scientific reports

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TdT/Cas12a-based biosensor for sensitive detection of DNA breakpoints in sperm cryopreservation

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With the advancement of assisted reproductive technology (ART), sperm cryopreservation has become an essential technique in both clinical and research applications. However, maintaining DNA integrity during the freezing and thawing process remains a significant challenge. This study introduces an innovative TdT/Cas12a-based biosensor to enable precise detection and quantification of sperm DNA breakages. Compared to traditional methods such as the sperm chromatin structure assay (SCSA), this biosensor exhibits exceptional sensitivity (capable of detecting DNA breakages at as low as 0.001 nM) and molecular-level resolution, allowing for the precise localization and quantification of DNA breakpoints. By integrating TdT for nucleotide labeling and Cas12a for signal amplification, the system achieves high specificity and reliability. Additionally, the introduction of a standard strand as a quality control significantly enhances reproducibility. Furthermore, the study explores the protective effects of natural antioxidants on sperm DNA integrity during the freeze–thaw process. The results reveal a marked increase in DNA breakpoints caused by cryopreservation, with the TdT/Cas12a system outperforming traditional methods in providing detailed and accurate assessments. Notably, the addition of antioxidants effectively mitigates DNA damage during the freeze–thaw process, safeguarding sperm DNA integrity. This platform not only represents a significant advancement in DNA damage detection and monitoring but also supports the optimization of cryopreservation protocols and contributes to improving the success and safety of ART. It offers valuable insights and innovative tools for the field of reproductive medicine.

Keywords Biosensor, DNA damage, SCSA, TdT/ Cas12a, Cryopreservation, Mean number of DNA breakpoints

Abbreviations

PR	Progressive motility
MDB	Mean number of DNA breakpoint
ART	Assisted reproductive technology
TdT	Terminal deoxynucleotidyl transferase
SCSA	Sperm chromatin structure assay
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
RES	Resveratrol
OAT	Oligoasthenoteratozoospermia
DFI	DNA fragmentation index

In recent years, the widespread application of cryopreservation technology for sperm cell storage has led to increased research focus on sperm DNA damage in reproductive medicine and life sciences. As a vital carrier of genetic information, sperm DNA integrity directly influences male fertility and has significant implications for offspring health and genetic stability[1–5]. However, cryopreservation can induce DNA damage due to adverse

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factors such as oxidative stress, freezing stress, and ice crystal formation, which significantly impair sperm function and fertilization capability[4–8]. Sperm DNA damage primarily manifests as single-strand and double-strand breaks, with oxidative stress being the primary cause during the freeze–thaw process. Reactive oxygen species (ROS) can damage the sperm DNA structure, resulting in fragmentation and breaks[9]. This damage diminishes natural fertilization ability, reduces in vitro fertilization success rates, and may pose risks to offspring health[7,10]. In assisted reproductive technologies, such as intracytoplasmic sperm injection (ICSI), although fertilization may occur with damaged sperm, excessive DNA damage may exceed the repair capacity of the oocyte or zygote, leading to embryonic developmental abnormalities and increased genetic disorder risks[7].

Current methods for detecting sperm DNA damage include the comet assay, sperm chromatin dispersion (SCD) test[11], SCSA[12–14], and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay[15,16]. Each of these techniques has limitations regarding sensitivity, operational complexity, and result interpretation. For instance, while the comet assay and SCD test offer intuitive damage visualizations, they are susceptible to operator subjectivity. SCSA uses acid treatment to denature damaged DNA into single strands and employs acridine orange (AO) fluorescence staining to assess chromatin sensitivity, representing an indirect detection method. The TUNEL assay directly assesses DNA breakage by identifying 3'-hydroxyl ends of breaks, but it may underestimate damage levels due to the compact chromatin structure of sperm[17]. Overall, existing methods fail to fully meet the demand for precise and efficient assessment of sperm DNA damage during the freeze–thaw process.

Therefore, it is essential to develop efficient and accurate detection methods to assess sperm DNA damage, enhance cryopreservation efficiency, and ensure reproductive health.

Rapid advancements in molecular biology techniques have led to the development of CRISPR-Cas-based detection methods, providing new opportunities to overcome these technical bottlenecks. CRISPR-Cas12a, characterized by its unique trans-cleavage activity, has emerged as a highly sensitive and specific tool for nucleic acid detection[18–22]. Leveraging this, we combined terminal deoxynucleotidyl transferase (TdT) with the CRISPR-Cas12a system to develop a novel fluorescence-based DNA breakpoint detection technology. TdT possesses unique template-independent nucleotide extension capability, which efficiently generates poly-A tails at DNA 3′-OH ends to provide specific recognition sites for Cas12a-mediated signal amplification. The CRISPR-Cas12a detection mechanism uses crRNA to guide Cas12a in specifically recognizing the poly-A tails extended at DNA breakpoints. Once activated, Cas12a's trans-cleavage activity indiscriminately cleaves the fluorescent reporter probe (FAM-BHQ1), releasing a fluorescent signal that enables highly sensitive quantitative detection of DNA breaks.

