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Original Article

Aconitate 2 (ACO2) and pyruvate kinase M2 (PKM2) are good predictors of human sperm freezability

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Abstract: The aim of this study was to find the useful and reliable biomarkers to predict human sperm freezability. According to progressive motility of the thawed sperm, ejaculate samples were classified as good freezability ejaculates (GFE) and poor freezability ejaculates (PFE). Before starting cryopreservation protocols, we chose ACO2 and PKM2 as probable markers of sperm freezing capacity to compare their expressions between GFE and PFE group using quantitative real-time PCR (qRT-PCR), western blot and immunofluorescence. Moreover, Pearson's linear correlation was used to assess the association between ACO2 and PKM2 expressions before cryopreservation and the sperm motility before cryopreservation. The expressions of ACO2 and PKM2 were significantly higher in GFE group compared to PFE group in both qRT-PCR and western blot analysis. Besides, the immunofluorescent staining confirmed these results and showed that ACO2 localized in the sperm midpiece, and PKM2 was located in the body and tail of spermatozoa. Pearson's linear correlation demonstrated a significant positive correlation between sperm freezability and normalized band volume of ACO2 and PKM2 before cryopreservation. In this study, the expressions of ACO2 and PKM2 before cryopreservation were higher in GFE group than PFE group, which suggested that these two proteins could be used as predictive markers of human sperm freezability.

Keywords: Sperm freezability, predictors, aconitate 2, pyruvate kinase M2

Introduction

Sperm cryopreservation has been carried out as a secure and efficient technique for preserving sperm samples and the treatment of human infertility for long-term time. Importantly, it is widely applied in some infertile patients, especially in patients with defective genitals, oligozoospermia or asthenospermia and cancer patients which are given potentially sterilizing therapies, such as chemotherapy or radiotherapy [1, 2]. Although the cryopreservation of spermatozoa is an important routine method, freeze-thawing protocols are subjected to affect human sperm function and survival, which may lead to a series of drastic alterations in acrosome and nucleus integrity, plasma membrane, and the quality and fertility of spermatozoa [3-5]. Moreover, sperm fertilizing ability obviously decreases after freezing and thawing in a certain amount of samples [6, 7].

Because there are great variations between different individuals in the ability of sperm to withstand the stresses, not all sperm are resistance to cold shock [8]. Thus, ejaculates can be classified into good freezability ejaculates (GFE) and poor freezability ejaculates (PFE) based on their resistance to cryoinjury [9]. In addition, although sperm motility and percentage of normal morphology play a vital role in the prediction of human ejaculate freezability, studies have found that the routine analysis of semen quality before freezing has a low value to predicte freezability, and cannot be considered as a freezability marker [10]. Previous works have found that some freezability markers have

linked this aspect with genetic differences in human semen samples, and amplified fragment length polymorphisms have also been suggested as freezability predictors in terms of variation of post-thaw sperm quality [11].

Proteomic techniques have been extensively applied in terms of human sperm cryopreservation, and some proteins have been found to be probable predictive markers of sperm freezability, like enolase1 (ENO1), vimentin (VIM) and tektin-1 (TEKT1) [12]. Therefore, these findings suggested that we could find specific proteins of human ejaculates as good predictive markers to assess human sperm freezing capacity. Sperm motility need adequate supply of energy in the form of ATP and glycolysis plays a vital role in energy metabolism. Mitochondrial aconitate (ACO2) and pyruvate kinase M2 (PKM2) are closely related to glucose metabolism and our previous study showed these two proteins had marked differences between the fresh and freeze-thawed sperm samples [12-14]. Therefore, we selected ACO2 and PKM2 as research objects to find specific proteins of human sperm freezing capacity.

Materials and methods

Sample collection

Prior to sample collection, approval for this study was granted by the Ethics Committee of Nanjing Medical University. The study was performed in accordance with national and international guidelines. The present study included 40 ejaculate samples from 40 normozoospermic donors obtained at Human Sperm Bank, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China. Before enrollment, all the donors received a thorough explanation of this study's purpose, benefit, and possible risks by well-trained interviewers and were required to sign an informed consent to participate in the present study. All semen samples harvested in sterile containers were collected through masturbation after 3-5 days of sexual abstinence. All candidates were given routine semen assessments in our sperm bank, according to the fifth edition of the World Health Organization (WHO) laboratory manual for the examination and processing of human semen [15]. Besides, all semen samples met the following criterias: liquefaction time (min) < 60; volume (ml) > 2; sperm concentration (× 10^6/ml) > 20; motility ("a+b", progressive) > 60%; and normal morphology > 5%. Patient demographic information and clinic feature was showed in Table S1. We divided each liquefied semen sample into two aliquots, one part was processed for fresh-related experiments and the other one was immediately mixed with freezing medium and frozen for 7 days before further investigation.

