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Non-invasive assessment of stem cell DNA integrity in response to environmental stressors

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The maintenance of DNA integrity in spermatogonial stem cells (SSCs) is essential for ensuring genetic stability and proper function in male germline cells. Here, we established a non-invasive approach to evaluate SSC DNA integrity and investigated the impact of detrimental environmental stressors on genomic stability. SSCs were isolated and cultured from mouse testes. SSCs identity was confirmed through immunofluorescence staining and flow cytometric analysis of specific molecular markers. DNA strand breaks were detected using a novel TdT enzyme-Endo IV-fluorescent probe biosensor, confirming the methodological feasibility. Heat stress and cryopreservation models were established, with the biosensor quantifying stress-induced DNA damage in SSCs. Additionally, extracellular free DNA fragments in culture supernatants were quantified as a non-invasive measure of SSCs DNA integrity.

Keywords Spermatogonial stem cells, Non-invasive detection, Heat stress, Probe biosensor, Mean number of DNA breakpoint, Reactive oxygen species

Abbreviations

ROS	Reactive oxygen species
TdT	Terminal deoxynucleotidyl transferase
LBP	Lycium barbarum polysaccharides
SSCs	Spermatogonial stem cells
MDB	Mean number of DNA breakpoint

Spermatogonial stem cells (SSCs), also referred to as male germ cell line stem cells, are essential for species continuity as they are responsible for sperm production[1,2]. These cells have unique capabilities for self-renewal and multi-directional differentiation. They can be cultured, cryopreserved, and transplanted back into the testes, positioning them as a crucial focus of male reproductive research[2-8].

In modern society, the rising prevalence of oxidative stress and reactive oxygen species from various sources presents significant challenges to male reproductive health. This study aims to investigate the impact of oxygen-free radicals on the DNA integrity of SSCs, which is crucial for the continuity of species. Despite increasing interest in this research area, the interaction between reactive oxygen species and SSCs—particularly their effects on genetic material—remains relatively underexplored.

The integrity of stem cell DNA is of utmost importance for maintaining genetic stability and functional characteristics. DNA damage, particularly double-strand breaks (DSBs), represents a severe form of impairment[9]. Reactive oxygen species, commonly known as oxygen-free radicals, play a significant role in inducing DNA damage. Unresolved DSBs not only impair the self-renewal and differentiation capacity of SSCs but also lead to genomic instability and cell apoptosis, potentially resulting in disease[10].Therefore, accurately assessing the DNA integrity of spermatogonial stem cells (SSCs) under various stress conditions is crucial for SSC research[11,12]. Currently, a common method used to assess DNA integrity is the comet assay[13-15]. Although the comet assay is relatively simple and feasible, its results can be influenced by subjective factors, leading to reduced accuracy. In addition, this method depends on costly fluorescence microscopy, which limits its widespread application[16]. Another frequently employed technique is the TUNEL assay, which employs terminal deoxynucleotidyl

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transferase (TdT) to catalyze the transfer of fluorescein-labeled dUTP to the 3'-hydroxyl ends of DNA damage molecules, subsequently allowing for analysis through microscopy or flow cytometry. However, this method is complex, time-consuming, and susceptible to interference from unbound dyes that have not been thoroughly washed off. Additionally, it shows low binding efficiency to densely packed chromatin, limiting its widespread application.

Recent advancements in nucleic acid probe technology have led to exciting discoveries in assessing DNA integrity. We[17] employed a TdT -Endo IV-fluorescent probe biosensor to evaluate human sperm DNA integrity and established a new, more sensitive assessment parameter called Mean number of DNA breakpoints (MDB). This technique utilizes TdT to identify 3'-hydroxyl ends (3'-OH) generated during DNA breakage, extends the 3'-OH with the assistance of dATP (Deoxyadenosinetriphosphate), forming a polyadenine (poly A) sequence. The self-designed DNA fluorescent probe binds to the poly A sequence, and an Endo IV recognition probe is added to cleave the probe, generating a noticeable fluorescence signal. The MDB can be obtained by comparing the fluorescence increase rate with a standard curve. Building on this biosensor technology, we further optimized enzyme concentrations and probe designs for more sensitive detection, applying it to assess the DNA integrity of SSCs under diverse stress conditions, thereby achieving non-invasive detection (see Fig. 1).