This innovative technique utilizes TdT to precisely identify and label DNA breakpoints, followed by the activated CRISPR-Cas12a system to efficiently cleave fluorescent probes, enabling highly sensitive, specific, and quantitative analysis of sperm DNA breakpoints. Demonstrating superior performance in detection efficiency, sensitivity, and specificity compared to conventional methods, this approach provides a powerful new tool for assessing sperm DNA damage. In our study, this detection system was successfully applied to evaluate DNA damage in cryopreserved-thawed sperm samples. The results demonstrate the technology's capability to accurately detect cryopreservation-induced DNA damage, providing crucial data for optimizing reproductive cell cryopreservation protocols. Furthermore, our investigation revealed the protective effects of natural antioxidants (resveratrol and Lycium barbarum polysaccharides) on sperm DNA integrity during the freezing process.

The advancement of this innovative technology establishes a solid foundation for creating cryopreservation standards and enhancing assisted reproductive technologies. In the future, this technology offers promising applications in infertility treatment, sperm quality assessment, and reproductive medicine research. It is anticipated to significantly contribute to increasing the success rates of assisted reproduction and ensuring offspring health.

Materials and methods

All methods were carried out in accordance with relevant guidelines and regulations.

Materials and reagents

All DNA and RNA oligonucleotides (sequences listed in Table S1) were synthesized by Sangon Biotech (Shanghai, China). LbCas12a, TdT, Buffer, and other reagents were purchased from New England Biolabs (NEB, USA). The DNA extraction kit (DP304) was obtained from TIANGEN Biotech (Beijing, China).

DNA breakpoint detection and fluorescence reporting system

The sperm DNA concentration was adjusted to 5 ng/μL for breakpoint detection. A fixed volume of 2 μL was loaded per reaction. The total reaction system volume was 15 μL, containing the following components: 1 × TdT buffer, 1 × $CoCl_2$, 0.1 mM dATP (dTTP/dCTP/dGTP), 0.5 U TdT, and substrate DNA (L001). The reaction system was incubated at 37 °C for 30 min, followed by heat inactivation at 85 °C for 10 min. After heat inactivation, the CRISPR-Cas12a fluorescence reporting system was immediately added, which included 20 nM Cas12a, 10 nM crRNA (CrRNA-1/CrRNA-2/CrRNA-3/CrRNA-4), $MgCl_2$, Fluorescent probe (P001, 200 nM), and 1 × NEBuffer 2.1. The final reaction was conducted at 37 °C, with the PCR program set to 37 °C for 30 s per cycle, for a total of 60 cycles.

Validation of the TdT/Cas12a biosensor

The standard DNA was diluted to 0–0.3 nM concentrations for calibration. Reactions (15 μL) containing TdT buffer, $CoCl_2$, dATP, TdT, and DNA substrate were incubated at 37 °C for 30 min, then heat-inactivated at 85 °C for 10 min. The CRISPR-Cas12a detection system (Cas12a, crRNA, $MgCl_2$, FAM-BHQ1 probe P001, and

NEBuffer 2.1) was added, followed by isothermal fluorescence measurement at 37 °C (60 cycles, 30 s/cycle) using a real-time PCR instrument (QuantReady 9600, Life-Real).

Sample collection and selection criteria

Semen samples for this study were collected from the Reproductive Medicine Center of the Ningxia Medical University General Hospital and the Reproductive Center of the Yinchuan Maternal and Child Health Hospital. 32 male volunteers, aged between 25 and 45 years, were recruited, with an average age of 31.53 ± 4.89 years. Recruitment was based on the standard semen parameters established by the World Health Organization (WHO, 2021), and all participants were confirmed to have normal results in routine semen analysis. The study was approved by the Ethics Committee of the Ningxia Medical University General Hospital (Approval No. KYLL2022-1081), and written informed consent was obtained from all participants.

Semen sample analysis

After 2–6 days of abstinence, semen samples were collected by masturbation into sterile containers. After liquefaction for 30 min at 37 °C, the semen samples underwent detailed routine analysis according to the standard methods specified in the WHO Laboratory Manual for the Examination and Processing of Human Semen (6th edition). Sperm concentration and motility were measured using a computer-assisted sperm analysis system (CASA, WLJY-9DOO version 2.0, Beijing Future New Century Science & Technology Development Co., Ltd., Beijing, China).

Sample grouping

The semen samples were categorized into four groups: the Fresh group, the Cryoprotectant group, the CryoProtec + LBP group, and the CryoProtec + RES group. The Fresh group included semen samples that were not subjected to freezing or any treatment.

The Cryoprotectant group utilized only the standard cryoprotectant (Sperm CryoProtec, Nidacon, SCP-020). The CryoProtec + LBP group incorporated 1 mg/mL Lycium barbarum polysaccharide (LBP) into the standard cryoprotectant. The CryoProtec + RES group incorporated 30 μmol/L resveratrol (RES) into the standard cryoprotectant.

Sperm freezing and thawing process

The specific procedures for sperm freezing and thawing are as follows: First, Sperm CryoProtec was utilized as the cryoprotectant. The cryoprotectant was added dropwise to a cryovial containing 0.3 mL of semen while gently rotating the cryovial to ensure thorough mixing. After allowing the mixture to stand, let it sit at room temperature for 3 min, then cool in liquid nitrogen vapor for an additional 10 min. Finally, fully immerse the cryovial in liquid nitrogen for freezing and store it in liquid nitrogen for 1 week. For thawing, the cryovial was removed from the liquid nitrogen tank and placed directly into a 37 °C water bath. The vial was gently rotated until the semen was completely thawed, a process that typically took approximately 5 min.

