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Selection of frozen–thawed stallion semen by microfluidic technology

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Abstract

The use of microfluidic technology is increasing in artificial reproduction technologies: With a small amount of semen, it allows for the selection of sperm with the best characteristics of kinetics, morphology and chromatin integrity. The ZyMot Multi (850 µl) is the most popular device of ZyMot Fertility Inc. To date, it was proven to be a valid instrument for sperm selection for in vitro fertilization and intracytoplasmic sperm injection in men. The aim of this study was to test the efficacy of the ZyMot Multi (850 µl) for stallion semen. Frozen–thawed semen from 15 stallions that were previously classified as being of 'good fertility' (GF; $n = 8$; pregnancy rate ≥ 40%) and 'poor fertility' (PF; $n = 7$; pregnancy rate < 20%), respectively, was used. Each ejaculate was assessed before and after microfluid recovery for kinetics (CASA), membrane integrity (MI) (SYBR14/PI), membrane functionality (MF) (HOS test), acrosome integrity (Spermac Stain Kit), morphology (Spermac stain kit), mitochondrial membrane potential (MMP) (JC-1) and chromatin integrity (aniline blue staining). Sperm concentration was reduced after sperm recovery in both groups, but more markedly in frozen–thawed semen of PF stallions ($p < .05$). Microfluid recovery increased total motility, MI, MF and MMP. While there was a significant increase in the percentage of progressively motile sperm after sperm microfluid recovery, there was a decrease in DAP, DSL, VAP, VSL, LIN, WOB and ALH ($p < .05$). A slight increase ($p < .05$) was detected in beat-cross frequency. The present results suggest that the ZyMot Multi (850 µl) device selects a specific sperm population from any stallion ejaculate with motile sperm and could therefore be a valid tool for in vitro testing with the aim to predict the fertility of frozen–thawed stallion semen.

KEYWORDS
frozen–thawed, microfluidics, sperm, stallion

1 | INTRODUCTION

The ideal sperm sorting technique should be rapid, cheap and easy to use. It should allow for isolation of sperm with high velocity, reduce reactive oxygen species (ROS) generation and morphological defects as well as the percentage of sperm with fragmented DNA (Asghar et al., 2014). Because centrifugation will increase the exposure of motile spermatozoa to ROS, this processing step should be avoided (Len et al., 2020). Swim-up and density gradient centrifugation are conventional sperm sorting techniques routinely used in artificial reproduction technologies (ART) because they meet many of these requirements. Both these techniques, however, include a

centrifugation step which may cause sperm damage due to ROS production and the consequent increment of plasma membrane peroxidation (Len et al., 2020).

As a more recently developed alternative for the selection of sperm in ART, microfluidic technology is suggested to mimic flow dynamics of the female reproductive system. Microfluidic systems consist of multiple laminar flow systems. These allow for selection of most functional sperm with high DNA integrity by swimming through pores of a membrane and thus being accumulated in one compartment of the system where they are finally collected (Gode et al., 2019). Microfluidic systems require low sample consumption. They allow for an easier manipulation of spermatozoa compared with traditional methods and without the drawbacks of centrifugation (Len et al., 2020). For these reasons, the use of microfluidic technology is of increasing interest within ART laboratories (Baldini et al., 2021). Such techniques may also attract interest as new approaches for the in vitro analysis of semen characteristics with the aim to predict in vivo fertility.