Assessment of spermatozoa motility and concentration

Motility of fresh and thawed sperm specimens was evaluated with a Makler counting chamber. Each semen was evaluated "rapid (a)", "progressive (a+b)", "total motility (a+b+c)", or "static (d)", according to the WHO laboratory manual [15]. We also evaluated the spermatozoa concentrations and morphology with a Makler chamber before and immediately after the cryopreservation procedure. In accordance with the standard of postthaw motility (a+b) > 40%, semen samples with motility (a+b) > 40% were grouped in good freezability ejaculates (GFE) and motility (a+b) ≤ 40% were considered as poor freezability ejaculates (PFE) [16].

Semen cryopreservation and thawing

The liquefied semen samples were mixed with an equal volume of 10% glycerol-yolk freezing medium. Then the equilibrated samples were transferred to cryovials (Greiner Bio-One, Germany) and a programmable freezer to obtain cooling from 20°C to -80°C. The cryovials were removed and then transferred directly to liquid nitrogen at -196°C. After 7 days, the cryovials was left at 37°C for 7 minutes in a thermostat and water bath for thawing.

Sample preparation for protein assessment

The method was performed as previously reported, fresh specimens were washed on a 60% Percoll gradient (GE Healthcare, USA) and centrifuged at 800 × g for 7 min. After discarding the supernatant, the suspension of the sperm was washed twice in Biggers-Whitten-Whittingham (BWW) medium (GenMed Scientifics, USA) and centrifuged at 500 × g for 5 min. The sperm pellets were resuspended using lysis buffer containing 1% protease inhibitor (Thermo Scientific, USA). Then sperms were disrupted by sonication at 20 joules for 2

Table 1. Parameters of sperm before and after cryopreservation

	Pre-freeze			Post-thawing		
	GFE	PFE	Total	GFE	PFE	Total
Case number	28	12	40	28	12	40
Volume (ml)	4.03 ± 1.49	4.12 ± 1.27	4.08 ± 1.09	1.00	1.00	1.00
Liquefaction time (min)	26.48 ± 3.45	26.67 ± 4.57	26.58 ± 3.74	-	-	-
Concentration (M/ml)	124.19 ± 21.43	102.09 ± 13.76	114.76 ± 18.54	42.37 ± 9.82	49.53 ± 12.93	45.74 ± 8.23
Normal morphology (%)	33.45 ± 3.82	31.63 ± 2.17	32.63 ± 3.48	14.89 ± 2.58	12.81 ± 1.94	13.85 ± 2.35
Progressive motility (%)	65.45 ± 5.28	62.83 ± 3.27	63.97 ± 4.82	52.05 ± 7.82	31.82 ± 3.78*	43.32 ± 7.53
VCL (lm/s)	57.18 ± 6.86	60.35 ± 7.45	58.78 ± 5.48	58.73 ± 7.69	53.09 ± 5.62	56.75 ± 6.83
VSL (lm/s)	38.98 ± 4.74	36.33 ± 3.91	37.69 ± 4.16	39.57 ± 5.94	36.44 ± 4.32	38.26 ± 5.42
VAP (lm/s)	41.33 ± 4.12	39.94 ± 4.18	40.73 ± 4.25	42.77 ± 5.18	38.39 ± 3.79	40.74 ± 5.03
MAD (°)	57.91 ± 4.48	59.86 ± 5.06	58.37 ± 5.26	51.79 ± 4.97	52.33 ± 3.43	52.30 ± 4.80
ALH (lm)	4.82 ± 1.40	4.63 ± 1.48	4.86 ± 1.43	3.73 ± 0.28	3.41 ± 0.37	3.60 ± 0.63
BCF (Hz)	5.03 ± 0.73	5.22 ± 0.39	5.17 ± 0.43	4.95 ± 0.36	5.29 ± 0.43	5.09 ± 0.38