Sperm DNA extraction

Sperm DNA extraction was conducted using a genomic DNA extraction kit following the manufacturer's instructions to ensure the standardization and reliability of the extraction process. The extracted DNA was stored at − 80 °C for subsequent experiments. Specific procedures and details can be found in Fig. S1.

DFI test

The DNA Fragmentation Index (DFI) of sperm was assessed using the SCSA method with a sperm flow cytometry kit (BKR802, Wuhan Biocarry Technology Co., Ltd.) The detection was performed strictly in accordance with the manufacturer's instructions.

Statistical analysis

Data were presented as mean ± standard deviation (mean ± SD). Statistical analysis was conducted using SPSS software version 24.0 (IBM Corp.). Due to the small sample size, the Shapiro–Wilk test was used to evaluate the normality of the data, and Levene's test was used to assess the homogeneity of variances. Welch's one-way ANOVA was employed to detect differences among groups, and Tukey's HSD test was used for post hoc comparisons. A significance level of $P < 0.05$ was considered statistically significant.

Results

Detection principle

In the described detection system, TdT is utilized to recognize the 3′-hydroxyl group (3′-OH) at sperm DNA break sites, which are formed during the freezing process due to adverse factors such as reactive oxygen species (ROS) and ice crystals (as shown in Fig. 1). The TdT enzyme catalyzes the polymerization of dATP, which extends a poly-A tail at the 3′-OH ends of the DNA break sites. Subsequently, the crRNA of the CRISPR-Cas12a system specifically recognizes the poly-A tail at the extended DNA break sites, activating the trans-cleavage activity of Cas12a. This activation results in the rapid cleavage of single-stranded DNA (ssDNA) probes, generating a significantly amplified fluorescent signal. Consequently, the number of sperm DNA break sites (including single-strand and double-strand breaks) is positively correlated with the fluorescence signal intensity. By analyzing the fluorescence signal intensities of different samples and referencing a standard curve generated using DNA standards, the number of DNA break sites corresponding to each sperm sample can be accurately calculated. This method offers a sensitive and quantitative approach to assess sperm DNA integrity.