In this innovative technology, the TdT enzyme is also utilized to recognize the 3'-OH at DNA breakpoints, similar to the detection principle of the TUNEL assay. However, after recognition, it is further extended to form a poly A sequence. In addition, a self-designed oligonucleotide fluorescent probe and the endonuclease IV are used for signal amplification. In this technical approach, a standard chain is designed as a quality control sample for each experiment, ensuring more accurate detection each time. Consequently, this technology offers a more direct and accurate assessment of DNA breakpoints.

Our research primarily focuses on the impact of heat stress and freezing stimulation on stem cell DNA integrity, specifically examining their relationship with oxygen-free radicals. Studies have reported a strong association between oxygen-free radicals and DNA oxidative damage[18,19]. However, heat stress and freezing stimulation result in an increased generation of intracellular oxygen-free radicals[20-25], leading to oxidative DNA damage, which compromises the genetic stability and functionality of stem cells. Therefore, it is essential to carefully consider the potential impact of oxygen-free radicals.

We utilized the improved TdT enzyme-Endo IV-fluorescent probe biosensor to quantify the extent of DNA damage by measuring the fluorescence signal intensity, achieving greater sensitivity and accuracy. By measuring changes in fluorescence signals under stress conditions, we gain a more precise understanding of the impact on the DNA integrity of SSCs, thus enhancing our comprehension of the relationship between oxygen-free radicals and DNA integrity. Furthermore, through this biosensor, we precisely measured the number of DNA fragments released by stem cells into the culture medium under stress conditions. This non-invasive method provides insights into the extent of DNA damage experienced by stem cells under stress conditions and whether antioxidants can mitigate such damage.

In conclusion, comprehending the interplay between oxygen-free radicals and DNA integrity is essential for evaluating the DNA integrity of SSCs. Our optimized technology offers researchers a more accurate, sensitive, and non-invasive tool to assess stem cell DNA integrity under stress conditions, thereby enhancing our understanding of how stem cells respond to oxygen-free radicals.

Materials and methods

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

Experimental animals

Male C57BL/6 mice aged 6 days, with the day of birth considered as 0 days, were chosen as the experimental subjects. Six mice were obtained from Ningxia Medical University and maintained under specific standard conditions. Prior to the surgical procedures, the mice were housed in controlled environmental conditions

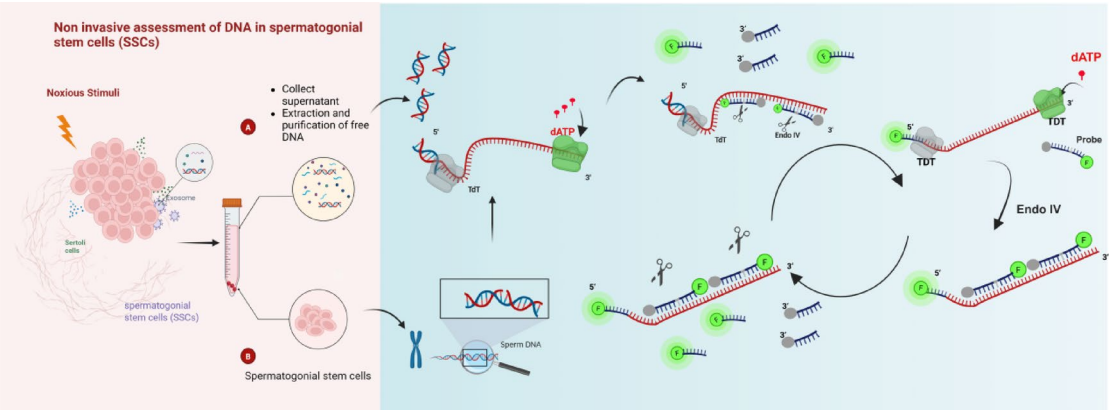

Fig. 1. Schematic diagram of the process of spermatogonial stem cell selection, identification, heat and freeze stress, and DNA breakpoint detection. (Created with BioRender.com).

characterized by an ambient temperature of 25 °C, a relative humidity of 65%, and a 12/12-hour light-dark cycle. During this period, the mice were provided with ad libitum access to both food and water. The animals were randomized into groups based on individual experiments to avoid the potential bias. In order to minimize the pain and discomfort experienced by the mice during the surgical procedures in this project, we deeply anesthetized the mice with intraperitoneal injection of pentobarbital sodium before collecting their bilateral testes for the extraction of SSCs.

Ethics statement

All animal experiments were performed in strict accordance with the the Guide for the Care and Use of Laboratory Animals. The use of animals in this study was approved by the Ethics Committee of Ningxia Medical University (NGY2020052). This study was performed in accordance with ARRIVE guidelines (https://arriveguidelines.org).

