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Short communication

Comparisons of the hypo-osmotic swelling and water tests to assess functional membrane integrity (FMI) of dog sperm from the sperm-rich fractions (SRFs) and whole ejaculates (WEs)

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Abstract

The aim of this study was to compare the hypo-osmotic swelling (HOS) and water-induced hypo-osmotic tests (Water test) by assessing the functional membrane integrity (FMI) of dog sperm from the sperm-rich fractions (SRFs) and whole ejaculates (WEs). ANOVA results showed that only sperm source had significant effects on the percentages of sperm cells with FMI. Both the HOS and Water tests indicated that sperm from the WEs exhibited significantly higher FMI than those from the SRFs. Scatter plot regression analysis confirmed highly positive significant relationships between the HOS and Water tests, particularly for sperm originating from the SRFs. Although the Bland-Altman method showed that the average discrepancy of the measurement of the FMI for the SRFs was higher than the WEs, the findings of the present study indicate that both HOS and the Water tests provided measurements that were in close agreement. Such findings confirm that both the HOS and Water tests detected similar populations of sperm cells with FMI either from the SRFs or WEs. We suggest that the significantly higher proportions of sperm with FMI from the WEs, detected by both the HOS and Water tests, reaffirm the important role of the sperm-coating components of the prostatic fluid in protecting the membrane structures of sperm exposed to hypo-osmotic conditions.

Keywords: dog, HOS test, sperm, water test

Introduction

The biochemical integrity of the sperm membrane is important for various sperm functions, such as motility and events associated with the sperm-egg fertilization processes (Jeyendran et al. 1984, Check et al. 2023). Different methods have been used to assess the structural and functional membrane integrity of sperm following different biotechnological processes (Abah et al. 2025). The hypo-osmotic swelling (HOS) test is simple and easily performed to assess the functional membrane integrity (FMI) of sperm cells (Bahamondes et al. 2001, Strzeżek and Fraser 2009, Check et al. 2023), and has been correlated with the *in vitro* fertilizing ability of sperm (Jeyendran et al. 1984). The basic principle of the HOS test is based on the swelling and coiling of sperm tails under hypo-osmotic conditions (Jeyendran et al. 1984, Quintela et al. 2010). If the sperm tails do not coil under hypo-osmotic conditions, the sperm membrane is considered to be damaged, suggesting that the sperm is dead (Jeyendran et al. 1984, Check et al. 2023). The water-induced HOS test is suggested to be based on the same basic principle as the HOS test, and has been used to assess the FMI of human sperm (Lomeo and Giambersio 1991, Bahamondes et al. 2001), and dog and feline sperm (Quintela et al. 2010, Prochowska et al. 2022). Moreover, the dog ejaculate consists mainly of the pre-sperm fraction, sperm-rich fraction (SRF) and post-sperm fraction (PSF), and the SRF, which is characterized by a higher sperm concentration compared with the other fractions, is commonly used in cryopreservation (Strzeżek and Fraser 2009). In contrast, the PSF comprises mainly the prostatic fluid, which contains specific factors that could reduce the sperm susceptibility to cryo-induced cold shock and osmotic damage (Strzeżek and Fraser 2009).

The aim of this study was to compare the relationships between the hypo-osmotic swelling (HOS) and water-induced hypo-osmotic tests (Water test) by assessing the FMI of dog sperm originating from the sperm-rich fractions (SRFs) and whole ejaculates (WEs). Scatter plot correlation analysis and the Bland and Altman agreement method were used to analyze the relationships between the HOS and Water tests (Bland and Altman 2010).

Materials and Methods

Animals

Four sexually mature dogs of mixed breed (4-10 yrs old) were used in this study. The SRFs and WEs (pre-sperm fraction, SRF and PSF) were collected by digital manipulation at a 1-week interval (Strzeżek and Fraser 2009). The SRFs and WEs were collected alternately from the same dogs, giving a total number of 20 SRFs and 20 WEs.