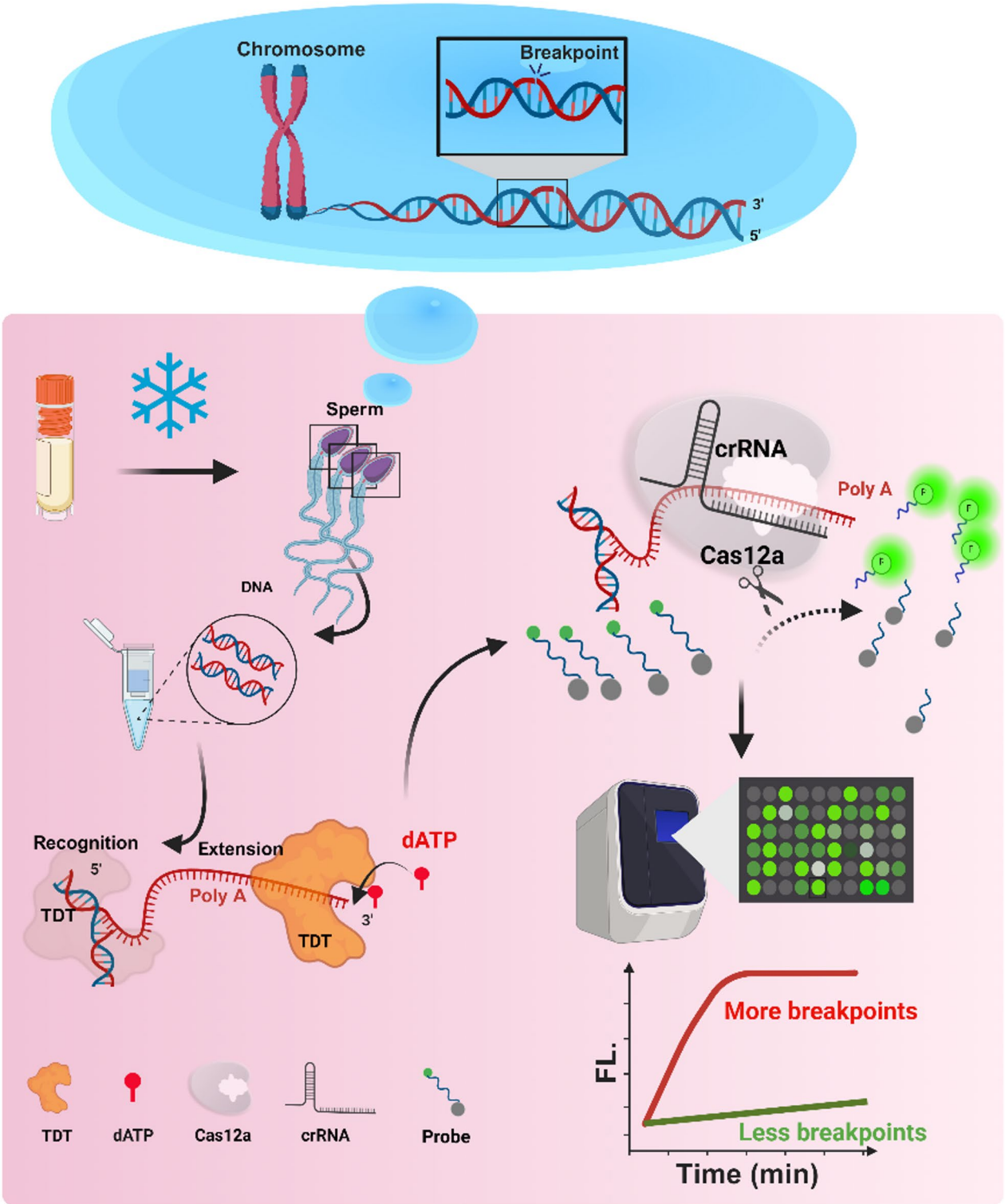

Fig. 1. Detection principle of TdT/Cas12a Cascade Amplification System.

Basic sperm parameters

Supplementary Table 2 presents the results for 32 patients, including their age, abstinence duration, semen volume, sperm concentration, progressive motility (PR), and sperm motility.

All study samples met WHO (2021) standards for sperm concentration and motility, confirming their clinical relevance and homogeneity. This standardization ensured reliable DNA breakpoint analysis by eliminating semen quality variations, with CASA-verified parameters providing a valid baseline for cryopreservation studies.

The critical role of TdT in recognizing 3′-hydroxyl DNA breakpoints and extending polynucleotide sequences

The ability of TdT to recognize 3'-hydroxyl DNA breakpoints and extend polynucleotide sequences is fundamental to the functionality of our biosensor. Therefore, we first investigated TdT's capacity to recognize 3'-hydroxyl DNA breakpoints and to extend PolyA, PolyT, PolyC, and PolyG sequences individually. As shown in Fig. 2A, after TdT recognized the 3'-hydroxyl DNA breakpoints, the PolyA sequences exhibited the longest and most stable extension, with a more concentrated band pattern. In addition, Fig. 2B demonstrates that using ssDNA (L001, 0.1 nM) as a substrate, the PolyA extension generated the highest signal-to-noise ratio (SNR) of 62.8 during fluorescence detection. These results confirm that PolyA extension offers superior sensitivity and stability for fluorescence-based detection, establishing it as the optimal choice for our TdT/Cas12a biosensor system.

