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ORIGINAL ARTICLE

Semen Analysis

Multivariate model for predicting semen cryopreservation outcomes in a human sperm bank

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Semen cryopreservation is widely used in assisted reproductive technologies, but it reduces sperm quality dramatically. The aim of this study was to develop a model using basal semen quality to predict the outcome of postthaw semen parameters and improve the efficiency of cryopreservation in a human sperm bank. Basal semen parameters of 180 samples were evaluated in the first stage, and a multiple logistic regression analysis involving a backward elimination selection procedure was applied to select independent predictors. After a comprehensive analysis of all results, we developed a new model to assess the freezability of sperm. Progressive motility (PR), straight-line velocity (VSL) and average path velocity (VAP) were included in our model. A greater area under the receiver operating characteristic curve was obtained in our model when compared with other indicators. In the second stage of our study, samples that satisfied the new model were selected to undergo freeze–thawing. Compared with the first stage, the rate of good freezability was increased significantly (94% vs 67%, $P = 0.003$). By determining basal semen quality, we have developed a new model to improve the efficiency of cryopreservation in a human sperm bank.

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INTRODUCTION

Cryopreservation involves the freezing of living cells and tissues and storage at -196°C or below. At this temperature, all metabolic processes are arrested including some cellular changes that can result in cell death. Sperm cryopreservation has a history of more than 70 years. It helps keep spermatozoa alive indefinitely, enables the conservation of male fertility and is widely used in assisted reproductive technologies (ARTs). It is of great importance for patients undergoing medical or surgical treatments that could induce sterility, such as those with cancer about to undergo chemotherapy or radiotherapy, as it offers them the hope of future fertility and psychological support in the therapeutic process.¹ In some cases, couples cannot use the male partner's own spermatozoa for ART, so they might benefit from using donor spermatozoa obtained from a bank of frozen samples.

However, cryopreservation adversely affects spermatozoa in terms of standard semen parameters and fertilizing ability.^{2,3} The recovery of functionally intact spermatozoa from thawed samples varies between individuals and is dependent on the cryopreservation process but also on the initial quality of the semen sample. One aspect of great importance in Andrology is the identification of men whose ejaculates are more suitable to undergo cryopreservation procedures. Although a protocol is indicated in the World Health Organization (WHO) manual for semen analysis,⁴ the procedure is not standardized. To date, there is a lack of markers for predicting the quality of semen samples following the freeze–thawing procedure.

Many studies have tried to find predictors of sperm freezability success through evaluating prefreezing characteristics. Lee *et al.* reported that, compared with conventional semen analysis, Kruger strict morphology criteria were better predictors of the postthaw progressive motility recovery.⁵ Others found that higher concentration and prefreeze motility and fewer days of abstinence before producing a semen sample were associated with an increased sperm recovery rate through evaluating the relationship between prefreezing and postthawing semen characteristics.^{6–8} In general, it appears that the postthawing recovery is associated with basal semen quality, but previous studies only used single parameters to predict the outcome after thawing without considering the possibility of combining parameters.

Therefore, the aim of this study was to evaluate basal semen indexes to develop a new diagnostic tool for predicting freezability. This new model should help to minimize cryoinjury, maximize sperm survival, and contribute to the development of an optimized cryopreservation protocol.

MATERIALS AND METHODS

Sample collection

This study was approved by the Ethics Committee of Nanjing Medical University. All subjects gave written consent with regard to the storage of their information for the purpose of research. The study was performed in accordance with national and international guidelines.

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The first stage included 180 semen samples from 180 normozoospermic donors contributing to our Human Sperm Bank at The First Affiliated Hospital of Nanjing Medical University. Semen samples were collected in sterile containers by masturbation after 3–7 days of sexual abstinence. All the semen samples underwent clinical and laboratory evaluations in our sperm bank in accordance with the WHO laboratory manual for the examination and processing of human semen, Fifth Edition.⁴ Normozoospermic samples needed to have the following characteristics: volume >2 ml; liquefaction time <1 h; sperm concentration >60 × 10⁶ ml⁻¹; progressive motility (PR) >60% and normal morphology >5%. The ages of donors, days of sexual abstinence, semen volumes and liquefaction time, and other clinical data were recorded. Each liquefied semen sample was divided into two aliquots: one was analyzed fresh for seminal parameters, and the other was frozen for 7 days and analyzed soon after thawing for sperm motility and vitality.