Velocities (VCL, curvilinear; VSL, straight line; VAP, average path); GFE, good freezability ejaculates; PFE, poor freezability ejaculates. MAD, mean angular displacement; ALH, amplitude of lateral head deviation; BCF, beat cross frequency. Significant differences are indicated as *P < 0.01 between GFE and PFE.

s × 10 at intervals of 15 s. We maintained the samples on ice for more than 1 h and shaked them for every 15 min. After centrifuging at 20,000 × g for 1 h at 4°C to remove insoluble material, the supernatant was collected. The extracted protein concentration of each sample was determined by Bradford microprotein assay (Beyotime Institute of Biotechnology, China). The sperm lysates were ready for western blot.

Quantitative real-time PCR (qRT-PCR)

Fresh specimens were lysed by the TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, RNA to cDNA from 1 mg of total RNA was reverse transcribed in a final volume of 10 mL using random primers and a Reverse Transcription Kit (Takara, China). Subsequently, 1 μl of cDNA was amplified as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 10 s. The reactions were made and analyzed by ABI 7900 system (Applied Biosystems, Carlsbad, USA).

The PCR primers were as follows: ACO2: Forward: 5'-GAGCGAGGCAAGTCGTACC-3', Reverse: 5'-GGCTTCAATCAGATGGTCACAG-3'; PKM2: Forward: 5'-ATGTCGAAGCCCCATAGTGAA-3', Reverse: 5'-TGGGTGGTGAATCAATGTCCA-3'; β-actin: Forward: 5'-TCACCCACACTGTGCCCATCTACGA-3', Reverse: 5'-CAGCGGAACCGCTCATTGCCAATGG-3'.

Western blot analysis

Specimens containing 50 μg of protein from fresh sperms were electrophoresed on a 12% SDS polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Bio-Rad, USA). The membranes were blocked with blocking solution (TBS containing 5% nonfat milk powder) for 1 h and then incubated overnight at 4°C with agitation with primary antibodies, polyclonal anti-ACO2 (1:1000, Abcam, USA), PKM2 Antibody (1:1000, Abcam, USA) and anti-β-actin (1:1000, Abcam, USA). β-actin was used as a protein control to normalize protein expression. After membranes were washed with TBS 10 min for three times, they were incubated for 1 h with HRP-conjugated goat-anti-rabbit IgG or goat-anti-mouse IgG (1:2000; Abcam, USA). Protein bands from scanned images were quantified using Image Lab Software.

Sperm immunofluorescence

The fresh sperms were washed three times by centrifugation for 5 min at 300 × g resuspended in phosphate-buffered saline (PBS), and air-dried onto poly-L-lysine-coated coverslips. The sperm cells were fixed with 4% paraformaldehyde for 1 h. After washing, the slides were treated with PBS containing 0.5% Triton X-100 at room temperature for 15 min. The slides were then treated with a 5% BSA blocking solution for 2 h at room temperature and incubated overnight with primary antibody, anti-ACO2

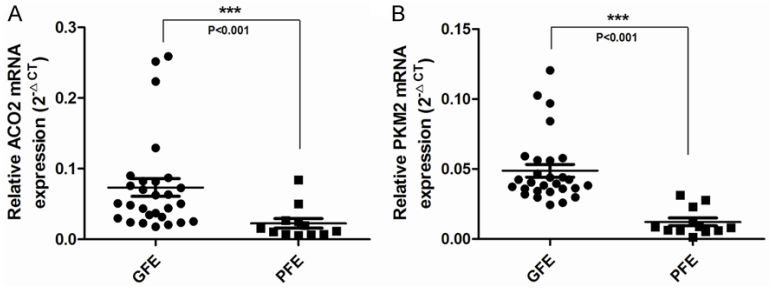

Figure 1. mRNA expression levels of ACO2 (A) and PKM2 (B) in GFE and PFE groups. Quantifications of ACO2 and PKM2/β-actin protein ratios are shown and the data were expressed as mean ± SD. ***Significant differences are indicated at P < 0.001.