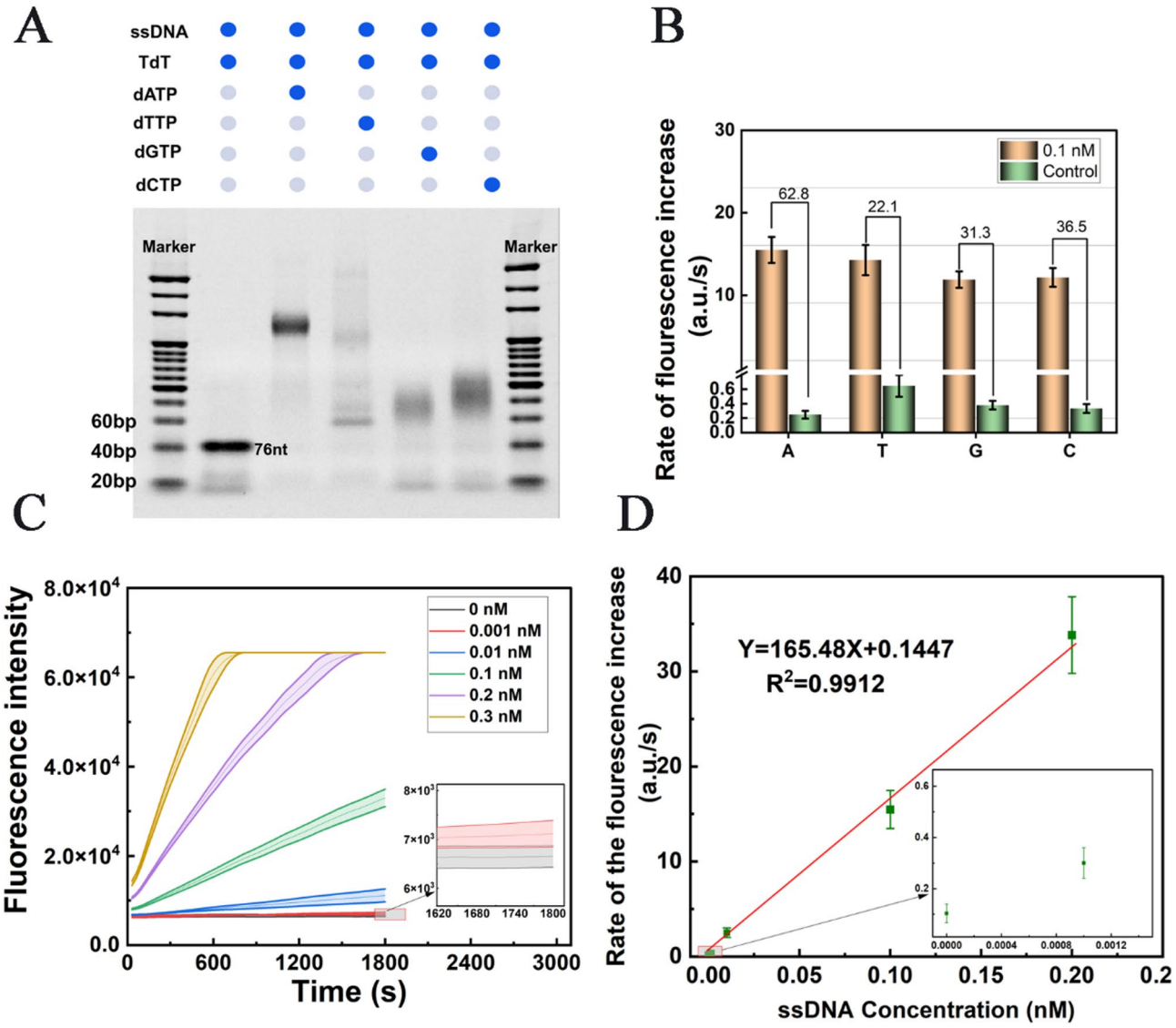

Fig. 2. Verification of the Working Principle of the TdT/Cas12a System. (**A**) PAGE Analysis of TdT's Ability to Recognize 3′-Hydroxyl DNA Breakpoints and Extend PolyA/T/C/G Tails. Lane 1: Single-stranded DNA (ssDNA, L001) + TdT; Lane 2: L001 + TdT + dATP; Lane 3: L001 + TdT + dTTP; Lane 4: L001 + TdT + dGTP; Lane 5: L001 + TdT + dCTP. (**B**) The fluorescence rate of the L001 sample (0.1 nM), after TdT recognition and extension of polyA/T/C/G tails at DNA 3'-hydroxyl breakpoints and subsequent Cas12a system recognition, was compared to the fluorescence rate of the blank control group (nuclease-free water replacing DNA substrate). (**C**) Fluorescence intensity of the biosensor at varying concentrations of ssDNA: 0 (nuclease-free water replacing DNA substrate), 0.001 nM, 0.01 nM, 0.1 nM, 0.2 nM, and 0.3 nM. (D) Standard curve illustrating the relationship between the rate of fluorescence signal increase and ssDNA concentration.

Validation of the working principle of the TdT/Cas12a biosensors

CRISPR-Cas12a has been widely and effectively utilized for nucleic acid detection[20–22]. In this study, we employed the optimized reaction ratio proposed by Zhang et al.[23] (LbaCas12a: 20 nM, gRNA: 10 nM, probe: 200 nM) to conduct our experiments. We successfully established the TdT/Cas12a detection system and measured the fluorescence signal corresponding to various standard concentrations of single-stranded DNA (0, 0.001 nM, 0.01 nM, 0.1 nM, 0.2 nM, and 0.3 nM), as illustrated in Fig. 2C. We investigated the relationship between the rate of fluorescence signal increase at various standard concentrations (0, 0.001 nM, 0.01 nM, 0.1 nM, and 0.2 nM) and the number of 3'-hydroxyl groups in single-stranded DNA (L001). The results indicated that, within the range of 0.001 nM to 0.2 nM, the number of 3'-hydroxyl groups exhibited a linear correlation with the rate of fluorescence signal increase, yielding a linear correlation coefficient of 0.9912. The linear regression equation is: $Y = 165.48X + 0.1447$ (see Fig. 2D).

Calculation of the mean number of DNA breakpoints (MDB) in sperm

Based on the linear equation provided above ($Y = 165.48X + 0.1447$), MDB can be calculated. For example, when 10 ng of sperm DNA is added to the system, the fluorescence rate for this sperm sample in the system is measured as 20 (represented as Y in the equation). By substituting this fluorescence rate value into the linear equation, the corresponding DNA break concentration (X) in the system is calculated to be 0.12 nM. This indicates that, under the given system conditions, the sample contains 0.12 nM of DNA breaks. To further calculate the MDB for this sperm sample, the method outlined in Supplementary Calculation Formula was referenced. By substituting 0.12 nM as C_0 into the formula provided in the appendix, the MDB for this sperm sample was determined to be 2.93×10^5. This indicates that there are 2.93×10^5 breakpoints present in a single complete DNA strand.

MDB test

After completing the principle validation, standard curve construction, and establishment of the detection formula using standard concentrations of single-stranded DNA, we further investigated the feasibility of the TdT/Cas12a detection system in analyzing sperm DNA.

First, we randomly selected the DNA from one sperm sample and applied different gradient input amounts (10 ng, 20 ng, 30 ng, and 40 ng) to verify the detection capability of the system. As shown in Fig. 3A, the fluorescence rate increased proportionally with the doubling of the input amounts, confirming the sensitivity and linear response capability of the system in detecting sperm DNA breakpoints. Furthermore, we performed breakpoint quantification on the sperm samples. Figure 3B displays the fluorescence curves of five representative sperm samples. By analyzing the fluorescence rise rates of these samples, we calculated the corresponding MDB values. This provides a reliable basis for evaluating the integrity of sperm DNA. Additionally, the stability and reproducibility of this biosensing technology were evaluated. As shown in Table S3, the intraday relative standard deviation (RSD) for the same sperm DNA ranged from 2.27 to 3.91%, whereas the interday relative standard deviation (RSD) was 1.57%.