In the present investigation, we compared semen characteristics of frozen–thawed stallion semen that previously proved to be of low or good fertility when used for insemination although it had been shown to meet minimal requirements with regard to sperm motility (Aurich et al., 2020) as suggested previously (Loomis & Graham, 2008). We hypothesized that the sperm populations from the two groups recovered by a microfluid separation device would differ with regard to at least some of their semen characteristics. We also hypothesized that the percentage of sperm recovered by the device from frozen–thawed semen would be larger in stallions with good fertility. This would support the idea that characteristics of the sperm population selected by microfluid technology might be beneficial to predict the fertility of semen samples.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Frozen–thawed semen from 15 stallions with two repetitions per stallion was included into the study. Semen had been produced for commercial purposes at the Centre for Artificial Insemination and Embryo Transfer of Vetmeduni Vienna that is approved according to European Union regulations (Directive 65/92 EEC). Semen was included in the study if it had been used for insemination in at least five different mares (age 3–15 years) with no previous history of reproductive problems. All mares underwent a breeding soundness examination (BSE) including negative uterine culture and cytology before insemination. Mares were inseminated by veterinarians known to have ample experience in horse reproduction and insemination. Mares were always inseminated shortly before or within 6 h after ovulation that was confirmed by transrectal ultrasound examination. In accordance with WBFSH recommendations ('WBFSH, World Breeding Federation for Sport Horses: Semen standards'; accessed on 19 May 2020; available online: http://www.wbfsh.org/files/Semen%20standards.pdf), semen was classified acceptable for insemination when progressive motility after thawing was ≥35%. Semen was classified according to previously achieved pregnancy rate as 'good' (GF; $n = 8$; pregnancy rate ≥ 40%) or 'poor' (PF; $n = 7$; pregnancy rate < 20%). In the present investigation, straws from one semen batch of each of these stallions were included. Characteristics of frozen–thawed semen were analysed before and after microfluid recovery. All stallions underwent a BSE before semen production and were only included into the semen cryopreservation programme when raw semen characteristics at BSE met minimal requirements (Aurich et al., 2020). Semen was cryopreserved following European Union regulations (Directive 65/92 EEC), and all stallions met the respective health requirements. Semen cryopreservation followed an established protocol (Aurich et al., 2020). Based on this protocol, the mean concentration of frozen–thawed semen samples included into the present experiment irrespective of group varied between 112.1 (minimum) and 921.0 (maximum) $\times 10^6$/ml with a mean of $370.6 \pm 209.0 \times 10^6$/ml (± SD). According to the Austrian law for the protection of experimental animals (Law Decree n. 26 issued on 4 March 2014, art. 2), the approval by an ethical committee for the experiment is not required under the circumstances that this trial was carried out. The owner's informed consent was always collected.

2.2 | Semen collection and cryopreservation

Semen freezing was performed as described previously (Aurich et al., 2020). Briefly, ejaculates were collected on a breeding dummy using an artificial vagina (Hannover model; Minitube, Tiefenbach, Germany) fitted with an inlaying filter (Minitube) for the removal of the gel fraction. Semen was diluted 1:1 (v:v) with EquiPlus extender (Minitube) centrifuged at $700 \times g$ for 12 min. The supernatant was removed, and the pellet was resuspended with Ghent freezing extender (Minitube) at a ratio of 1:1 (v:v) irrespective of the final sperm concentration. The semen was filled into 0.5 ml straws automatically sealed at room temperature (RT) (MPP Uno, Minitube). Straws were frozen using a computer-controlled rate freezer at 20°C (IceCube 14 M; Sylab, Purkersdorf, Austria). Semen was first cooled to 5°C at a cooling rate of 0.3°C/min, then to −25°C within 3 min (10°C/min) and finally to −140°C (25°C/min). Straws were removed from the freezing chamber and immediately plunged into liquid nitrogen. Before semen was used for breeding, from each ejaculate, at least one straw was used for analysis of post-thaw semen characteristics (total and progressive motility, sperm membrane integrity (MI)) as described previously (Aurich et al., 2020). Based on this analysis, the number of straws recommended for one insemination was calculated to provide at least 250×10^6 progressively motile sperm after thawing.

2.3 | Samples evaluation

Sperm characteristics from each ejaculate were assessed after thawing (control) and after microfluid recovery (treatment). For each stallion ejaculate, treatment was assessed in two repetitions: Two 0.5 ml

straws from the respective semen batch were thawed in a water bath at 38°C for 15 s, and the content of the straws was transferred into one 2 ml Eppendorf tube and gently mixed. Semen was then processed with the ZyMot Multi (850 µl) Sperm Separation Device (ZyMot Fertility, Gathersburg, MD, USA) according to the manufacturer instructions (Figure 1). Briefly, 850 µl of semen was gently injected into the device using a 1 ml syringe. Then, the upper collection chamber was filled with 750 µl of PBS until the membrane was completely covered. After filling, the device was incubated at 37°C for 30 min. Immediately thereafter, sperm-containing fluid was aspirated from the upper collection chamber using a fresh 1 ml syringe and transferred into a 2 ml Eppendorf tube. Sperm concentration was measured using a NucleoCounter (ChemoMetec, Allerød, Denmark).

