions (A, B and C) with a Missouri model of artificial vagina using mares in behavioural oestrus or phantom. Forty-one ejaculates were used for the analysis: 14 for stallion A, 16 for B and 11 for C, corresponding to those requested by the owners of the mares for the insemination. As it was not possible to standardize the sperm collection frequency for the three stallions, due to constraints of the Centre, sperm collection for stallion A was performed every 3 days, while for stallions B and C 4 and 5 days passed between two collections respectively with a frequency of collection varying from 1 to 2. × week.

After collection, ejaculates were filtered through a sterile gauze to remove the gel and gel-free semen was evaluated for gross appearance and volume, then stored at 35°C in an incubator for few minutes (approximately 5′), until they were analysed and diluted. Semen concentration was determined by using a spectrophotometer, calibrated for the species. Visual motility was assessed by a phase-contrast microscope equipped with a warm stage both on raw and diluted semen. The semen was diluted for AI with a commercial skim milk extender (EZ-Mixin BF, Animal Reproduction Systems, Chino, CA, USA), to obtain a total motile sperm count of approximately 1×10^9 in a total dose volume of 48 ml. Moreover, an aliquot of semen was diluted 1 : 2 and 1 : 3, using the same extender, for motility and staining evaluation.

Total sperm number per insemination dose was calculated by multiplying dose volume and concentration of diluted sperm.

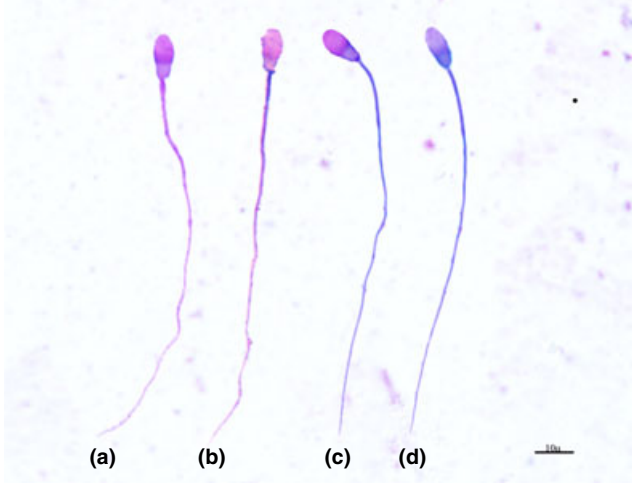

Fig. 1. Different categories of sperm stained with Trypan blue/Giemsa: Live Sperm with Intact Acrosme (a), Live Dead Sperm (b), Dead Sperm with Intact Acrosme (c), Dead Dead Sperm (d)

Trypan blue/giemsa staining

Samples of all dilutions of semen were analysed by Trypan blue/Giemsa staining at collection and after 24 h stored at 4°C as reported by Kovács et al. (2000) with minor modifications. For staining, Trypan blue was used at a concentration of 0.27%. Spermatozoa were diluted 1 : 10 with 0.9% NaCl, one drop (5 μl) of diluted semen and one drop (5 μl) of Trypan blue were mixed on a slide and two smears were prepared by using a single semen droplet. Slides were air-dried in vertical position then put into a fixative consisting of 86 ml 1N HCl and 14 ml of 37% formaldehyde solution with the addition of 0.2 g Neutral Red (Sigma N-2880) in a slide staining jar for 2 min and then rinsed with distilled water. Slides were put into jars containing the Giemsa solution and placed in a water bath at 37°C for 2 h. We introduced the use of a water bath, not reported by Kovács et al. (2000), for improving the Giemsa staining quality, that is reported to be more effective at 25–40°C (Kútvölgyi 2012), and reducing the length of the procedure, avoiding the Giemsa staining overnight. The Giemsa staining solution was freshly made by adding 7.5% (v/v) of Giemsa stock solution (Sigma GS-500) to distilled water. Slides were rinsed again in distilled water, air-dried in vertical position and coverslipped with Entellan (Merck 107). Dead spermatozoa stained dark blue while live spermatozoa appeared pink. Intact acrosomes were purple, loose and damaged acrosomes were lavender, and the anterior part of the sperm head with no acrosome was pale gray (Fig. 1). Live cells have sky-bluish postacrosomal region while dead cells are dark blue (Kovács et al. 2000). The term 'live sperm' indicated spermatozoa with intact membrane while 'dead sperm' those with disrupted membrane as it is considered more appropriate to refer to the membrane intactness (Love 2012).