Isolation and culture of SSCs

Please refer to the supplementary material for detailed methods and protocols for the isolation, culture, and identification of SSCs.

Cryopreservation and thawing of cells

Cryopreservation

Freezing process: the cells and cryoprotectant were gently mixed, and then added into a 1.5 mL cryopreservation tube. The cryopreservation tube was put into a programmed cooling box, and then put into a refrigerator at minus 80 ℃ overnight and stored in liquid nitrogen. The formula of frozen liquid: complete culture liquid about SSCs: serum (FBS): DMSO = 7:2:1; adding LBP (Cat:6420004, Ningxia Wolfberry Biological and Food Engineering Co., Ltd, China), according to the final concentration of LBP, the groups were 0 mg/mL, 0.1 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL, 3 mg/mL, 4 mg/mL.

Thawing procedure

The frozen tube was defrosted quickly at 37 °C water bath, then centrifuged at 1200 rpm and resuspend.

Determination of cell survival rate after thawing of SSCs

The cell survival rate was measured with trypan blue solution. After thawing, SSCs cells were stained in 1% trypan blue solution for 3–5 min, then the cells were counted by hemocyte counter. The number of dead cells and living cells were counted and cell viability was calculated. The cell survival rate were obtained from the group treated with 0 mg/mL, 0.1 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL, 3 mg/mL, or 4 mg/mL, the group with the highest cell survival rate in the LBP groups was selected as the LBP group (LBP-1), while the 0 mg/mL group was used as the control group (Control-1), and the unfrozen SSCs were used as the fresh group for subsequent experiments (Fresh-1).

Establishment of heat stress model

Counted by cell counter 1.6×10^4 cells were inoculated into 6-well plates with three parallel holes in each group. The heat stress treated cells were incubated at 43 ℃ for 15 min, 30 min, and 45 min respectively and then transferred to 37 ℃ incubator for 2 h for subsequent experiments. SSCs were divided into Fresh group (Fresh-2), Heat stress treatment (Control-2) group and LBP intervention group (LBP-2). Select the group with the most obvious DNA damage in the heat stress model as the positive control group, and add a certain concentration of LBP as the intervention group in the reorganization mode.

Determination of cell proliferation by cell counting Kit-8 (CCK-8) assay

The CCK-8 kit (Meilun Bio, Dalian, China) was used for cell detection. Approximately 10,000 cells were seeded into each well of a sterile 96-well plate, with approximately 100μL of SSCs suspension added to each well. The plate was gently shaken to ensure even distribution of the cells. Three replicate wells were set up in the 96-well plate.Under subdued lighting, 10 μl of CCK-8 solution was added to each well in the sterile hood. The plate was then placed in a 37 °C, 5% CO_2 incubator and incubated for 2 h. The absorbance of each well was measured at 450 nm using a spectrophotometer.

DNA breakpoint detection

DNA extraction from SSCs

DNA extraction kit (catalog number DP304, Cellpro, Beijing TIANGEN BIOTECH Co., Ltd., Beijing, China.) was used. The DNA extraction process of SSCs is carried out in strict accordance with the instructions of the kit. Measure the concentration of extracted DNA on the Nanodrop 2000 instrument, and then put it in the − 80 ℃ low-temperature refrigerator for later detection.

Free DNA extraction from culture medium

Free DNA extraction kit (catalog number DP339, Cellpro, Beijing TIANGEN BIOTECH Co., Ltd., Beijing, China.) was used. The DNA extraction process of Free DNA is carried out in strict accordance with the instructions of the kit. Measure the concentration of extracted DNA on the Nanodrop 2000 instrument, and then put it in the − 80 ℃ low-temperature refrigerator for later detection.

DNA breakpoint detection (Free DNA fragment count detection)

The number of DNA breakpoints of SSCs was detected according to the detection method published on Clinical Chemistry[17], with the omission of the enzyme inactivation step. We adjusted the concentration of the sample

DNA (50 ng/μL, 100 ng/μL, 150ng/μL, 200 ng/μL, 250 ng/μL), and the final loading concentration was 100 ng/μL, and the loading volume was 2 μL. The experimental group was blind to both the two behavioral performers to avoid the potential bias.