Semen motility and sperm kinetic parameters were analysed by computer-assisted sperm analysis (CASA; SpermVision, Minitube) as described previously (Aurich et al., 2020) with the modification that analysis was performed on pre-warmed glass slides filled by placing the exact volume of 10 µl on the designated place, followed by immediate application of the coverslip (WHO prepared motility slide, [Hoogewijs et al., 2012]). The CASA characteristics total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), distance average path (DAP, µm), distance curved line (DCL, µm), distance straight-line (DSL, µm), amplitude of the lateral head displacement (ALH, µm), straightness (STR, %), linearity (LIN, %), wobble coefficient (WOB, %) and beat-cross frequency (BCF, Hz) were considered. For each sample, seven fields and a total of at least 700 sperm were analysed, and the mean values were calculated by the CASA system.

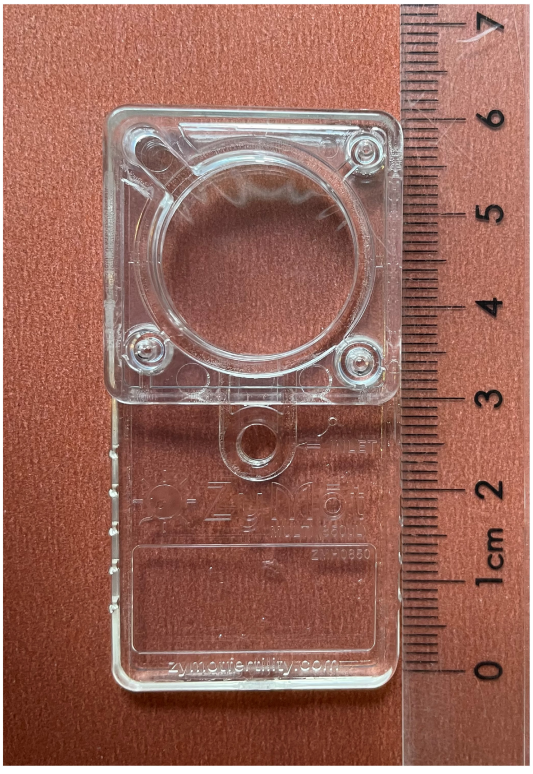

FIGURE 1 ZyMot Multi (850 µl) Sperm Separation Device (ZyMot Fertility). Picture by C. Aurich, Vetmeduni Vienna, Austria

Membrane integrity was assessed using SYBR14/PI fluorescent stain (Minitube) as described (Aurich et al., 2020): 100 µl of semen was mixed with 2 µl of SYBR-14/PI and incubated for 10 min at RT in in the dark. One droplet was placed onto a glass slide, covered with a glass coverslip and evaluated by fluorescence microscopy at magnification x 40 (Olympus AX70; Olympus, Vienna, Austria; U-MWB filter block, BP420–480 excitation filter, BA515 suppressor filter, dichromatic mirror: DM500). Green spermatozoa were considered membrane-intact (alive), while dead spermatozoa were stained red. Spermatozoa were recognized and counted according to their colour by the CASA system (15 fields).

In addition, the hypo-osmotic swelling (HOS) test was used for the evaluation of membrane functionality (MF): 10 µl of semen was mixed with 100 µl of fructose solution (100 mOsm) and incubated at 37°C for 1 h. After incubation, one droplet of the solution was placed onto a glass slide and analysed by light microscopy at x 40 magnification (Pinto & Kozink, 2008).

Acrosome integrity and sperm morphology were evaluated using the Spermac Stain Kit (Minitube) as described by Runcan et al. (2014)). A drop of the sample was smeared onto a glass slide, left to dry at RT and then dipped into the fixative solution for 5 min. The slide was then washed in distilled water and left to dry at RT. Once dried, it was stained for 3 min in stain A, 1 min in stain B and 30 s in stain C. After each staining passage, the slide was washed in distilled water. At the end of the staining procedure, the slide was left to dry at RT and examined with light microscopy at x 100 magnification. Spermatozoa were considered to have an intact acrosome when the anterior acrosomal region stained green and the posterior post acrosomal region was red-pink. For morphology, spermatozoa were classified as normal or with head, midpiece or tail anomalies.