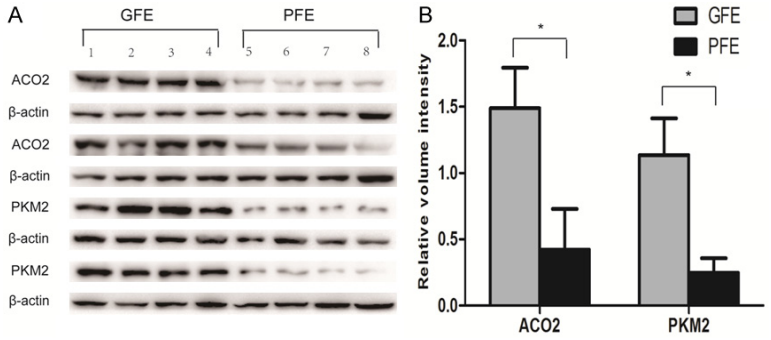

Figure 2. A. Western blot analysis of ACO2 and PKM2. Expression of ACO2 and PKM2 was evaluated by western blot and anti-β-actin antibodies were performed on about 50 μg of total protein extracts prepared from sperm proteins of GFE and PFE. B. Relative volume intensity of ACO2 and PKM2. Bars represent the density of gel bands determined from three samples. Quantifications of ACO2 and PKM2/β-actin protein ratios are shown and the data were expressed as mean ± SD. *Significant differences are indicated at P < 0.05.

(1:200, Abcam, USA) and anti-PKM2 (1:200, Abcam, USA) at 4°C. Spermatozoa were incubated again with the secondary antibody labeled with Alexa Fluor 555 (Jackson, USA) or labeled with FITC (Santa Cruz) at 1:200 dilution for 1 h at room temperature. Immunofluorescent staining was visualized using a confocal microscope (LSM 710, Zeiss).

Statistics

Differences between experimental groups were assessed by using analysis of variance (ANOVA) followed by the paired Student's t test with the SPSS software (version 22.0). Pearson's linear correlation analysis was performed to investigate the association between sperm freezability and the expression of ACO2 and PKM2. Data were expressed as means ± standard deviation (SD). Statistically significant differences were determined at $p < 0.05$.

Results

Classification of frozen-thawed ejaculates

As shown in **Table 1**, 40 ejaculates from 40 sperm donors had no significant differences of sperm concentration, normal morphology, motility ("a+b", progressive) between two groups of samples before freeze-thawing process. According to the progressive motility of the sperm after thawing, the ejaculates were separated into two groups, 28 of them were classified as GFE group and the other 12 belonged to the PFE group. As expected, the GFE group presented a higher progressive motility percentage of thawed sperm than the PFE group, while sperm concentration and normal morphology percentage had no significant difference.

The relative expression of ACO2 and PKM2

As shown in **Figure 1**, qRT-PCR was used to investigate the mRNA expression levels of ACO2 and PKM2 from fresh samples. Compared with the PFE group, ACO2 mRNA expression level was significantly higher in GFE group ($P < 0.05$). When investigating mRNA levels of PKM2, a significantly increased PKM2 expression level was also observed in GFE group ($P < 0.05$).

Western blot was performed to determine the protein expression levels of ACO2 and PKM2 between GFE and PFE group. The results demonstrated that the expression of the two proteins was signifcantly higher in GFE group than in PFE group (**Figure 2**).

The location of ACO2 and PKM2 by immunofluorescence analysis

According to immunolocalization, we identified that ACO2 localized in the sperm midpiece, also called the mitochondrial sheath (**Figure 3**).