Detection of parameters related to reactive oxygen species

Detection of malondialdehyde (MDA) total superoxide dismutase (T-SOD) glutathione peroxidase (GSH-Px) and Reactive oxygen species (ROS). Thiobarbituric acid (TBA) colorimetric method was used to determine the content of MDA in each group in strict accordance with the instructions of MDA kit (Jiancheng, Nanjing, China) provided by Nanjing Jiancheng Institute of Bioengineering. The levels of T-SOD and GSH-Px in fresh group, control group and LBP group were detected according to the instructions of T-SOD kit (Jiancheng, Nanjing, China) and GSH-Px kit (Jiancheng, Nanjing, China) provided by Nanjing Jiancheng Institute of Bioengineering. The level of ROS in cells were detected by flow cytometry using DCFHDA fluorescent probe (Solarbio, Beijing, China), Green fluorescence emission was measured between 504 and 529 nm using flow cytometry.

Western blot

Protein was extracted with a whole protein extraction kit (Keygentec, Nanjing, China) and protein was then quantified using the bicinchoninic acid method (Keygentec, Nanjing, China). In each lane, Protein lysate was separated by 10% SDS-PAGE and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Massachusetts, USA) and the membranes were blocked in 5% non-fat milk (blocking solution) for 1 h. Then the membrane was incubated with antibodies against Bcl-2, Bax, CytC, and Caspase-3 in control group and LBP group. Primary antibodies: Caspase-3 (ProteinTech Group, Chicago, IL, USA, 1:10000), Bax (ProteinTech Group, Chicago, IL, USA, 1:5000), Bcl-2 (ProteinTech Group, Chicago, IL, USA, 1:10000), CytC (Protein Tech Group, Chicago, IL, USA, 1:1000), β-ACTIN (Protein Tech Group, Chicago, IL, USA, 1:10000). Secondary antibody: goat anti-rabbit IgG (H&L) HRP (Abcam, 1:5000).

Statistical methods

All data were expressed as the mean ± standard deviation (SD) and analyzed by SPSS24.0 statistical software. Differences among multiple groups were analyzed by Welch's one-way ANOVA, following by Tukey's HSD test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Isolation, culture, and identification of mouse SSCs

We successfully established a co-culture system with Sertoli cells for the in vitro cultivation of spermatogonial stem cells (SSCs). The Sertoli cells were identified through Sox9 immunostaining, which showed specific nuclear localization of the marker (Supplementary Fig. 1). After culturing freshly isolated SSCs in this system for 48 h, differential interference contrast microscopy revealed that the cells maintained typical round or oval morphologies and formed distinct grape-like colonies (Fig. 2A). Immunofluorescence analysis indicated specific staining for the stem cell markers ID4, CD90, and CD49f; although there was some background non-specific

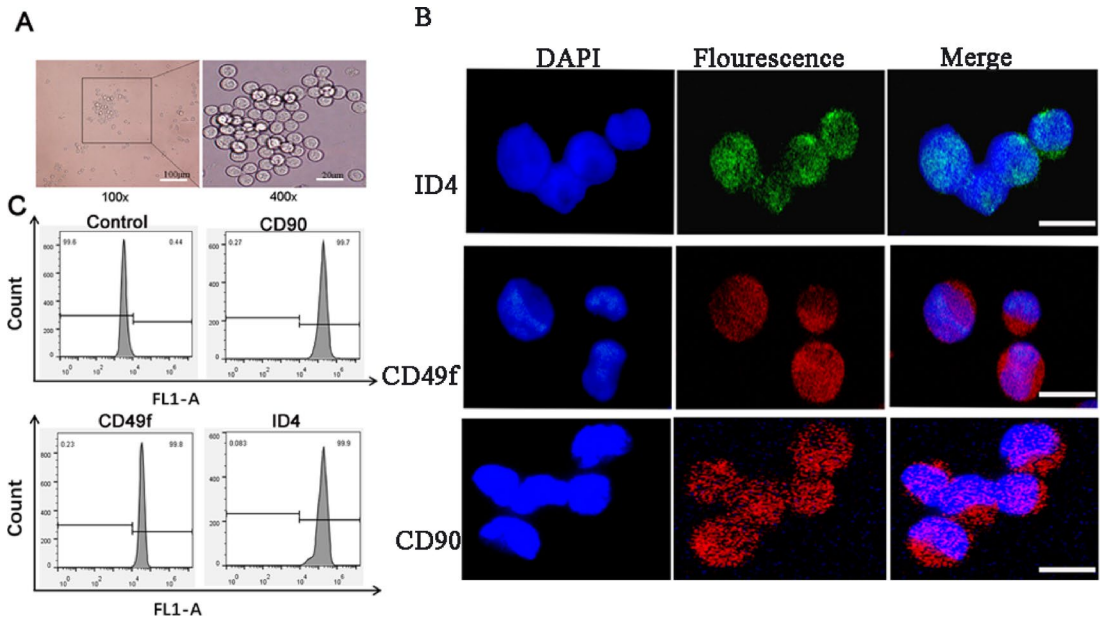

Fig. 2. Characterization of SSCS. (**A**) Morphological images of stem cells captured at varying magnifications (bright field). (**B**) Immunofluorescence staining outcomes for the SSCS markers ID4, CD90, and CD49f. (Scalebars, 20 μm) C Flow cytometry-based expression analysis of the SSCS markers ID4, CD90, and CD49f. Note: *SSCs* spermatogonial stem cells.