Mitochondrial membrane potential (MMP) was assessed using JC-1 fluorescence staining (Gravance et al., 2000): 150 µl of semen was incubated for 8 min in the dark with 3 µl of JC-1 (1530 µM). After incubation, a drop of the solution was put onto a glass slide, covered with a glass coverslip and analysed by fluorescence microscopy at x 40 magnification (Zeiss Observer Z1, Jena, Germany). Spermatozoa with bright orange midpiece were considered to have a high MMP.

Sperm chromatin condensation was assessed with aniline blue staining as described previously (de Oliveira et al., 2017). Semen samples were centrifuged twice in PBS (1:1) for 10 min at 1500 rpm. The pellet was resuspended with 1% SDS in sodium citrate and incubated for 15 min at 4°C, then spread onto a glass slide and allowed to dry. The smears were fixed in 3% buffered glutaraldehyde in 0.2 mol/L phosphate-buffered saline (pH 7.2) for 20 min. The slides were finally stained with 5% aqueous aniline blue mixed with 4% acetic acid (pH 3.5) for 15 min at RT. The slides were assessed with brightfield microscopy at x 40 magnification (Zeiss Observer Z1). Spermatozoa with blue-stained head were considered to have abnormal chromatin condensation.

For each of these tests, at least 100 cells were counted.

2.4 | Statistical analysis

Statistical analysis was performed using SPSS version 27.0 (SPSS-IBM; Armonck, NY, USA). Data were tested for normality and homogeneity of variances using the Kolmogorov–Smirnov and the Levene test, respectively. Semen characteristics were compared by General Linear Model ANOVA with group (good vs. poor fertility) and treatment (with vs. without microfluid sorting) as within-subject factor and stallion as covariate. The percentage of sperm recovered by the device was calculated (number of sperm in the selection compartment divided by number of sperm injected into the lower compartment multiplied with 100); percentages for good and poor fertility semen were compared by the non-parametric Mann–Whitney *U*-test. For all statistical comparisons, a *p*-value <.05 was considered significant. Values are given as mean ± SEM if not stated otherwise.

3 | RESULTS

Sperm concentration in the fluid aspirated from the microfluid selection device was reduced ($p < .001$) in comparison with frozen–thawed semen. This reduction was more in frozen–thawed semen of the PF than of the GF group ($p < .05$; Figure 2a). As a result, the percentage of sperm recovered by the microfluid device was greater in GF than in PF, respectively ($p < .05$; Figure 2b). Microfluid selection of frozen–thawed sperm increased total motility, MI, MF and MMP, but no influences of group (GF vs. PF) or stallion were detected (Tables 1 and 2). While there was a significant increase in the percentage of progressively motile sperm after microfluid selection, there was a decrease in DAP, DSL, VAP, VSL, LIN, WOB and ALH ($p < .05$; Table 1). With regard to BCF, a slight increase ($p < .05$; Table 1) was detected. For all these parameters, no differences between GF and PF were determined (Table 1). In some parameters (concentration, DAP, DCL, VAP, VCL, ALH and chromatin integrity), a stallion effect ($p < .05$) was present (Tables 1 and 2).