The slides were examined by bright field microscopy at 100× magnification. For all samples, two slides were examined and 100 spermatozoa were counted for each slide. According to the different presence of dye in various cellular compartment, the following categories were assessed: live sperm with intact acrosome (LSIA), considered as normal, dead sperm with intact acrosome (DSIA), acrosome reacted sperm (ARS), as well as sperm with detached head, live sperm with proximal droplets (LSPD), bent midpiece and coiled tails, swollen/roughed/broken tails (Dag-like).

AI and pregnancy rate

Thirty-five mares were examined daily during oestrus by rectal palpation and transrectal ultrasonography to monitor follicular growth, uterine fluid accumulation and uterine oedema, until ovulation was detected. Examination was always performed using a SonoSite Titan ultrasound system equipped with a multifrequency linear probe. Uterine swabs and smears were collected at the beginning of the breading season for each mare, eventually treated if positive with an appropriate therapy. When a follicle of at least 35 mm in diameter together with uterine oedema was detected (D0), ovulation was induced with human chorionic

gonadotropin (2000 IU) and mares inseminated after 24 h (D1). When the mare, monitored by ultrasonographic exams, did not ovulate within 36 h after the first AI, insemination was performed (D2) and repeated at 36 h interval until ovulation occurred. In case an anovulatory follicle was detected, insemination was not performed. Semen from stallions was collected on D1 at the Regional Center of Equine Improvement and processed for the insemination. AI doses were prepared with 1×10^9 total motile sperms of in a total volume of 48 ml, with a mean concentration of 20×10^6 motile sperm/ml; mean dilution rate per stallion was: 2.6 ± 0.4 for A, 2.4 ± 0.5 for B and 2.5 ± 0.5 for C.

The mares were checked ultrasonographically 14 days after ovulation for early pregnancy diagnosis and after 30 days.

A total of 14 mares were inseminated with stallion A, 10 mares with B and 11 mares with C, with a mean cycle/mare of 1.8 ± 0.8 and a mean number of doses/stallion used to inseminate the mares of 20.6 ± 1.5.

Pregnancy rate per cycle was calculated as the percentage of number of cycles diagnosed positive (at day 14), divided by the total number of cycles inseminated. Moreover, pregnancy rate per season was considered as the number of mares detected pregnant by the end of the breeding season, divided by the total number of mares inseminated during the breeding season.

All the practices on the animals were carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

Statistical analysis

Data on volume, sperm concentration, visual motility and membrane integrity parameters were analysed by ANOVA and percentage data were arcsin transformed when appropriate. The model used to analyse sperm quality parameters included the effect of stallion (3 levels) and month (2 level: April–May and June–July) and their interaction. The ANOVA model used to analyse membrane integrity data included the main effects of month (2 levels), stallion (3 levels) and dilution (2 levels) and their first- and second-order interaction. In particular, tail alterations were analysed separately and grouped (Bent + Coil + Dag-like alteration; BCD). Differences between fresh diluted semen and semen cooled for 24 h were analysed by ANOVA for repeated measures with a model including the effects of the stallion, dilution and month groups and their first- and second-order interaction. Correlation analysis was performed among sperm membrane integrity categories. Fertility data were analysed using the Chi-square test.

Receiver operating characteristic curve (ROC curve) analysis was used to evaluate sensitivity and specificity of the Trypan blue/Giemsa dye test. ROC curve is a plot of the true positive rate against the false-positive rate for the different possible cut-off of a diagnostic test. The area under the curve measures the accuracy of the test that is the ability of the test to correctly classify the cases according to the positive value of the state variable (Akobeng 2007). In our data case, pregnancy was considered as the positive value of the state variable and the different sperm parameters as test variables. The sperm parameter data used in the ROC analysis were those evaluated in the doses prepared for the last AI before ovulation, at the same dilution rate.

The sensitivity was assessed as the number of cycles followed by a pregnancy that were correctly detected by the test in relation to the total cycles followed by a pregnancy and specificity as the number of cycles not followed by a pregnancy wrongly detected by the test out of the total cycles not followed by a pregnancy.

All statistical analyses were performed by IBM SPSS Statistics 20.0 (2012).