In the second stage, we selected 75 samples that fulfilled the original standards. Within these, 31 samples satisfied our new model and underwent freezing–thawing. Finally, we compared the characteristics of good freezability ejaculates between the two stages.

Pre- and post-treatment semen analyses

Samples were analyzed before and after thawing for volume, morphology, concentration and motility, using the WHO guidelines.⁹ Morphology was evaluated using an optical microscope (Axioskop 2 Plus, Zeiss, Jena, Germany) with × 1000 magnification under oil immersion. Sperm motility and concentrations were determined using computer-aided sperm analysis (CASA, CFT-9201, Rich, Xuzhou, China). In each analysis, a minimum of 200 spermatozoa were assessed and the following sperm motility parameters were recorded: progressive motility (PR, %), nonprogressive motility (NP, %), immotility (IM, %), total motility (TM, %), curvilinear velocity (VCL, μm s⁻¹), straight-line velocity (VSL, μm s⁻¹), average path velocity (VAP, μm s⁻¹), percentage of straight paths (% STR = VSL/VAP × 100), motility parameter wobble (% WOB = VAP/VCL × 100), percentage of linearity (% LIN = VSL/VCL × 100), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), mean moving angle (MAD°) and sperm concentration (10⁶ ml⁻¹). The computer settings for detecting progressively motile spermatozoa were VSL >25 μm s⁻¹; or 5 μm s⁻¹ < VSL ≤ 25 μm s⁻¹, STR >80% and LIN >50%. Three replicates per sample were evaluated before calculating the corresponding mean ± s.d.

Freezing and thawing procedures

The liquefied semen samples were mixed with an equal volume of 10% glycerol–10% yolk freezing medium.¹⁰ The equilibrated samples were transferred to 2 ml cryovials (Greiner Bio-One GmbH, Frickenhausen, Germany) and a programmable freezer (Kry320-1.7, Planer PLC, Sunbury-on-Thames, UK) to obtain cooling from +20°C to –80°C. Then, the cryovials were removed and stored in liquid nitrogen (–196°C). After 7 days, the frozen samples were thawed in a water bath at 37°C for 10 min.

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics (version 20.0; IBM Corp., Armonk, NY, USA) and R, version 3.2.1 (<http://www.r-project.org/>) for Windows. The Kolmogorov–Smirnov test was used to test sample distributions. Differences between the means of parameters were analyzed using Student's *t*-test for normally distributed variables and the Mann–Whitney nonparametric U-test for nonnormally distributed variables. Multiple logistic regression analysis with a backward elimination selection procedure was applied. Parameters showing significant differences (*P* < 0.05) were included into

a nomogram for good sperm freezability. An equation for the freezability rate (FR) was developed on the basis of the final logistic regression model. Receiver operating characteristic (ROC) curves were generated as a binary classifier system to identify the accuracy of prefreezing semen parameters in predicting the success rate of cryopreservation. In all analyses, the significance level was set at *P* < 0.05.

RESULTS

Effect of cryopreservation on sperm parameters

In accordance with the standard of postthaw PR ≥40%,^{11,12} samples were grouped into poor freezability ejaculates (PFE, *n* = 59) and good freezability ejaculates (GFE, *n* = 121), of which 67.2% (121/180) showing good freezability results. Basic semen characteristics in the first stage are shown in **Table 1**. The median age of both groups of donors was 22 years (range: 19–35) with no statistically significant difference. Days of sexual abstinence, volume, and liquefaction time also had no statistically significant differences between the PFE and GFE groups.

The 14 sperm parameters before and after cryopreservation are shown in **Table 2**. After thawing, the PR, NP, TM, VCL, VAP, MAD, and ALH values, concentration and percentage of normal morphology of all samples decreased dramatically. Significant increases in LIN, WOB and STR values were observed. However, cryopreservation had no effect on VSL or BCF.

Prognostic value of sperm parameters

As shown in **Table 2**, the PR, TM, VCL, VSL, VAP, and ALH values were dramatically higher in the GFE than in the PFE samples among all the evaluated parameters of prefreezing samples. These parameters were included into our multivariate logistic analysis. Sperm concentration was also higher in GFE samples but not significantly (*P* = 0.081). Considering the important role of concentration in semen quality, we included it in our logistic analysis. After a backward elimination selection procedure, PR, VSL, and VAP showed significant differences indicating that they were potential predictors for freezability (**Table 3**).