The TdT/Cas12a detection system demonstrated excellent linear response in gradient validation, confirming its successful application for quantitative analysis of sperm DNA breakpoints. With intraday/interday precision

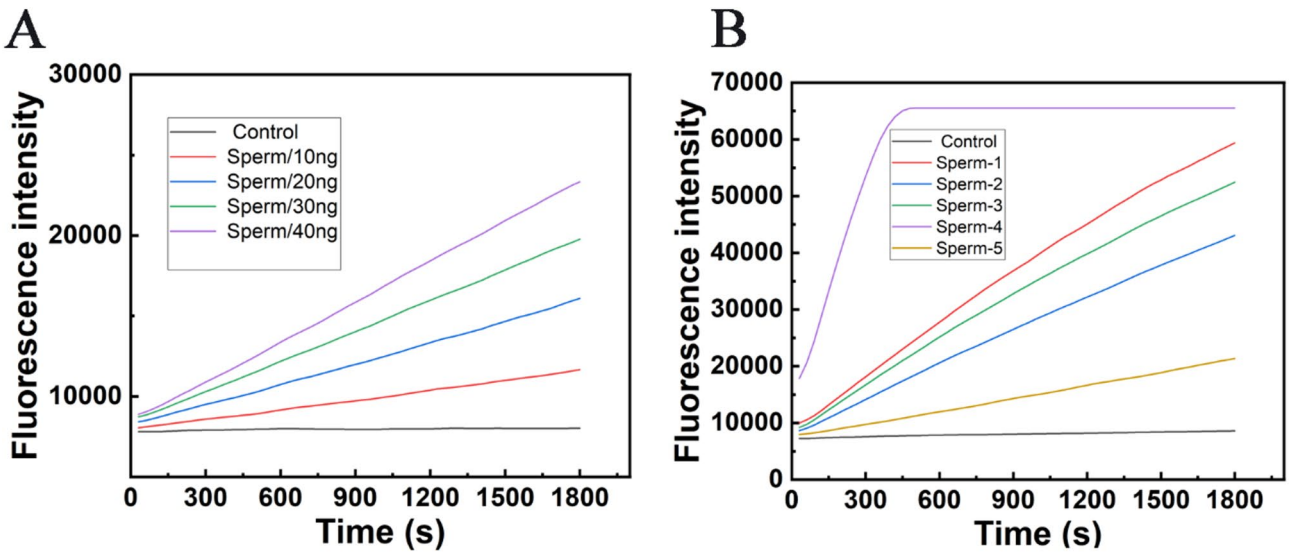

Fig. 3. Fluorescence Curves of DNA Breakpoints Detected by the TdT/Cas12a System. (**A**) Real-time fluorescence trajectories showing dose-responsive detection of DNA breakpoints across serial sperm DNA inputs (10, 20, 30, and 40 ng). Negative controls (nuclease-free water substituted for DNA template) established baseline fluorescence levels (gray line). (**B**) Fluorescence curves of actual sperm samples detected by the TdT/Cas12a system, illustrated using five sample examples. Negative controls (nuclease-free water substituted for DNA template) established baseline fluorescence levels (gray line).