Results

Dilution effect

No differences were found in semen quality between dilutions and also the Trypan blue/Giemsa staining did not evidence any difference on sperm viability on fresh and after 24-h storage at 4°C. Data of the 2 dilutions were pooled to evaluate stallion effect.

Stallion effect

Results on semen parameters are reported in Table 1 for each stallion. Differences among stallions were found in volume ($p < 0.05$), sperm concentration ($p < 0.05$) and visual motility ($p < 0.01$).

Trypan blue/Giemsa staining evidenced similar percentages of sperm with intact acrosome (LSIA) and sperm acrosome reacted (ARS) among stallions (Table 2). The percentage of proximal droplets was higher in stallion A compared to B and C ($p < 0.01$) while Bent + Coil + Dag-like abnormalities (BCD; Table 2) were higher in stallion C compared to stallion A ($p < 0.01$). No differences were found among stallions

Table 1. Semen volume, concentration and visual motility among stallions and months (mean ± SE)

Stallion	Volume (ml)	Concentration (10^6/ml)	Visual Motility (%)	Total sperm (10^6)
A	106.4 ± 21.7^{a}	124.5 ± 7.7^{a}	80.4 ± 3.3^{A}	10408.3 ± 836.8
B	108.7 ± 3.9^{a}	86.7 ± 5.2^{b}	63.5 ± 3.3^{B}	9145.0 ± 552.8
C	86.1 ± 8.0^{b}	98.2 ± 4.3^{ab}	70.9 ± 2.9^{AB}	8222.9 ± 456.4
Month				
April–May	104.2 ± 7.4	110.1 ± 5.8^{a}	78.2 ± 3.1^{A}	11573.6 ± 621.1
June–July	95.3 ± 9.9	96.1 ± 3.5^{b}	65.0 ± 2.2^{B}	6943.8 ± 392.2
Total	98.5 ± 6.8	98.4 ± 23.1	71.6 ± 2.0	9258.7 ± 367.3

Values with different letters differ significantly (a, b = $p < 0.05$) (A, B = $p < 0.01$).

in detached head percentage with an average value of 0.62 ± 0.51%.

A positive correlation was found between semen concentration and LSPD percentage ($r = 0.48$; $p < 0.05$). A higher incidence of Bent abnormality was associated with a higher incidence of Dag-like spermatozoa ($r = 0.51$; $p < 0.05$) and to a lower incidence of ARS ($r = -0.44$; $p < 0.05$). Visual motility of fresh semen was positively correlated to the live sperm with intact acrosome (LSIA) ($r = 0.372$; $p < 0.05$) and to LSIA + LSPD ($r = 0.486$; $p < 0.01$).

After 24 h, sperm motility was on average 56.4% with a decrease of 21.2% compared to that of fresh semen. Also, LSIA (55.5 ± 1.7% vs 49.8 ± 1.6%; $p < 0.01$) and LSPD (11.1 ± 0.7%vs 8.6 ± 0.5%; $p < 0.01$) significantly decreased on average after 24 h of cooling, while ARS and BCD alterations increased respectively by 43% (6.7 ± 0.5% vs 9.6 ± 0.9%; $p < 0.01$) and 11.5% (10.4 ± 0.8% vs 11.3 ± 0.7%; $p < 0.01$). However, a significant interaction was found between stallion and storage effects, showing an increase in BCD alterations after 24 h only for the two stallions (A and B) with the lower BCD values in the fresh semen (6.9 ± 1.5% vs 8.6 ± 1.5%, 9.4 ± 1.1% vs 12.1 ± 1.1% and 14.5 ± 0.9 vs 13.4 ± 1.1 in A, B and C stallions respectively; $p < 0.01$).

Different percentages of LSIA after 24 h were found among stallions, with a higher value in stallion C ($p < 0.05$; Table 3), while the percentage of LSPD 24 h was higher in stallion A compared to B and C ($p < 0.01$). BCD after 24 h was different among stallions ($p < 0.05$; Table 3) with higher values in stallion C. Percentages of detached heads were similar among stallions after 24 h of storage with an average value of 0.72 ± 0.89%.

Month effect

Months had no influence on sperm volume, but in June–July, a significant decrease in sperm motility ($p < 0.01$) and concentration ($p < 0.05$) were registered, with a lower total sperm number (Table 1; $p < 0.01$).