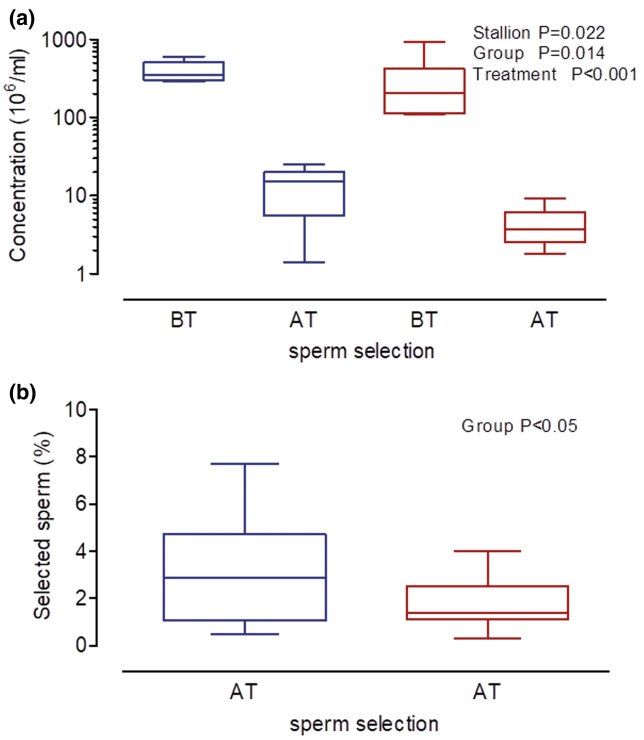

FIGURE 2 Sperm concentration before and after treatment (i.e. microfluid sperm selection) and of selected sperm after treatment in frozen–thawed stallion semen from ejaculates characterized being of good or poor fertility. (a) Box plots representing sperm concentration (10^6/mL) in good fertility (blue) (GF; $n = 16$) and poor fertility (red) (PF; $n = 14$) stallion semen samples before (BT) and after treatment (AT). (b) Box plots representing the percentage of selected sperm with the ZyMot multi (850 µl) device in good fertility (blue) and poor fertility (red) samples

4 | DISCUSSION

To the best of our knowledge, differences in microfluid selection of frozen–thawed semen between stallion ejaculates with good and poor fertility have not been investigated so far. It is important to note that semen from both groups was produced by the same laboratory and clearly met minimal requirements for frozen–thawed semen before being used for commercial purposes, that is for insemination of reproductively healthy mares. There, the semen resulted in either good or poor conception rates. The present experiment aimed at searching 'novel' semen characteristics after microfluidic selection that may help to predict fertility in frozen–thawed semen known to meet minimal requirements with regard to semen motility. This approach was chosen because it is well-known that determination of semen characteristics is not enough to predict fertility of cryopreserved stallion semen (e.g. Greiser et al., 2020). Further research may help deciphering why PF semen resulted in lower pregnancy rates despite presenting good semen characteristics. The difference between sperm selected in the two groups (Figure 2b), however, somehow confirms the accuracy of this distinction. Interestingly, in the present investigation, semen concentration before and after selection as well as the percentage of sperm selected by the microfluid device were the only characteristics with differences between the fertility groups. This does not only suggest that greater concentration of frozen–thawed semen is of benefit for fertility but also for the recovery by the microfluid device. A recent study (Gode et al., 2019) confirms the high selectivity of the sperm separation device when compared to density gradients. Yet, this is the first study on microfluidics in which differences in samples grouped by fertility were assessed.

In the present study, microfluid recovery improved not only the percentage of motile sperm, but also sperm with intact plasma membrane, normal morphology, MMP and chromatin integrity. This is in agreement with findings from studies not only in horses (Gonzalez-Castro & Carnevale, 2019), but also in other species like humans (Asghar et al., 2014) or cattle (Nagata et al., 2018). So far, however, the influence of microfluid selection on motion parameters has not been investigated in horse sperm. In the present

TABLE 1 Results for the characteristics concentration and motility (see Note) of frozen–thawed stallion semen from ejaculates characterized being of good or poor fertility, before microfluid selection (BT) and thereafter (AT)