ROC analysis was used to identify the accuracy, sensitivity and specificity of PR, VSL and VAP (**Figure 1**) in predicting good freezability. The areas under the curve (AUC) of PR, VSL, and VAP were 0.746, 0.589, and 0.612 respectively, and the PR percentage had the highest predictive value for good freezability.

Establishment of multivariate prediction model

The model was created based on the results of our logistic analysis. PR, VSL, and VAP values were included to establish the model. The equation for freezability rate (FR) was defined as follows:¹³

$$FR = \frac{e^{-10.178 + 0.18 \times PR - 0.79 \times VSL + 0.7 \times VAP}}{1 + e^{-10.178 + 0.18 \times PR - 0.79 \times VSL + 0.7 \times VAP}}$$

We then developed a nomogram resulting from the graphical representation of multivariate regression analysis of the studied

Table 1: Basic information of semen samples in Stage 1 (mean±s.d.)

Variables	Total (n=180)	PFE (n=59)	GFE (n=121)	<i>P</i>
Age (years)	22.19±3.01	22.14±2.78	22.21±3.13	0.857 ^a
Day	4.3±0.93	4.34±0.98	4.28±0.92	0.842 ^a
Volume (ml)	3.91±1.46	3.99±1.51	3.87±1.44	0.685 ^a
Liquefaction time (min)	26.84±5.62	26.51±5.74	27.01±5.58	0.548 ^a

^aMann–Whitney U-test for age, day, volume, and liquefaction time between PFE and GFE. s.d.: standard deviation; Day: day of sexual abstinence; PFE: poor freezability ejaculates; GFE: good freezability ejaculates



Table 2: Basic parameters of sperm before and after cryopreservation in Stage 1 (mean±s.d.)

Variables	Prefreeze		P ^a	Prefreeze (n=180)	Postthawing (n=180)	P ^b
	PFE (n=59)	GFE (n=121)				
PR (%)	61.34±6.71	67.66±6.96	<0.001	65.59±7.48	41.92±12.71	<0.001
NP (%)	17.42±5.97	18.01±5.29	0.504	17.82±5.51	7.67±3.94	<0.001
TM (%)	79.15±6.44	85.97±6.11	<0.001	83.74±6.98	50.07±14.40	<0.001
VCL (µm s ⁻¹)	51.99±6.05	55.22±6.76	0.002	54.16±6.70	50.62±6.89	<0.001
VSL (µm s ⁻¹)	34.32±3.96	35.71±4.57	0.046	35.26±4.41	35.07±5.15	0.642
VAP (µm s ⁻¹)	37.86±3.95	39.69±4.76	0.007	38.09±4.58	37.76±5.16	0.001
MAD (°)	55.40±7.76	57.16±6.65	0.117	56.58±7.06	51.72±6.24	<0.001
ALH (µm)	4.21±1.02	4.56±0.95	0.028	4.45±0.99	3.44±0.94	<0.001
BCF (Hz)	5.12±0.51	4.99±0.44	0.088	5.03±0.47	4.98±0.59	0.287
LIN (%)	63.63±6.67	62.66±5.88	0.320	62.98±6.15	67.65±5.79	<0.001
WOB (%)	71.47±5.83	70.53±5.05	0.264	70.84±5.32	74.18±4.96	<0.001
STR (%)	86.95±2.77	86.76±2.59	0.653	86.82±2.64	89.38±3.31	<0.001
Concentration (10 ⁶ ml ⁻¹)	130.42±51.34	141.85±48.02	0.081	138.1±49.28	58.70±22.50	<0.001
Normal morphology (%)	29.08±5.19	30.10±4.37	0.170	29.77±4.66	13.82±2.18	<0.001