staining (possibly due to insufficient washing of the secondary antibody), the specific signals for the three markers were clearly distinguishable. Further flow cytometry analysis showed a significant upregulation of ID4, CD90, and CD49f expression levels in the cultured spermatogonial stem cells (Fig. 2C).

The proliferation of SSCs under different stimuli

To investigate the growth and proliferation of SSCs under different stimuli, we conducted CCK-8 experiments to assess the proliferation of SSCs under hydrogen peroxide (H_2O_2) stimulation, freezing stimulation, and heat stress. The results revealed that as the duration of reactive oxygen species (ROS) stimulation increased, cell proliferation weakened. Additionally, both freezing and heat stimulation resulted in a decreased proliferation of SSCs (Supplementary Fig. 2).

Principle verification of TdT-endonuclease IV-fluorescent probe sensor for detecting the integrity of stem cell DNA

Initially, we extracted DNA from SSCs using an optimized protocol, which resulted in high DNA concentration and purity (Supplementary Table 1). We then investigated the optimal DNA loading amount for assessing the integrity of spermatogonial stem cell DNA using the TdT-Endonuclease IV-Fluorescent Probe sensor (Fig. 3A), Our findings indicated that an increase in the DNA loading amount led to a corresponding rise in the slope of the line, signifying a significant increase in the number of DNA breaks in the stem cells. This confirmed the feasibility of using this method to detect DNA breakages in SSCs. Moreover, as the loading amount increased proportionally, the rate of fluorescence signal elevation (i.e., the slope of the line) also showed a proportional increase. For subsequent experiments, we selected a DNA loading amount of 200 ng for detecting DNA breakpoints.

We successfully employed the TdT-Endonuclease IV-Fluorescent Probe sensor to establish a standard curve for DNA breakpoint detection using a series of standard concentrations of single-stranded DNA (0 nM, 0.01 nM,