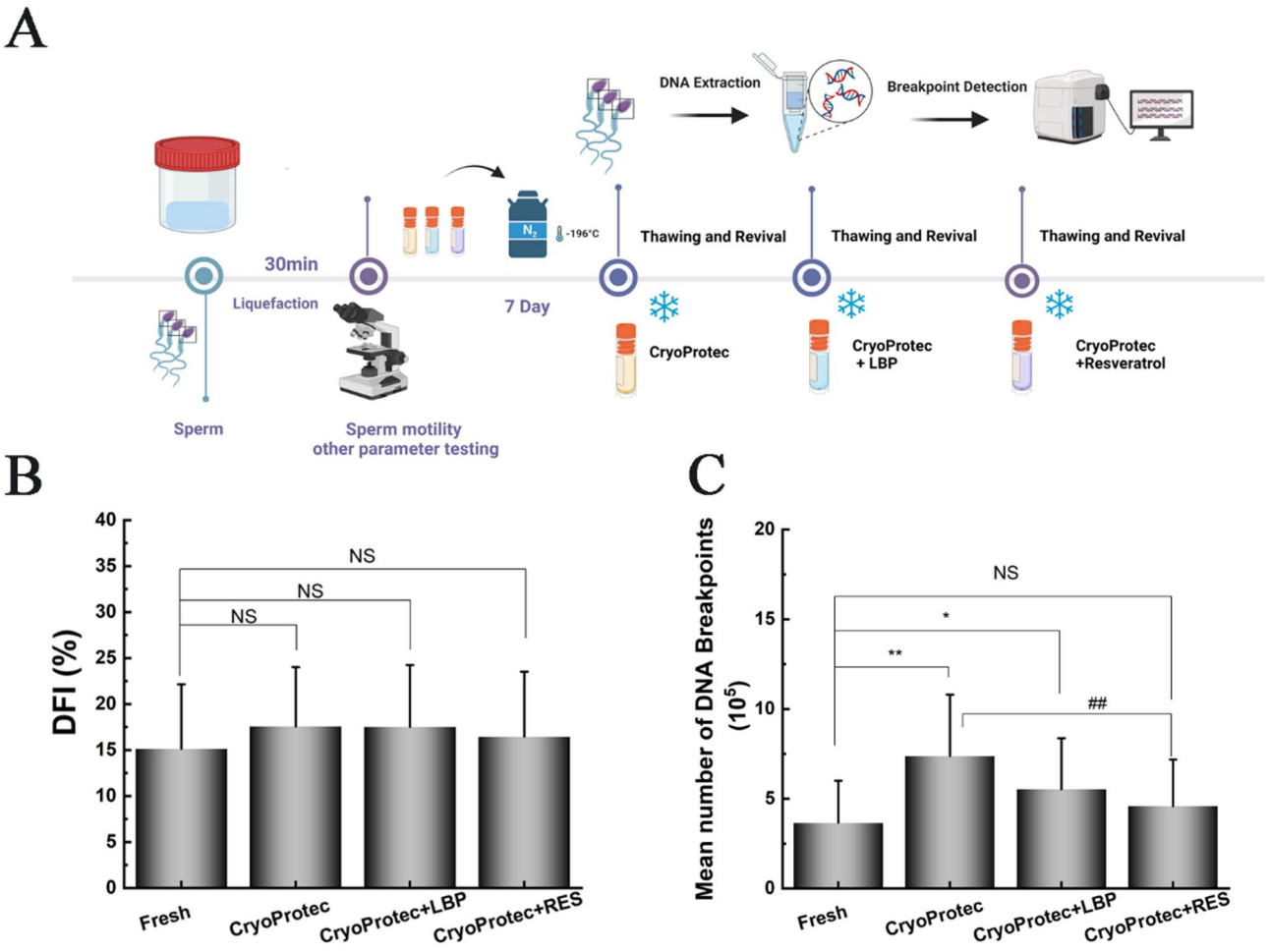

Fig. 4. Detection of DFI and MDB Values in Sperm Samples Before and After Freezing. (**A**) Schematic diagram of the workflow for sperm freezing detection in this experiment. (**B**) Bar chart of DFI values for sperm samples in different groups detected by the SCSA (n = 32). (**C**) Bar chart of MDB values for sperm samples in different groups evaluated by the TdT/Cas12a detection system (n = 32). *NS* not significant, $^{*}p < 0.05$, $^{**}p < 0.01$ versus Fresh group; $^{\#\#}p < 0.01$ versus CryoProtec group.

meeting clinical testing standards, this system provides a novel molecular diagnostic tool for reproductive medicine.

Detection of cryopreservation-induced sperm DNA damage and antioxidant protection using TdT/Cas12a system

To validate the potential application of the TdT/Cas12a detection system in identifying sperm DNA breaks and its utility in sperm cryopreservation research, this study incorporated natural antioxidants (LBP[24,25] and RES[26]) into the sperm cryopreservation media to evaluate the system's ability to assess improvements in cryoprotectants. A total of 32 sperm samples were divided into different treatment groups, cryopreserved, and thawed after seven days (as shown in Fig. 4A). Subsequently, the DNA Fragmentation Index (DFI) and Mean Number of DNA Breaks (MDB) were assessed before and after freezing using the SCSA technique and the TdT/Cas12a detection system. The results (Fig. 4B) indicated that the DFI values of all frozen groups increased compared to those of the fresh group, but these differences were not statistically significant ($p > 0.05$). However, as shown in Fig. 4C, the MDB values of the CryoProtec group and the CryoProtec + LBP group were significantly higher than those of the fresh group ($p < 0.05$), while the CryoProtec + RES group exhibited an increase that was not statistically significant ($p > 0.05$). Notably, the CryoProtec + RES group showed a significant decreasing trend in MDB values compared to the CryoProtec group ($p < 0.01$).

The TdT/Cas12a system effectively quantified cryopreservation-induced sperm DNA damage, demonstrating RES antioxidant protection while outperforming conventional SCSA in sensitivity.

Discussion

With the continuous development of assisted reproductive technologies (ART), individuals can preserve their fertility through sperm cryopreservation. However, the freezing process may cause DNA damage to sperm[7,8,27].

In this study, we successfully developed the TdT/Cas12a biosensor based on TdT enzyme and Cas12a, which can precisely assess the number of DNA breakpoints in sperm. This biosensor shows great potential for evaluating sperm damage caused by cryopreservation. Using this biosensor, we found that the number of DNA breakpoints in sperm increased after freezing, but the increase was not significant in the CryoProtec + RES group, showing no statistically significant difference compared to the fresh group. Additionally, our experiments revealed that although the number of DNA breakpoints changed significantly during the freezing–thawing process, the post-thaw sperm DNA fragmentation index (DFI) did not show significant changes.

The TdT/Cas12a biosensor identified DNA breaks occurring during the freezing process, and the addition of the antioxidant resveratrol (RES) to the cryoprotectant effectively reduced DNA breaks. These breaks may represent minor, localized damage, whereas DFI typically evaluates overall DNA fragmentation. The TdT/Cas12a biosensor is more sensitive and can detect subtle DNA breaks that traditional DFI assessment methods might overlook, resulting in insignificant changes in DFI values.