^aBefore freezing, PR, TM, VCL, VSL, VAP and ALH were significantly different between GFE and PFE among all the evaluated parameters; ^bAfter thawing, PR, NP, TM, VCL, VAP, MAD, ALH, concentration and normal morphology percentage of the entire population decreased significantly; LIN, WOB, and STR increased significantly. s.d.: standard deviation; PR: progressive motility; NP: nonprogressive motility; TM: total motility; VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; MAD: mean moving angle; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; LIN: percentage of linearity; WOB: motility parameter wobble; STR: percentage of straightness; PFE: poor freezability ejaculates; GFE: good freezability ejaculates

Table 3: Multivariate analysis of the predictors of freezability ejaculates rate^a

Variables	B	OR	95% CI for OR		P
			Lower limit	Upper limit	
Intercept	-10.178				<0.001
PR (%)	0.177	1.193	1.115	1.277	<0.001
TM (%)	0.069	1.072	0.972	1.182	0.166
VCL (µm s ⁻¹)	0.078	1.081	0.878	1.330	0.464
VSL (µm s ⁻¹)	-0.786	0.456	0.293	0.710	0.001
VAP (µm s ⁻¹)	0.697	2.008	1.318	3.058	0.001
ALH (µm)	-0.075	0.927	0.563	1.528	0.767
Concentration (10 ⁶ ml ⁻¹)	-0.009	0.991	0.978	1.004	0.157

^aPR, TM, VCL, VSL, VAP, ALH and concentration were included in our logistic analysis with a backward elimination scheme. PR, VSL, and VAP showed significant difference (P<0.05) and were included into an equation for freezability rate. PR: progressive motility; TM: total motility; VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; ALH: amplitude of lateral head displacement; OR: odds ratio; CI: confidence interval

variables (Figure 2). As shown in Figure 1, the new model had a higher AUC (0.789) than did single parameters. Considering that a higher Youden's index (YI = sensitivity + specificity - 1) should be maintained, we determined the cutoff FR value at 0.8324, which provided a sensitivity of 52.9% and a specificity of 93.2%. Donors with an FR >0.8324 were entered into the high-rate group, whereas others were entered into the low-rate group. The actual rate of good freezability in the high-rate group was significantly higher than in the low-rate group (94% vs 51% of donors, P < 0.001).

In the second stage, 75 candidate semen donors were recruited and evaluated using our new model. Of these, 31 high-rate samples were chosen and subjected to the standard freeze-thawing program. The characteristics of the donors in both stages are shown in Table 4. No significant difference in basic conditions was found between the two stages. The rate of good freezability among samples was improved from 67% to 94% (P = 0.003) compared with the retrospective cohort.

DISCUSSION

Sperm cryopreservation in ART normally involves the freezing and storage of semen samples at -196°C in liquid nitrogen. It has potentially

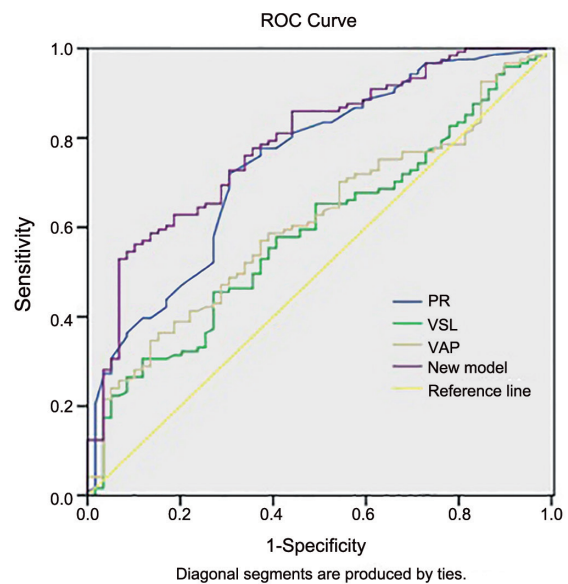


Figure 1: Receiver operating characteristic (ROC) curve analysis of PR, VSL, and VAP values and the new model. The areas under the curve (AUC) of these predictors were 0.746, 0.589, 0.612, and 0.789, respectively. PR (%): progressive motility; VSL (µm s⁻¹): straight-line velocity; VAP (µm s⁻¹): average path velocity.

opened opportunities for fertility preservation in a variety of situations including the gonadotoxic effects of chemotherapy or radiotherapy, anatomical and pathological defects of the male reproductive system, cryptozoospermia, transient azoospermia, and for rare occupational reasons.