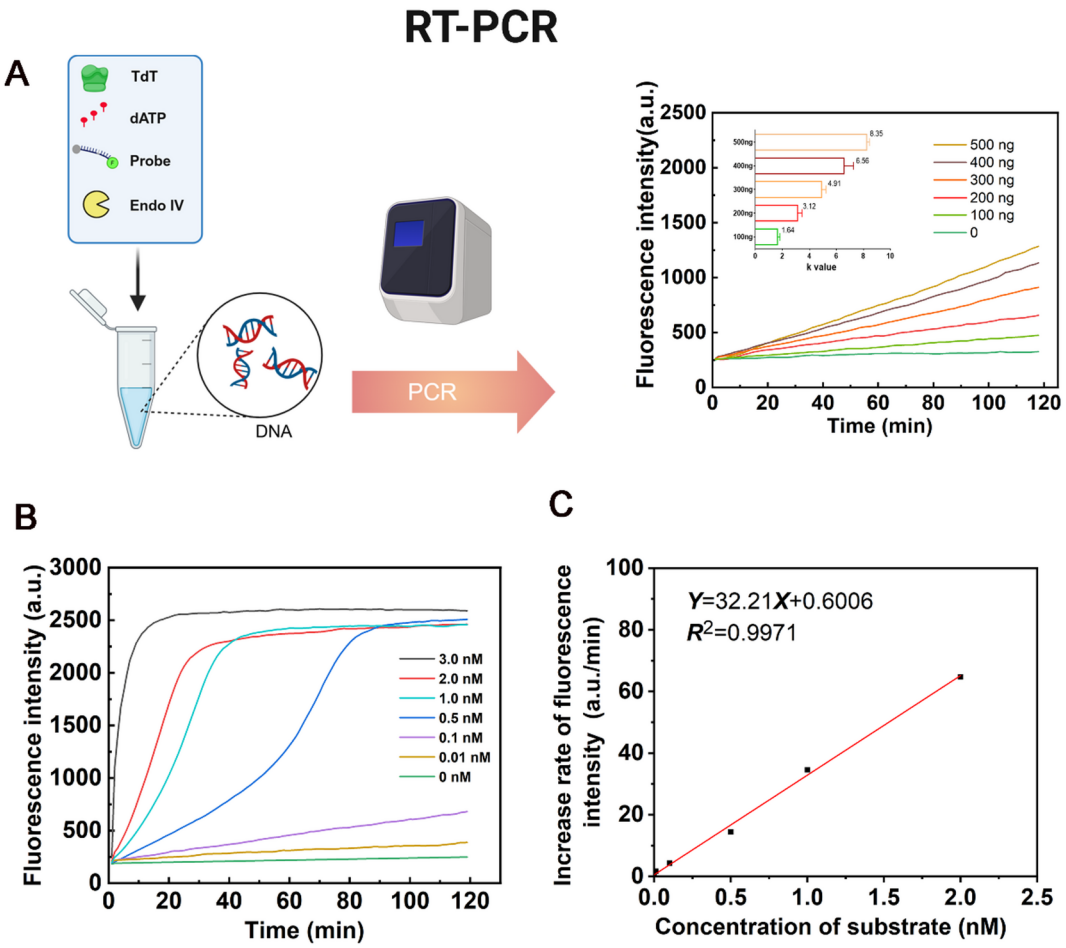

Fig. 3. SSCS DNA Breakage Detection: Principle Validation and Loading Concentration Optimization (**A**) Fluorescence signals of spermatogonial stem cell DNA breakage at different loading amounts (0 ng, 100 ng, 200 ng, 300ng, 400 ng, 500 ng). The rate of fluorescence signal increase = slope of the line (n=3). (**B**) Standard curve for DNA breakage detection using single-stranded DNA at specific concentrations (0 nM, 0.01 nM, 0.1 nM, 0.5 nM, 1.0 nM, 2.0 nM, 3.0 nM). (**C**) Linear correlation between the rate of fluorescence signal increase and the standard concentrations of single-stranded DNA in Figure A. **Note:** SSCs: Spermatogonial Stem Cells.

0.1 nM, 0.5 nM, 1.0 nM, 2.0 nM, 3.0 nM). The results of this standard curve (Fig. 3B-C). Importantly, the curve demonstrated a highly linear relationship with a linearity exceeding 0.99, within a linear range of 0.01 to 2.0 nM.

Effects of reactive oxygen species on SSCs

To examine the effects of reactive oxygen species (ROS) on SSCs, we employed hydrogen peroxide (H_2O_2) as a stimulant. In the unstimulated control group (0 h), the number of DNA breakpoints in the stem cells remained at a very low level. However, after a 1-hour intervention, there was a significant increase in the number of breakpoints, and this trend continued as the duration extended (Fig. 4A).

Cryopreservation experiment and heat stress experiment of SSCs

In the cryopreservation-induced stress experiment, we utilized the optimized novel sensor to assess DNA integrity in SSCs. We investigated the intervention effect of Lycium barbarum polysaccharides (LBP). The results showed that cryopreservation led to an increase in the number of DNA breakpoints in SSCs (Fig. 4B). Following cell viability experiments and the selection of the optimal concentration of LBP (2 mg/mL) (Supplementary Fig. 3), we examined its effect during the cryopreservation process. The results demonstrated that LBP effectively reduced DNA damage (Fig. 4B).

Next, we used the sensor to detect DNA breakpoints in SSCs subjected to heat stress at different time points. The results indicated that after 45 min of heat stress, the number of DNA breakpoints in SSCs was significantly higher compared to the other control groups (Supplementary Fig. 4). Therefore, we selected 45 min of heat stress as the duration for subsequent experiments. Throughout the entire heat stress process, we administered LBP as an intervention. The results showed that heat stress significantly increased the number of DNA breakpoints in SSCs, but LBP effectively reduced the number of DNA breakpoints during heat stress ($p < 0.05$, Fig. 4C).