On fresh semen (Table 2), in the June–July period, the percentage of LSIA and LSPD were lower ($p < 0.01$), while ARS percentage increased ($p < 0.05$). Dag-like, bent and coil abnormalities (Table 2) were not influenced by the month effect.

After 24 h of cooling (Table 3), LSIA and LSPD remained lower in the June–July period ($p < 0.01$), with a significant interaction between stallion and month effects for LSPD, showing a decrease mainly in stallion A, the one with the highest values. ARS increased after 24 h of cooling in June–July period ($p < 0.01$) compared to the fresh semen, while BCD did not show any differences between month groups.

Fertility data and sperm membrane integrity

No difference in fertility was found among stallions and months, with a mean value of 44.6% fertility/cycle and 68.6% pregnancy rate per season (pregnant mare/inseminated mares).

Total sperm number per insemination dose (on average $1.6 \times 10^9 \pm 0.04$) and the motility values (on average 70.6 ± 3.5) were similar in pregnant and non-pregnant mares. Significant differences were found in the percentage of LSIA among ejaculates used in mares that resulted pregnant compared to the non-pregnant; the mean percentage values of LSIA were 57.1 ± 2.9% for the pregnant mares compared to 48.1 ± 2.4% for the non-pregnant ones ($p < 0.05$). The number of LSIA sperm in an AI dose was calculated as the number of

Table 2. Live sperm with intact acrosome (LSIA), dead sperm with intact acrosome (DSIA), sperm with proximal droplets (LSPD), acrosome reacted sperm (ARS) and tail abnormalities (bent coil and daga; BCD) among stallions and months

Stallion	LSIA (%)	LSPD (%)	DSIA (%)	ARS (%)	BCD (%)
A	57.2 ± 2.9	16.3 ± 1.2^{A}	12.1 ± 2.9	6.3 ± 0.9	6.9 ± 1.5^{A}
B	51.8 ± 2.9	9.9 ± 1.2^{B}	19.0 ± 3.2	7.7 ± 0.9	9.4 ± 1.1^{AB}
C	57.4 ± 2.3	7.2 ± 1.0^{B}	12.4 ± 2.9	6.0 ± 0.7	14.5 ± 0.9^{B}
Month					
April–May	63.4 ± 2.7^{A}	13.2 ± 1.1^{A}	7.4 ± 2.7^{A}	6.0 ± 0.8^{a}	9.9 ± 1.2
June–July	49.1 ± 1.8^{B}	8.6 ± 0.8^{B}	21.5 ± 1.5^{B}	7.3 ± 0.6^{b}	10.8 ± 0.8
Total	55.5 ± 1.7	11.1 ± 0.7	14.5 ± 1.5	6.7 ± 0.5	10.4 ± 0.7

Values with different letters differ significantly (a, b = $p < 0.05$) (A, B = $p < 0.01$).

Table 3. Sperm morphology and motility after 24 h from collection: live sperm with intact acrosome (LSIA24h), dead sperm with intact acrosome (DSIA24h), sperm with proximal droplets (LSPD24h), acrosome reacted sperm (ARS24h) and tail abnormalities, bent coil and daga (BCD24h) among stallions and between months

Stallion	LSIA24h (%)	LSPD24h (%)	DSIA24h (%)	ARS24h (%)	BCD24h (%)	Motility24h (%)
A	45.4 ± 2.6	14.9 ± 1.0^{A}	21.0 ± 2.2^{a}	7.6 ± 1.5	8.6 ± 1.5^{a}	63.3 ± 2.6^{A}
B	48.8 ± 2.5	5.4 ± 0.7^{B}	18.4 ± 2.4^{a}	10.4 ± 1.4	12.1 ± 1.1^{ab}	50.0 ± 2.6^{B}
C	55.1 ± 2.3	5.5 ± 0.7^{B}	13.1 ± 1.6^{a}	10.8 ± 1.3	13.4 ± 1.1^{b}	55.8 ± 2,3^{AB}
Month						
April–May	56.4 ± 2.9^{A}	12.9 ± 0.9^{A}	11.3 ± 2.1^{A}	7.1 ± 1.6^{A}	10.3 ± 1.3	61.6 ± 2.4^{A}
June–July	43.2 ± 1.7^{B}	5.6 ± 0.5^{B}	23.7 ± 1.1^{B}	12.6 ± 1.0^{B}	12.1 ± 0.7	51.2 ± 0.7^{B}
Total	49.8 ± 1.6	8.9 ± 0.5	17.5 ± 1.2	9.6 ± 0.9	11.3 ± 0.7	56.4 ± 1.6