However, cryopreservation can reduce sperm fertility through impairing sperm DNA integrity, motility and viability.^{2,3} Many studies on human and animal semen have attempted to define predictors of freezability. These suggested that sperm freezability is associated with basal prefreezing semen qualities. Dong *et al.* found that sperm

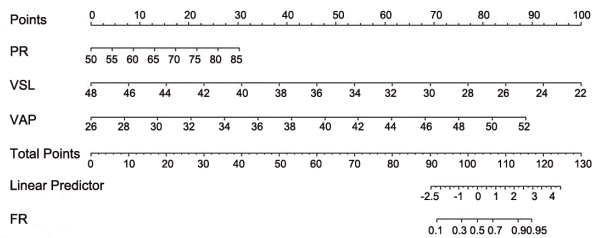


Figure 2: Nomogram for predicting the probability of good freezability ejaculates. Locate semen values on each axis, and compare these with the “Point” axis to determine how many points are attributed to each variable. Then, locate the sum of the points for all variables on the “Total Points” line to determine the possibility of good freezability ejaculates on the “FR” line. PR (%): progressive motility; VSL ($\mu\text{m s}^{-1}$): straight-line velocity; VAP ($\mu\text{m s}^{-1}$): average path velocity; FR: freezability rate.

Table 4: Comparison of candidates’ characteristics between two stages^a

Variables	Before new model (n=180)		Using new model (n=75)		P
	Mean \pm s.d.	Median	Mean \pm s.d.	Median	
Age (years)	22.19 \pm 3.01	21	22.39 \pm 3.05	21	0.634
Day	4.3 \pm 0.93	4	4.28 \pm 0.88	4	0.874
Volume (ml)	3.91 \pm 1.46	3.8	3.93 \pm 1.37	4	0.924
Liquefaction time (min)	26.84 \pm 5.62	30	27.55 \pm 5.53	30	0.362
PR (%)	65.59 \pm 7.48	65.00	66.97 \pm 7.58	67.00	0.183
NP (%)	17.82 \pm 5.51	17.705	17.78 \pm 5.96	17.53	0.957
TM (%)	83.74 \pm 6.98	84.11	84.90 \pm 6.69	84.81	0.222
VCL ($\mu\text{m s}^{-1}$)	54.16 \pm 6.70	54.035	55.81 \pm 5.94	56.12	0.065
VSL ($\mu\text{m s}^{-1}$)	35.26 \pm 4.41	35.165	36.08 \pm 4.32	35.35	0.174
VAP ($\mu\text{m s}^{-1}$)	38.09 \pm 4.58	39.05	39.94 \pm 4.39	39.49	0.173
MAD ($^{\circ}$)	56.58 \pm 7.06	57.065	58.10 \pm 7.41	58.07	0.123
ALH (μm)	4.45 \pm 0.99	4.395	4.71 \pm 1.00	4.63	0.059
BCF (Hz)	5.03 \pm 0.47	4.96	5.00 \pm 0.43	4.99	0.589
LIN (%)	62.98 \pm 6.15	62.42	62.44 \pm 6.38	61.66	0.531
WOB (%)	70.84 \pm 5.32	70.515	70.00 \pm 5.51	69.78	0.268
STR (%)	86.82 \pm 2.64	86.71	87.17 \pm 2.95	87.22	0.350
Concentration (10^6 ml^{-1})	138.1 \pm 49.28	129.35	131.83 \pm 59.07	121.96	0.398
Normal morphology (%)	29.77 \pm 4.66	30.59	29.35 \pm 4.59	30.45	0.510

^aThere was no significant difference in basic conditions between stage one and two. s.d.: standard deviation; Day: day of sexual abstinence; PR: progressive motility; NP: nonprogressive motility; TM: total motility; VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; MAD: mean moving angle; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; LIN: percentage of linearity; WOB: motility parameter wobble; STR: percentage of straightness

cryosurvival was affected by the original sperm concentration in semen from rhesus monkeys,¹⁴ while Dorado *et al.* found that the percentages of sperm motility and abnormal morphology of fresh semen were the best parameters to predict postthaw motility in goats.¹⁵ In the study by Lee *et al.* the postthaw recovery of progressive motility of human spermatozoa showed no correlation with prefreezing semen parameters, such as sperm concentration, progressive motility or WHO-based criteria morphology, but it was significantly correlated with the percentage of spermatozoa with normal morphology assessed by strict application of Kruger criteria.⁵ Others found that the recovery of progressive motility of frozen spermatozoa was significantly and positively affected by sperm concentration, progressive motility and the percentage of normal morphology.^{6,8,16} These results were confirmed

in a large-scale investigation by Zhang *et al.*⁷ and suggest that we can use basal semen qualities to predict the outcome of cryopreservation.