Detection of free DNA fragments

We successfully extracted free DNA from the culture medium of SSCs (Fig. 5A) and obtained DNA with good concentration and purity (Supplementary Table 2). We applied this method to the H_2O_2 model, cryopreservation model, and heat stress model, and detected free DNA in the H_2O_2 (0, 1 h, 2 h) groups, fresh group (Fresh),

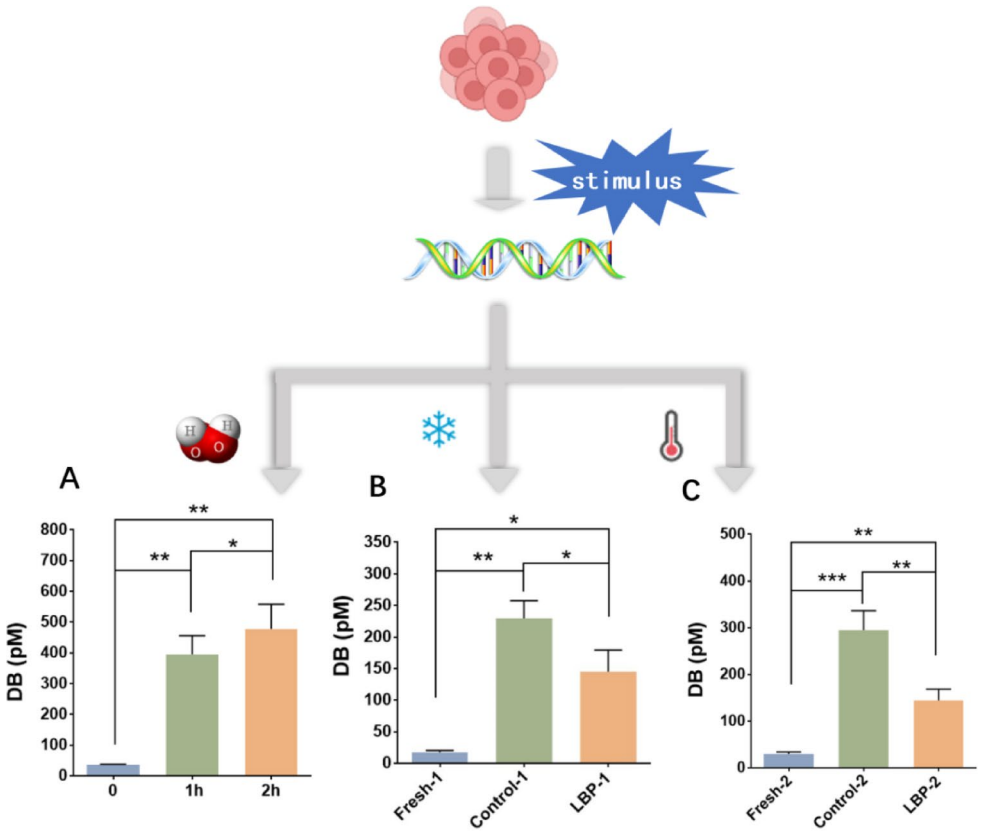

Fig. 4. Fluorescence Signal Detection of DNA Breakpoints in SSCs Exposed to Different Stresses. (**A**) Fluorescence signal detection of DNA breakpoints in SSCs treated with H2O2 (100 μmol/L). (**B**) Bar chart illustrating the frequency of DNA breakpoints in SSCs subjected to freeze stress. (**C**) Bar chart illustrating the frequency of DNA breakpoints in SSCs subjected to heat stress. Note: The experiments were conducted utilizing the innovative sensor to evaluate the occurrence of DNA breakpoints in SSCs exposed to distinct stress conditions. All the above assays were independently performed in triplicate (n=3). *p<0.05, ** p<0.01, ** *p<0.001.