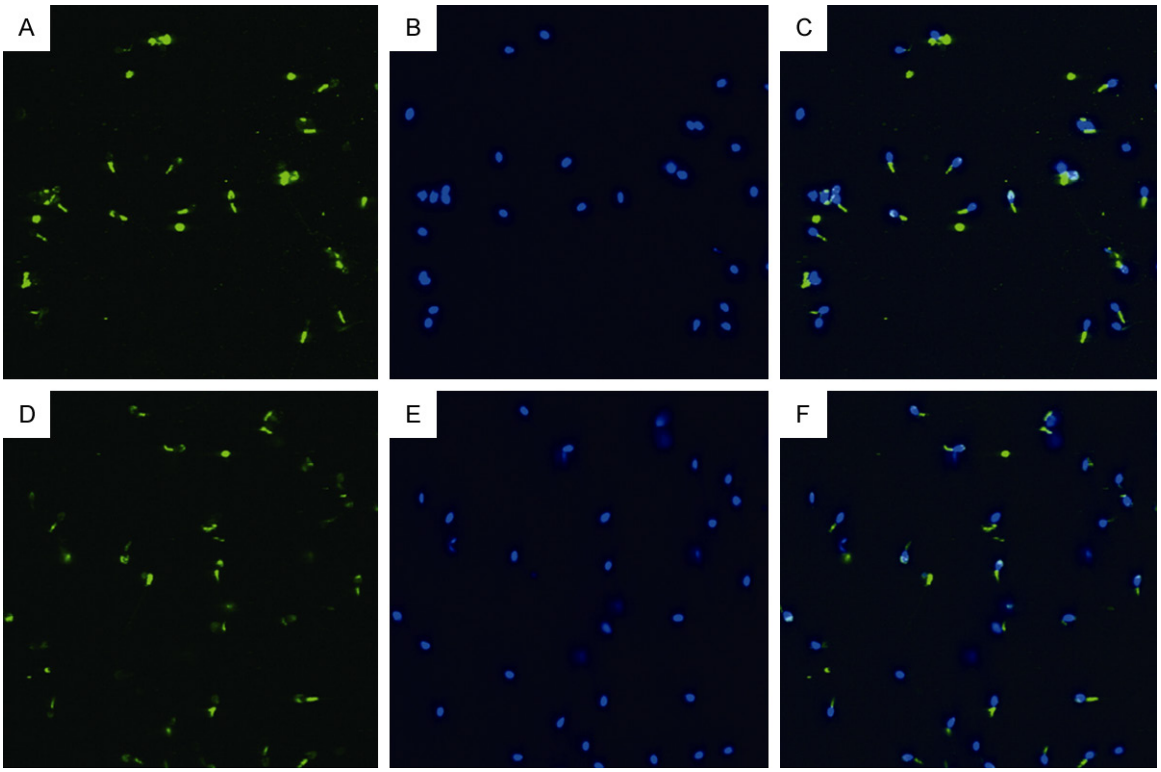

Figure 3. Immunolocalization analysis of ACO2. A, D. Represent the locations of ACO2 and the fluorescence intensity of GFE and PFE, respectively. C, F. Each represent a merged immunofluorescence double staining of Hoechst and DIC. B, E. Represent the negative controls of secondary antibody.

Moreover, PKM2 was most abundant in the body and tail of spermatozoa (**Figure 4**). Compared with PFE group, expression levels of the two proteins were obviously higher in GFE group. These findings were in line with the results of the qRT-PCR and western blot analysis.

Correlation analysis between the ACO2 and PKM2 expression and the sperm motility

The strength of the association between the sperm freezability capacity (described by progressive motility) and ACO2 and PKM2 protein expression was also studied. The correlation between freezability and normalized band volume of ACO2 was statistically significant (**Figure 5A**. $r = 0.806$; $P < 0.05$). Similarly, a significant positive correlation was detected between freezability and normalized band volume of PKM2 (**Figure 5B**. $r = 0.800$; $P < 0.05$).

Discussion

Sperm cryopreservation represents a valuable therapeutic option in the management of infertility including anatomical and pathological defects of male reproductive organ [1]. Besides, it has become an established method for the preservation of male fertility before cancer therapy, such as chemotherapy or radiotherapy, which can cause azoospermia or reduced spermatogenesis [2, 3]. The technology of sperm cryopreservation involved the cooling and storaging of semen samples at -196°C in liquid nitrogen, which could make all metabolic processes arrested at this temperature, including some biochemical activities resulting in cellular death. Despite its extensive use in assisted reproduction techniques, it has been suggested that sperm cryopreservation may cause cell injury, impair sperm DNA integrity and reduce the proportion of fully functional sperm in a sample, resulting in reduced sperm fertility [6]. New markers which can predict the ejaculate freezability before performing the freeze-thawing process were emergently needed.