From this study and related literature, it can be seen that the impact of the freeze–thaw process on sperm chromatin quality exhibits complexity and variability under different detection methods and experimental conditions. Overall, some studies using SCSA suggest that the freeze–thaw process has limited or even negligible effects on sperm chromatin quality. For instance, Evenson and Jost[28] found through SCSA that post-thaw sperm SCSA data (e.g., DNA fragmentation index, DFI) showed no significant difference compared to fresh sperm. Similarly, Lusignan's research[29] indicated that cryopreservation did not significantly affect the DFI values of either normal semen samples or samples with oligoasthenoteratozoospermia (OAT).

However, when using the TUNEL assay, the effects of cryopreservation on sperm DNA damage appear more pronounced. For example, Lusignan's TUNEL-based study[29] found a significant increase in the proportion of TUNEL-positive cells in both normal semen and OAT samples after cryopreservation. This observation aligns with our findings that sperm DNA breaks significantly increase after freezing.

It is worth noting that the differences between TUNEL and SCSA methods in evaluating sperm DNA damage may arise from their differing principles and evaluation parameters[30]. The SCSA[29,31] method detects potential DNA damage through an acid denaturation process, based on the higher sensitivity of damaged DNA to acid denaturation. In contrast, the TUNEL assay[32,33] directly incorporates labeled dUTP into 3'-OH ends of DNA single- or double-strand breaks via TdT enzyme, offering a more direct assessment of damage. Therefore, the TUNEL method has significant advantages in detecting DNA damage caused by cryopreservation and complements the SCSA method to some extent.

In this study, we used the advanced TdT/Cas12a biosensor to accurately evaluate DNA damage in sperm during the freeze–thaw process. Similar to the TUNEL assay, this system is based on the recognition of DNA breakpoints by the TdT enzyme. However, the TdT/Cas12a biosensor further quantifies the exact number of DNA breaks, providing a more precise assessment of DNA damage at the molecular level. Unlike the TUNEL assay, which only provides the proportion of TUNEL-positive cells (i.e., the percentage of cells with DNA damage), the TdT/Cas12a biosensor goes a step further by directly reflecting the number of DNA breakpoints and offering more intuitive and quantitative molecular data. Our TdT/Cas12a biosensor achieves 0.001 nM detection limit, demonstrating higher sensitivity than conventional methods (SCSA/TUNEL) and our prior TD-probe technique[34] (0.01 nM), enabling molecular-level quantification of cryopreservation-induced DNA breaks.

Our study confirmed that cryopreservation significantly increases DNA damage in sperm in the form of DNA breaks. This finding not only aligns with previous studies that used the TUNEL assay to identify an increase in TUNEL-positive cells after freezing but also further validates through the TdT/Cas12a biosensor the significant increase in the number of DNA breaks. This provides higher-resolution evidence regarding the impact of cryopreservation on sperm DNA damage. It highlights that while cryopreservation is a critical reproductive preservation technique, it may lead to increased sperm DNA breakage during the freezing and thawing process, potentially affecting sperm chromatin quality. Additionally, this study demonstrated that the addition of resveratrol to cryoprotectants effectively reduces the number of DNA breaks in post-thaw sperm.

Conclusion

This study innovatively developed a highly sensitive and high-resolution TdT/Cas12a biosensor and utilized it to conduct precise quantification and in-depth exploration of DNA damage dynamics in sperm during the freeze–thaw process. The study revealed that cryopreservation significantly increases the number of sperm DNA breakpoints, while traditional detection methods (such as SCSA and TUNEL) are limited in capturing these subtle changes due to their sensitivity and resolution constraints. In contrast, the TdT/Cas12a biosensor, with its outstanding sensitivity and molecular-level detection capability, precisely identifies and quantifies sperm DNA breakpoints, providing a groundbreaking and efficient tool for sperm quality assessment. Moreover, leveraging this biosensor, the study further uncovered the potential for optimizing cryoprotectants. Specifically, the addition of the antioxidant resveratrol (RES) to cryoprotectants significantly reduced DNA damage during the freezing process, resulting in genetic integrity in frozen sperm comparable to that of fresh sperm. This finding further highlights the critical role of the TdT/Cas12a biosensor in accurately evaluating and optimizing cryoprotectant formulations. Overall, this biosensor not only demonstrated, for the first time, its broad applicability in evaluating freeze–thaw technology but also showcased its immense potential in the field of assisted reproductive technology (ART). By employing the TdT/Cas12a biosensor, the study provides robust technical support for improving cryopreservation techniques and enhancing ART success rates, while also paving new avenues for sperm quality monitoring and genetic material preservation.

Data availability

Correspondence and requests for materials should be addressed to L.P. or J.W.

Received: 9 February 2025; Accepted: 30 June 2025
Published online: 13 July 2025

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Acknowledgements

The authors express their gratitude to the study participants for their valuable contributions to this research.

Author contributions

B.B.G and Z.Y.L. wrote the main manuscript text; B.Y., K.H.D. and L.G.P. prepared Figs. 1, 2, 3 and 4; L.G.P. and J.W., review and editing. All authors have read and agreed to the published version of the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (82260290, 82460295), the School-level key scientific research projects of Ningxia Medical University (XM2019074). Ningxia Natural Science Foundation (2022AAC03598, 2024AAC0508, 2024AAC03198), Key Laboratory of Fertility Preservation and Maintenance of Ministry of Education scientific research project (XZ2024013), Scientific research project of Ningxia Education Department (NYG2024136).

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-09768-4.

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