Multiple logistic regression analysis is widely used in clinical studies such as urology,¹⁷ osteology,¹⁸ and intensive care.¹⁹ On the basis of the observed data, this approach can establish appropriate dependent relationships between variables, and is usually applied in forecasting and control issues. In this study, we evaluated basal semen quality and used multiple logistic regression analysis to develop a new model as a diagnostic tool for predicting freeze–thawing success.

Cryopreservation resulted in decreases in PR, NP, TM, VCL, VAP, MAD, and ALH values, and in the percentage of spermatozoa with normal morphology. After eliminating the influence of dilution, sperm concentration still showed a significant decrease postthawing. Low VCL and ALH values are normally associated with the PR percentage, as are high LIN, WOB and STR values.²⁰ In general, the freeze–thawing procedure impairs the sperm motility parameters and causes a dramatic decrease in sperm quality.

Samples were classified as PFE or GFE according to postthaw progressive motility. After univariate analysis of prefreezing parameters, the PR, TM, VCL, VSL, VAP, and ALH values showed significant differences while sperm concentration showed a borderline significant difference ($P = 0.081$) between PFE and GFE samples. All these parameters were included into our multivariate logistic analysis, and the PR, VSL, and VAP values showed significant differences between the PFE and GFE groups.

Dynamic motility parameters as measured by CASA reflect the subtle movement characteristics of sperm objectively, and are significantly associated with fertilization rates.²¹ Only active spermatozoa can approach oocytes, and motility is essential to penetrate the cumulus oophorus and zona pellucida.²² CASA enables the rapid, quantitative analysis of sperm movement. Apart from the PR percentage, as shown in this study, VSL and VAP were also proved to be predictors for freezability. These are important parameters of sperm speed and reflect sperm motility effectively.²³ Unlike the LIN, and WOB measures, PR, VSL, and VAP are original measures rather than derived ones. We found that these parameters of sperm velocity were more accurate predictors of cryopreservation success. The results suggest that we can use these accessible CASA parameters to predict the outcome of cryopreservation.

According to the results of the logistic analysis, PR, VSL, and VAP values were included to construct our model. Compared with PR alone, this increased the AUC of the ROC analysis from 0.746 to 0.789. On the basis of the FR values, donors with an $\text{FR} > 0.8324$ were placed into the high-rate group while others were entered into the low-rate group. A notably higher percentage of good freezability ejaculates was revealed in 68 donors in the high-rate group than in 112 donors in the low-rate group (94.1% vs 50.9%, $P < 0.001$). Only 4/68 samples (6%) in the high-rate group turned out to have poor freezability, with a very low failure rate.

In the second stage of our study, we aimed to verify our new model prospectively in 75 donors who fulfilled the original standard. Of these, 31 samples underwent freeze–thawing procedure because of their higher FR values. The success rate of cryopreservation was improved significantly (94% of samples vs 67% in the original cohort; $P = 0.003$), which would minimize the use of manual labor and material resources. If the number of suitable sperm donors is inadequate, a lower FR target could be used.

CONCLUSIONS

We have developed an effective model based on sperm motility parameters derived by CASA to predict postthawing sperm motility from simple prefreezing variables. This model could predict the

sperm vulnerability to cryoinjury in human sperm banks, so that it could minimize the need for freeze–thawing of new semen samples to acquire data on thawing rate success. We should validate these findings in a larger cohort of semen donors to establish a more robust model. Further, more research is warranted to improve cryopreservation methods and diminish the impact of cryodamage on sperm quality.

AUTHOR CONTRIBUTIONS

ZJW and WZ conceived and designed the experiments. XPJ, WMZ, and SQW performed the experiments. WW, JYT, and ZX analyzed the data. ZXZ and CQ contributed reagents, materials and analysis tools. XPJ wrote the paper. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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