According to previous proteomics research in terms of human sperm cryopreservation, we have found that some proteins, including ACO2 and PKM2 have significant difference in the

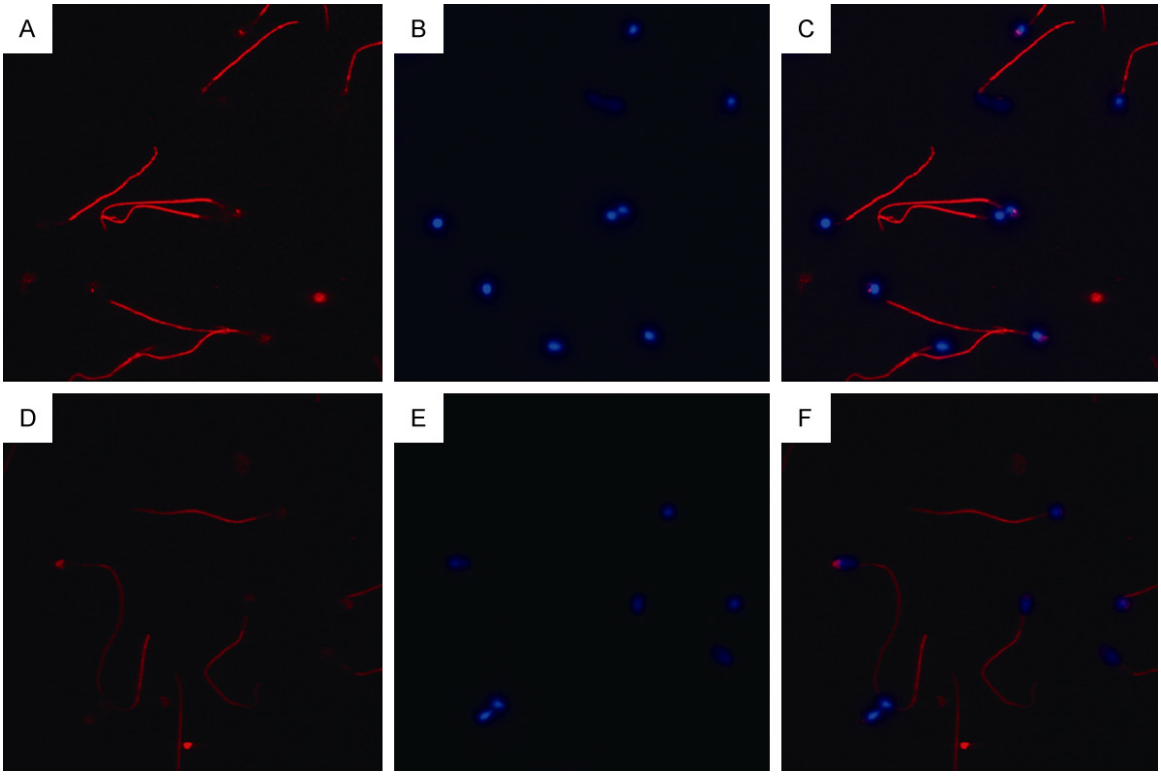

Figure 4. Immunolocalization analysis of PKM2. A, D. Represent the locations of PKM2 and the fluorescence intensity of GFE and PFE, respectively. C, F. Each represent a merged immunofluorescence double staining of Hoechst and DIC. B, E. Represent the negative controls of secondary antibody.

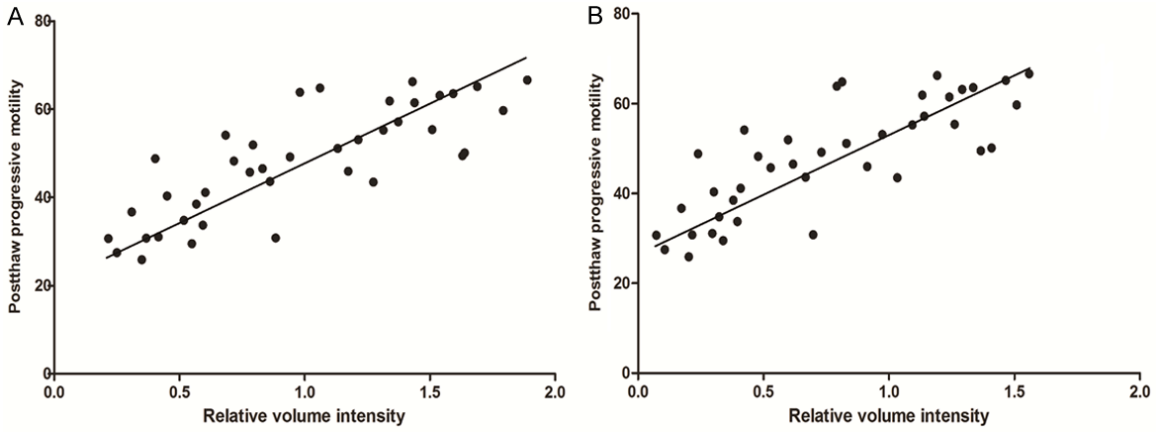

Figure 5. The correlation between freezability and relative volume intensity of ACO2 (A) and PKM2 (B).