Values with different letters differ significantly (a, b = $p < 0.05$) (A, B = $p < 0.01$).

sperm cells in one AI dose x the% of LSIA evaluated in the ejaculate. However, no difference was found between pregnancy groups for total LSIA sperm ($1105 \pm 480 \times 10^6$ vs $870 \pm 368 \times 10^6$ respectively for pregnant and not pregnant) and the minimum value of total LSIA sperm to obtain a pregnancy was 41×10^6.

Receiver operating characteristic curve showed a significant value for the area under the curve when LSIA was used as test variable (0.77; $p < 0.05$). The sensibility of the staining test resulted 100% for a LSIA value of 40% and a cut-off value of 48% LSIA was found for a test sensibility of 85%; at this values, the test specificity was 60%.

Discussion

The average sperm volume recorded was higher than that reported in other studies (Gamboa et al. 2009). Collection frequency has been reported to influence sperm volume, concentration and motility (Magistrini et al. 1987; Sieme et al. 2004). However, other reports did not show any influence on sperm volume at lower collection frequency (Pickett et al. 1976; Squires and Pickett 2011), but they agree with a reduction in sperm concentrations. In our study, collection frequency depended on the semen doses requested by the breeders: for stallion A, the collection was performed every 3 days, while for stallions, B and C 4 and 5 days passed between two collections, respectively. Moreover, stallions were not accustomed to sperm collection before the start of the project; hence, the high sperm volume found could be better explained by the prolonged reaction time and the higher numbers of mounts that often occurred to ejaculate that, as reported by Sieme et al. (2004), increased seminal volume and decreased sperm concentration.

Sperm concentration was not related to the volume, in contrast to other studies (Magistrini et al. 1987; Sieme et al. 2004), probably because of low and different collection frequency. The average of total sperm number obtained per collection was not different among stallions, with a mean collection frequency ranging between 1 and 2 per week, in agreement with Squires and Pickett (2011) that reported a variation in total sperm number at higher collection frequency (6× week).

In this study, on average, approximately 50% of total sperm was classified as normal, corresponding to live sperm with intact acrosome and without defects (LSIA), and their percentage was positively correlated with motility.

Sperm morphological defects have been classified traditionally as primary, secondary, or tertiary, according to their origin, as major and minor (Blom 1950a,b, 1977; Card 2005), according to their impact on fertility, or as compensable, when can be bypassed by increasing the number of sperm in a dose, and uncompensable deficiencies, considered independent of sperm dosage (Saacke 2008). The current trend is to record the numbers of specific morphologic defects, such as knobbed acrosomes, proximal protoplasmic droplets, swollen midpieces and coiled tails. In general, the percentage of morphologically normal sperm in a semen sample is similar to the percentage of progressively motile spermatozoa (Varner 2008).

A higher correlation coefficient, however, was found between visual motility and the percentage of LSIA + LSPD that seem to imply a role of LSPD sperms in increasing visual motility estimation, thus lowering the effectiveness of this parameter for sperm evaluation. Although proximal droplets may reflect a default in epididymal function, with severe effects on fertility in bulls (Amann et al. 2000; Thundathil et al. 2001; Pesch and Bergmann 2006), controversial reports have been found in stallions (Brito 2007) and they are commonly observed in young stallion spermatozoa (Jasko et al. 1990; Love et al. 2000). However, in our study, even if a different percentage of sperm with proximal droplets was found among stallions, there were no differences in fertility, suggesting that LSPD did not impair fertility in stallions, according to Love et al. (2000).

A different percentage of tail alteration was found among stallions that did not seem to influence the fertility rate, according to Hellander et al. (1991), and in contrast to negative correlations with fertility found by different authors (Jasko et al. 1990; Love et al. 2000; Love 2011).