	Good fertility ($n = 16$)		Poor fertility ($n = 14$)		*p*-value		
	BT	AT	BT	AT			
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Stallion	Group	Treatment
Concentration	400.48 ± 41.47	13.63 ± 2.01	336.56 ± 109.11	4.59 ± 0.63	.022*	.014*	<.001*
TM	70.59 ± 5.17	85.47 ± 3.35	65.74 ± 5.21	88.95 ± 1.5	.675	.793	<.001*
PM	58.36 ± 6.36	66.11 ± 4.84	55.62 ± 5.76	74.9 ± 2.96	.196	.161	.012*
DAP	38.13 ± 1.96	32.8 ± 1.13	33.77 ± 2.33	30.11 ± 2.04	.022*	.313	.018*
DCL	70.07 ± 3.44	65.55 ± 1.73	62.85 ± 3.99	60.23 ± 4.17	.031*	.348	.301
DSL	33.52 ± 4.81	28.15 ± 4.73	29.61 ± 2.16	26.91 ± 1.99	.05	.334	.03*
VAP	82.67 ± 1.70	70.32 ± 2.56	73.62 ± 5.15	64.04 ± 4.48	.024*	.318	.01*
VCL	151.58 ± 7.87	140.13 ± 3.99	136.29 ± 8.72	127.71 ± 8.81	.03*	.349	.184
VSL	72.73 ± 3.94	60.39 ± 2.65	64.63 ± 4.78	57.27 ± 4.37	.053	.338	.018*
STR	0.88 ± 0.01	0.85 ± 0.01	0.87 ± 0.01	0.88 ± 0.01	.506	.943	.497
LIN	0.48 ± 0.01	0.43 ± 0.01	0.47 ± 0.01	0.44 ± 0.02	.831	.735	.035*
WOB	0.54 ± 0.01	0.5 ± 0.01	0.53 ± 0.01	0.5 ± 0.02	.574	.7	.01*
ALH	3.48 ± 0.24	3.3 ± 0.09	3.35 ± 0.17	2.8 ± 0.19	.018*	.273	.034*
BCF	32.2 ± 1.4	33.15 ± 0.55	33.84 ± 0.8	37.34 ± 0.72	.474	.262	.014*

Note: Mean ± SEM for concentration and motility parameters of frozen–thawed good and bad fertility semen, before (BT) and after treatment (AT) with the ZyMot Multi (850 µl) device.

Abbreviations: ALH: amplitude of the lateral head displacement; BCF: beat-cross frequency; DAP: distance average path; DCL: distance curved line; DSL: distance straight-line; LIN: linearity; PM: progressive motility; STR: straightness; TM: total motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; WOB: wobble coefficient.

*$p < .05$.

TABLE 2 Results for various semen characteristic (see Note) analysed by microscopic evaluation of frozen–thawed stallion semen from ejaculates characterized being of good or poor fertility, before microfluid selection (BT) and thereafter (AT)

	Good fertility ($n = 16$)		Poor fertility ($n = 14$)		*p* - value		
	BT	AT	BT	AT			
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Stallion	Group	Treatment
MI	54.50 ± 4.51	84.81 ± 3.41	49.75 ± 2.89	86.05 ± 1.87	.519	.418	<.001*
MF	50.75 ± 3.37	70.41 ± 2.32	53.21 ± 3.85	68.50 ± 1.83	.517	.616	<.001*
MMP	56.19 ± 3.56	88.38 ± 2.56	49.71 ± 3.72	81.86 ± 2.51	.831	.218	<.001*
ACR	85.88 ± 1.67	94.38 ± 1.33	86.93 ± 1.82	92.00 ± 1.72	.673	.58	<.001*
Normal	77.44 ± 3.42	85.69 ± 2.37	79.86 ± 2.22	84.71 ± 2.05	.992	.884	.021*
Head	7.56 ± 2.06	1.69 ± 0.5	8.71 ± 2.28	3.64 ± 1.05	.984	.538	<.001*
Midpiece	2.75 ± 1.11	3.31 ± 0.83	3.86 ± 0.92	4.36 ± 1.39	.727	.458	.672
Tail	10.88 ± 1.4	9.38 ± 1.57	7.5 ± 1.52	7.29 ± 0.81	.339	.083	.571
SCC	88.81 ± 4.85	98.06 ± 1.22	92.86 ± 1.81	99.79 ± 0.15	.02*	.198	<.001*

Note: Mean ± SEM for membrane integrity (MI), membrane functionality (MF), mitochondrial membrane potential (MMP), acrosome integrity (ACR), morphology (normal; head anomalies; midpiece anomalies; tail anomalies) and sperm chromatin condensation (SCC) in frozen–thawed stallion semen. Parameters are divided by group in good fertility and poor fertility and by treatment in before (BT) and after treatment (AT).