Experimental procedure

At collections, the concentration and motility of sperm from the SRFs and WEs were analyzed before treatment (Strzeżek and Fraser 2009). Aliquots of sperm samples (100×10^6 sperm/ml) from the SRFs and WEs were used for the HOS and Water tests. The procedure for the HOS test was performed according to a previously described method (Bahamondes et al. 2001), with a slight modification. Briefly, sperm samples (100×10^6 sperm/ml) from the SRFs or WEs (were diluted with 1 ml Tris-fructose-citrate (TFC) solution (150 mOsm/kg) and held for 60 min at 37°C, prior to analysis. The Water test procedure was performed according to the method described by Lomeo and Giambersio (1991), with some modifications. Sperm samples (100×10^6 sperm/ml) from the SRFs or WEs were added to 1ml re-distilled water, and incubated for 5 min at 37°C. Following incubations, sperm suspensions from the SRFs or WEs for both the HOS and Water tests were placed on a glass slide, covered with a coverslip and at least 200 sperm were examined at 200× under a light microscope. The percentage of sperm with morphological swelling of tails (HOS-positive swelling) was considered as sperm cells with FMI (Jeyendran et al. 1984).

Data were analyzed using GraphPad Prism, version 10.4.1 for Windows (GraphPad Software, Boston, Massachusetts USA), or using the Statistica software package, (TIBCO Software Inc. Statistica, CA, USA; StatSoft Polska, Kraków, Poland). The distributions of the data were monitored usingthe Shapiro-Wilk test. The main effects of the sperm source (SRF and WE) and treatment (HOS and Water tests) on the FMI were analyzed using a 2-way ANOVA. Scatter plots with regression analysis were used to visualize the relationships between the HOS and Water tests. The Bland and Altman method was used to analyze the measurement agreement between the two FMI tests for the SRFs or WEs, with the 95% confidence interval (CI) limits (Bland and Altman 2010).

Results and Discussion

Motility of sperm from the SRFs and WEs averaged 78.42±8.23% (SD), ranging from 70% to 90% (Strzeżek and Fraser 2009). The normal distributions of the measurements by the HOS and Water tests for the SRFs or WEs were confirmed by the Shapiro-Wilk test ($p \geq 0.05$). It was found that only sperm source had a significant