frozen-thawed samples compared to the fresh samples [12]. Therefore, the main aims of present study were to determine the predictive value of ACO2 and PKM2 in terms of freezability before performing the freeze-thawing process. The content of ACO2 and PKM2 from fresh samples before cryopreservation was evaluated and the results showed the significant differences between GFE and PFE groups, which suggested ACO2 and PKM2 could be used as potential freezability markers. The most important parameter that might be affected by sperm freezing is the motility, which could assess the ejaculate freezability. In the present study, the reliability of these two freezability markers was also confirmed by Pearson corre-

lation analyses using protein expression and the progression motility as variables, which showed that ACO2 and PKM2 amounts were positively correlated with ejaculate freezability.

As an important regulatory enzyme of the tricarboxylic acid (TCA) cycle, ACO2 catalyzes the reversible hydration of cis-aconitate to yield the isomerization of citrate or isocitrat, which associates with adenosine triphosphate (ATP)-dependent sperm motility [17, 18]. As generally accepted, the TCA cycle is known to be a crucial metabolic pathway that contributes to produce ATP to protect spermatozoon against the cryopreservation process in mammalian spermatozoa mitochondria [19]. It is well known that an adequate supply of energy in the form of ATP is required to support sperm motility [20]. Previously, Wang et al showed that a lower ACO2 expression level was detected in human freeze-thawed spermatozoa compared with normal fresh semen [12]. In addtion, Tang et al also explained the role and mechanism of ACO2 in human sperm motility [21]. Our results showed that the expression level of ACO2 was significantly higher in GFE compared to PFE, which indicated that the degraded ACO2 lowered the production of isocitrate needed in the TCA cycle. In addition, ACO2 protein expression was significantly correlated with freezability capacity. These results suggested that ACO2 could become a good marker of freezability.

PKM2, also known as a key driver of aerobic glycolysis, controls the final and rate-limiting reaction in the glycolytic pathway [14]. Besides, the PKM2 gene, located on chromosome 15q22, encodes a pyruvate kinase that catalyzes the conversion of a phosphoryl group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP), thereby generating pyruvate and ATP [22-24]. Furthermore, it is well known that ATP plays a crucial role in sperm motility, along with intracellular free calcium $[Ca^{2+}]_i$ that is an important signaling molecule involved in the regulation of sperm motility [19]. When subjected to freezing or hypo-osmotic shock, spermatozoa releases less PKM2 to result in the injury of sperm mitochondria and decrease ATP production, this may be one of the mechanisms of cryopreservation-induced reduction of sperm motility [25]. Our experiment showed higher PKM2 expression in GFE group than PFE group, indicating that PKM2 was a vital factor in thawed sperm and could be used as a marker for predicting human sperm freezability.

The freeze-thawing injury was inevitable in sperm cryopreservation no matter what kind of technology was used. In order to minimize the damage to sperm function, more researches aimed to improve the cryopreservation technology are required. Meanwhile, we should look for more freezability markers of human sperm to optimize the procedure of cryopreservation.

Conclusion

In this study, the expression of ACO2 and PKM2 were higher in GFE group than PFE group which suggested that the two proteins could be used as predictive markers of human sperm freezability before starting the cryopreservation procedure, thereby avoiding unnecessary costs involved in this practice.

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Disclosure of conflict of interest

None.

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Table S1. Patient demographic information and clinic feature

Patient demographic	
No. of patients	40
Age, yr, mean (range)	27 (18-41)
BMI, kg/m^2, mean (range)	24.1 (17.9-31.0)
Mean abstinence days (range)	4 (3-5)
Clinic feature	
Smoking status (Never/Ever)	24/16
Drinking status (Never/Ever)	14/26
Hypertension (No/Yes)	17/33
Diabetes (No/Yes)	2/38

Continuous data are reported as median (range).