*$p < .05$.

study, parameters reflecting sperm motion velocity, velocity ratios and sperm wobble characteristics were included into the analysis. Interestingly, in the present study, all these motion parameters with the exception of BCF decreased although the overall motility increased after microfluid selection. This reflects that microfluid recovery as performed in the present investigation apparently selects for a sperm subpopulation of frozen–thawed semen that is characterized by an overall intactness but also by sperm with

slower motility. This may be associated with the recovery method of the microfluid device and suggests that intact sperm with a specific type of motion may be privileged. The changes with regard to sperm kinematics after microfluid recovery seen in the present investigation suggest that the microfluidic chamber preferably selects sperm that do not present hyperactivation-like motility that has been described as typically non-progressive in stallions (Romero-Aguirregomezcorta et al., 2018). In this context, it is of interest that microfluid selection of human sperm by the same device as used in the present study resulted in a slower sperm population in comparison with selection by swim-up (Alexa et al., 2021) and is thus in agreement with present findings. Similar results were obtained in bulls (Nagata et al., 2018), in which microfluidics not only selected progressively motile sperm but also identified two different subpopulations with different patterns of motility: one characterized by linear progressive trajectory and symmetrical flagellar waves, and the other one with decreased VSL and LIN, but still progressive because of a sufficient BCF.

Because the percentage of sperm that were selected by the system was greater in frozen–thawed semen with better fertility, it is tempting to speculate that microfluid selection devices may indeed simulate selection criteria also active in the female genital tract as has been suggested (Ahmadkhani et al., 2022; Asghar et al., 2014). Several studies show that microfluidics is ideal to re-create the anatomy of the female genital tract, which is fundamental not only to select sperm with the best quality characteristics, but also to ensure the physiological development of the embryo (Ma et al., 2011). Microfluid selection should therefore be considered a tool for the prediction of the fertility of semen and further experiments in this regard may be of interest.

The prominent decrement in sperm concentration makes the microfluid devices very difficult to use for AI, especially in species like the horse where hundreds of millions of sperm are required for successful insemination of a female (Sieme et al., 2004). In fact, low-dose intra-uterine insemination produced unsuccessful results in these species and more sophisticated methods like deep uterine horn insemination or hysteroscopic insemination are required to obtain acceptable pregnancy rates with low-dose insemination technique (Sieme et al., 2004). In cattle, where a total number of 2–8 million sperm is considered a satisfying insemination dose, selection of frozen–thawed sperm by microfluid devices for insemination might be worth considering, for example in sexed semen with low fertility outcome.

To date, in vitro fertilization has been quite ineffective in the horse, probably because of a deficit of sperm capacitation in vitro. Intracytoplasmic sperm injection (ICSI) is an expensive and time-consuming procedure, but produced satisfactory results for fertilization and embryo development, giving the opportunity to select the best spermatozoa and freeze them in small aliquots (reviewed by Briski & Salamone, 2022). The ZyMot Multi (850 µl) may certainly help in sperm selection. It is a matter of fact that ICSI is a micromanipulation technique that only requires DNA integrity to allow fertilization, since the chosen spermatozoon is injected directly into the oocyte; therefore, there is no need for acrosomal integrity or sperm motility (Briski & Salamone, 2022). It is precisely on these grounds that microfluidics are particularly recommended for ART. In clinical application, this device would be able to provide a sufficient number of sperm for ICSI in a short time (30 min) and with the least manipulation possible, that is avoiding centrifugation and iatrogenic sperm injury. All these factors contribute to a decrease in oxidative stress and therefore avoid DNA damage (Asghar et al., 2014).

In the equine breeding industry, stallions are generally because of their pedigree and athletic merits but often without considering their reproductive capacities. Therefore, we often find ourselves working with poor-quality semen. Selecting the best spermatozoa from poor quality ejaculates with the ZyMot Multi (850 µl) device may help to increase pregnancy rates after ICSI.

5 | CONCLUSION

The ZyMot Multi (850 µl) selects a specific sperm population in any ejaculate, and this selection is more evident in frozen–thawed horse semen proven to be of low fertility. This is a very important result recommending this microfluid device for choosing spermatozoa for ICSI in an easy and fast way. Because the microfluid device also selects for a specific sperm population and most likely simulates selection systems also present in the female genital tract, there is the recommendation to use it as a tool for in vitro testing with the aim to predict the fertility of frozen–thawed horse semen.

AUTHOR CONTRIBUTIONS

Christine Aurich and Maria Elena Falomo defined the study design and performed the statistical analysis of data. Veronica Vigolo and Camille Gautier executed the study and prepared the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

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

DATA AVAILABILITY STATEMENT

Data will be made available on request.

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