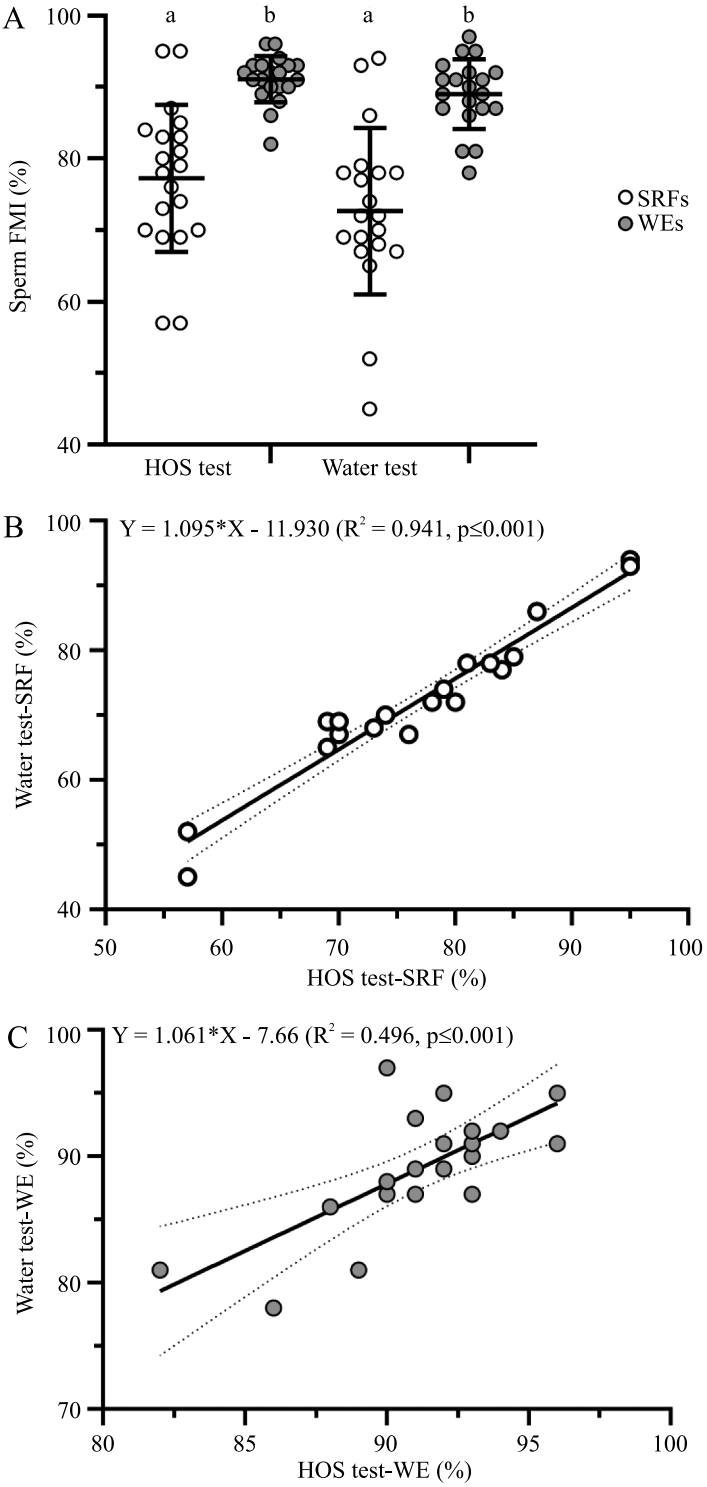

Fig. 1. Scatter plots and regression analysis of the functional membrane integrity (FMI) of dog sperm from the sperm-rich fractions (SRFs) and whole ejaculates (WEs). (A) Scatter plots showing the mean ± standard deviation (SD) of sperm FMI measured by the hypo-osmotic swelling (HOS) and Water tests (n=20 ejaculates, respectively). (B) Scatter plot with regression analysis of HOS and Water tests for the sperm-rich fraction (SRF). (C) Scatter plots with regression analysis of HOS and Water tests for the whole ejaculate (WE). Values with different letters are significant at p≤0.05. The solid line represents the linear regression correlation, while the 95% confidence intervals are shown by the broken lines.

effect (ANOVA Fisher test, 66.240, p≤0.001) on the percentages of sperm cells with FMI. Both the HOS and Water tests indicated that the WEs exhibited significantly higher (p≤0.05) percentages of sperm with FMI than those from the SRFs (Fig. 1A), thus reaffirming previous findings indicating that higher osmotic tolerance of sperm from the WEs might be attributed to the presence of specific sperm-coating components from the pros-