A decrease in sperm concentration, total sperm number and motility during summer was found, as reported by Heckenbichler et al. (2011). A lower concentration during summer was in accordance with previous studies (Pickett et al. 1976; Magistrini et al. 1987; Jasko et al. 1991; Janett et al. 2003a,b). The decrease in total sperm number was in contrast with that reported by Janett et al. (2003a), probably for the differences in latitude, breed, stallion management and frequency of semen collection. The decrease in sperm motility was in accordance with the results reported in Warmblood and Frenches-Montagnes stallions (Janett et al. 2003a,b) and with Heckenbichler et al. (2011) but differed from other authors (Pickett et al. 1976; Magistrini et al. 1987; Hoffmann and Landeck 1999). Further, Jasko et al. (1991) and Wrench et al. (2010) reported a higher percentage of motile and progressively motile sperm during summer compared to winter, assessed by CASA and by phase-contrast microscopy, respectively. Increased sperm output during the breeding season has been explained by endocrine changes associated with an elevated population of spermatogonia (Johnson 1991). It is worth to point out that in South Italy, summer temperature reaches and sometimes exceeds 30°C with high level of humidity (over 70%). Also, sperm membrane intactness was impaired by hot climate with a reduction of LSIA and an increase in acrosome reacted sperm (ARS) according to Janett et al. (2003a) in Warmblood but not in Frenches-Montagnes stallions (Janett et al. 2003b). The increase in ARS in boar has been related to a reduction of the stability of acrosome proteins due to heat stress, high hot and humidity level that could act together with an impaired endocrine activity under testosterone control and it is well known that spermatogenesis is highly affected by hot weather even in temperate countries (Murase et al. 2007).

Percentage of LSIA was higher in the insemination doses resulted in a pregnancy, while visual motility was similar between doses used on pregnant mares compared to non-pregnant ones. These results suggest, according to Love (2012), that sperm motility and sperm membrane intactness or viability measure clinically independent features of sperm quality.

The determination of Trypan blue/Giemsa test sensitivity by Roc analysis showed that it could be useful to evaluate sperm membrane integrity giving a valid indication on semen quality. A semen dose with <41% of LSIA failed to hesitate in pregnancy, while doses with more than 48% LSIA had a significantly better chance to give pregnancy. The test sensitivity using this value, as cut-off was 85%, showing that it could be used as an index of semen quality. The low level of test specificity found for this value was affected by the female effect (age, previous years of breeding, follicular growth, etc.) that had a heavy influence and could not be avoided.

At least 1×10^9 total spermatozoa were used per each insemination dose, a value considered optimal for frozen equine semen; the total LSIA sperm percentage was always above 400×10^6. As mentioned previously, it has been demonstrated that lack of pregnancy after AI could be due both to fertilization failure and to complication during zygote development, the first attributable to compensable defects that could be solved by a higher sperm concentration and the second independent from sperm dosage (Saacke 2008). Our results suggest that a simple evaluation of sperm visual motility cannot be considered a parameter to predict fertility. Moreover, a low percentage, fewer than 41%, of LSIA in an ejaculate could be considered a minimum threshold to achieve a pregnancy, which cannot be compensated by increasing the total sperm number in a dose. Considering that in this study the inseminations were always carried out using a high number of total sperm per dose, the problem of semen with low total number of LSIA did not occur. However, assessing the percentage of potentially fertile spermatozoa in an ejaculate could allow adjusting the total sperm number in a dose to reach a more suitable value to improve fertility.

In conclusion, Trypan blue/Giemsa could be considered a simple and reliable method that can be performed on field to assess different sperm attributes, as sperm membrane integrity, acrosome intactness and morphology, rapidly (in approximately 3 h) and cheaply. In fact, viability assessment by flow cytometry or fluorescence microscopy is generally not accessible to all practitioners, considering the cost of the equipment that limits their use in routine work. Furthermore, this test allows identifying poor-quality ejaculates that could be used for AI programme with appropriate semen processing to improve their low fertility potential through a more intensive veterinary management.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

R. Di Palo, R. Serafini and V. Longobardi conceived the experiment. R. Serafini, M. Spadetta and D. Neri were involved in semen collection and handling, mares' insemination and pregnancy diagnosis. V. Longobardi, R. Serafini and B. Ariota were involved in laboratory analyses. R. Di Palo performed the experimental design, data analyses and interpretation. R. Di Palo and R. Serafini wrote the manuscript. B. Gasparrini, and V. Longobardi were involved in the interpretation of data and writing of the manuscript. All authors read and approved the manuscript.

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