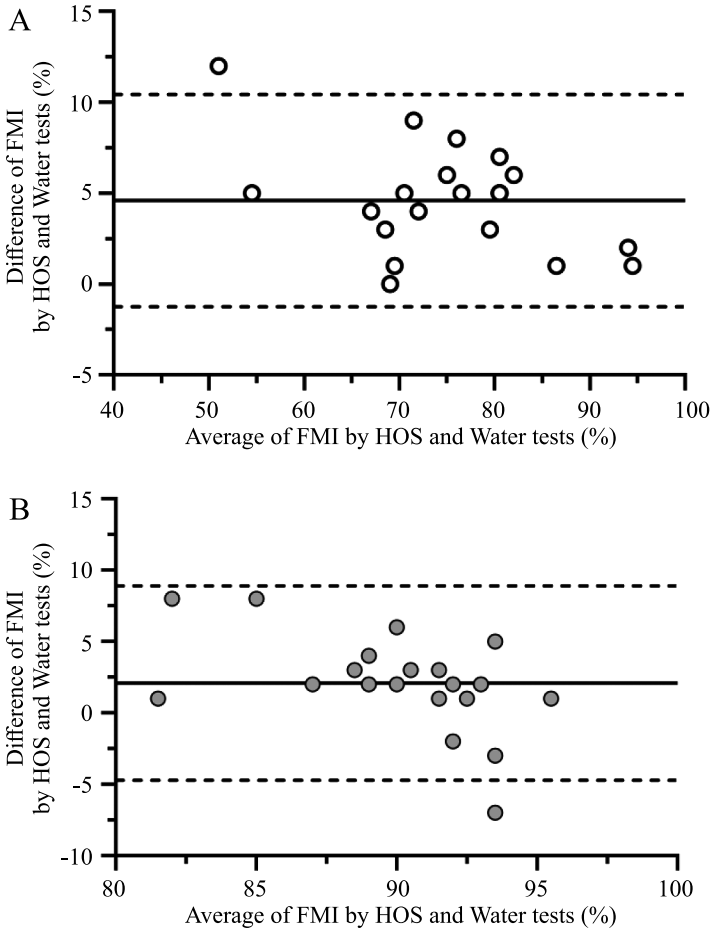

Fig. 2. Bland-Altman plots for the measurements of the functional membrane integrity (FMI) of dog sperm assessed by the hypo-osmotic swelling (HOS) and Water tests. (A) Sperm-rich fractions (SRFs). (B) Whole ejaculates (WEs). The solid line represents the average bias, while the 95% confidence intervals are shown by the broken lines.

tatic fluid (Strzeżek and Fraser 2009). Significant correlations were found between the HOS and Water tests for the SRFs and WEs (Fig. 1B and Fig. 1C, respectively).

Repeated measurements have been suggested as the best way to evaluate the repeatability of agreement analysis of two methods (Bland and Altman 2010). In this study the Bland and Altman method gives a visual representative of the quantitative measurements of the FMI assessed by the HOS and Water tests, and shows the degree of agreement between these two tests. The Bland-Altman plot demonstrated that the HOS test measured more than 4.60±2.98 (standard deviation, SD) of percentage points of FMI than the Water test (95% CI: 10.44 to -1.24) for the SRFs (Fig. 2A). Likewise, the Bland-Altman agreement confirmed that the HOS test detected more than 2.10±3.48 (SD) percentage points of FMI than the Water test (95% CI: 8.92 to -4.72) for the WEs (Fig. 2B). In the present study, both Bland-Altman plots show an outlier; however, the rest of the measurements of the FMI by the HOS and Water tests for either the SRFs or WEs were within the 95% CI limits. Although the average discrepancy (bias) of the measurements of the FMI for SRFs (Fig. 2A) was higher than the WEs (Fig. 2B), the findings of the present study indicate that both HOS and the Water tests provided measurements that were in close agreement, regardless of the sperm source. Such findings confirm the results shown in the column scatter plots (Fig. 1A), indicating that both the HOS and Water tests detected similar populations of sperm cells with FMI either from the SRFs or WEs. Similar findings were reported for human and dog epididymal sperm (Bahamondes et al. 2001, Hishinuma and Sekine 2003). However, our findings are not in accordance with those of Quintela et al. (2010), who demonstrated a significantly higher percentage of FMI of fresh dog sperm by the Water test compared with the HOS test. It seems that such a discrepancy might be attributed to the differences in the osmotic solutions used for the HOS test. In the current study, the TFC solution was adjusted to an osmolality of 150 mOsm/kg, whereas Quintela et al. (2010) used fructose solution with an osmolality of 60 mOsm/kg in their study. Notably, the HOS and

Water tests show a stronger relationship for sperm harvested from the SRFs (Fig. 1B) than those from the WEs (Fig. 1C). It is suggested that correlations measure the strength and direction between two variables, and that high significant correlation does not imply good agreement between the measurements of the two variables (Bland and Altman 2010).

Interestingly, it was reported that Na^+/K^+ ATP-ase was associated with the swelling reaction of dog sperm exposed to HOS conditions (100 mOsm/kg), whereas the sudden permeation of water through the sperm membrane was suggested to be a non-selective process (Hishinuma and Sekine 2003). The conditions employed in this study reaffirm that the HOS and Water tests give similar measurements, regardless of the source of the sperm, however, further studies on a larger animal population are required to elucidate the prognostic value of the two FMI tests for dog sperm subjected to different biotechnological techniques, such as cryopreservation.

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Author Declarations

Ethics approval

This study was approved by the Animal Experiments Local Ethics Committee, Olsztyn, Poland (approval number:11/WDZ/D on 30th November, 2005).

Use of generative artificial intelligence

The authors declare that no type of generative artificial intelligence was used for the preparation of this manuscript, creation of figures or their corresponding captions.

Conflict of interest

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

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