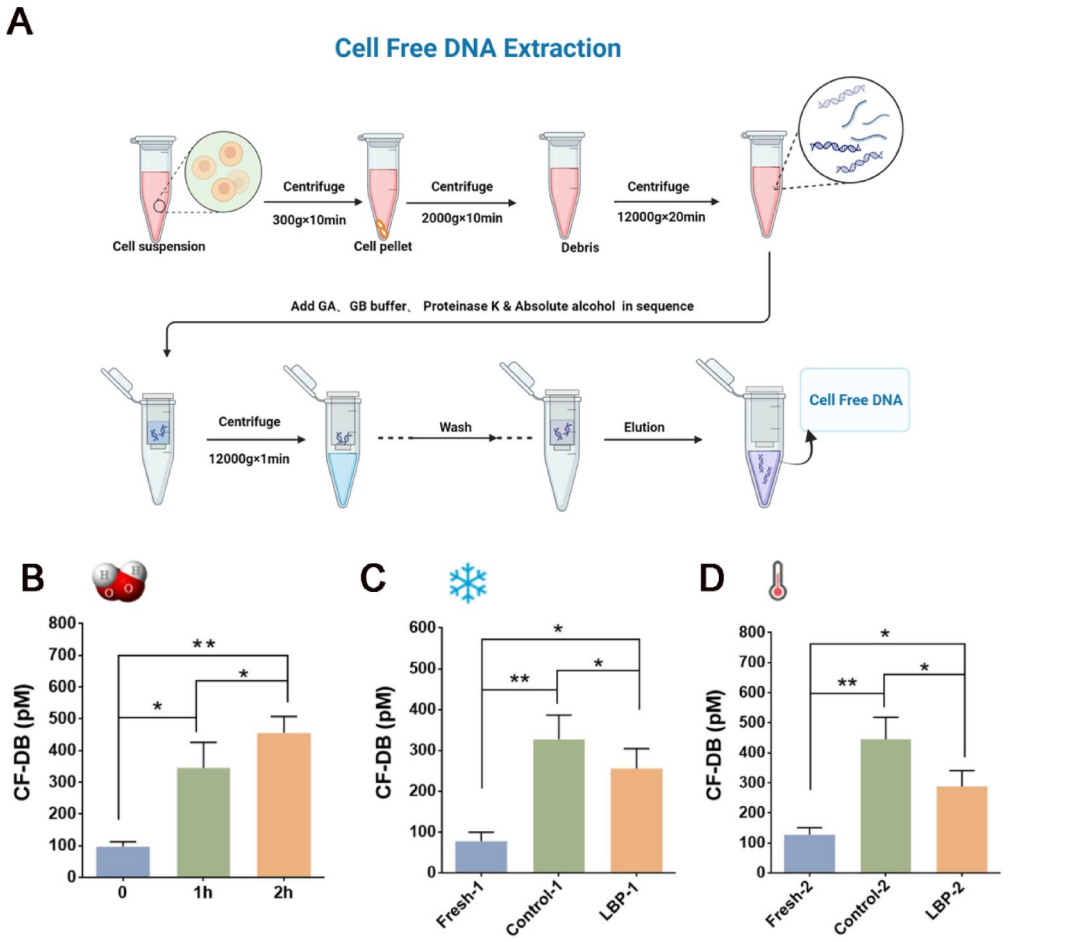

Fig. 5. Detection of Free DNA Fragment Equivalents using the Novel Sensor. (**A**) The process of extracting free DNA. (**B**) Bar graph showing the equivalent amount of free DNA fragments under freezing. (**C**) Bar graph showing the equivalent amount of free DNA fragments under heat stress. Note: All the above assays were independently performed in triplicate ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

stress group (Control), and antioxidant group (LBP). Importantly, as the exposure time to H_2O_2 increased, there was a significant increase in the number of free DNA fragments observed (Fig. 5B). Similarly, in the heat stress and cryopreservation stress models, the number of free DNA fragments released into the culture medium significantly increased compared to the unstimulated group. However, when the antioxidant LBP was added, there was a significant decrease in the number of free DNA fragments. (Fig. 5C-D)

Exploration of DNA damage pathways

In the stress model of SSCs, characterized by an increased number of DNA breakpoints and intervention with Lycium barbarum polysaccharides (LBP), we conducted an in-depth investigation into the molecular mechanisms underlying DNA damage under heat stress. The results indicated that heat stress resulted in a decrease in the levels of antioxidant substances GSH-PX (Fig. 6B) and T-SOD (Fig. 6C), along with an increase in MDA (Fig. 6A) and ROS (Fig. 6D-E). Following intervention with LBP, there was a significant increase in the levels of antioxidant substances and a notable decrease in ROS levels (Fig. 6B-E). Additionally, protein analysis related to apoptosis-related molecules showed that after LBP intervention, SSCs exhibited increased expression of the anti-apoptotic protein Bcl_2 and decreased expression of the apoptosis-related proteins Bax, Cytc, and Caspase-3 (Fig. 6F).

Discussion

Maintaining the integrity of stem cell DNA is crucial for normal proliferation and differentiation[26]. However, during cell growth and in response to stress, oxygen-free radicals (ROS) generated may lead to DNA breaks and damage, significantly affecting stem cells[27]. Timely repair of DNA breaks is essential to prevent genomic instability and disease. Traditional comet assays have limitations, necessitating advanced techniques for accurate assessment of DNA damage[28].

Inspired by the TdT enzyme-IV endonuclease-fluorescent probe sensor's capability to detect 3'-hydroxyl in DNA[17], we explored its application for assessing the DNA integrity of SSCs. Our method quantitatively measures extracellular free DNA fragments, providing a new approach to assess stem cell DNA damage. Using a two-step

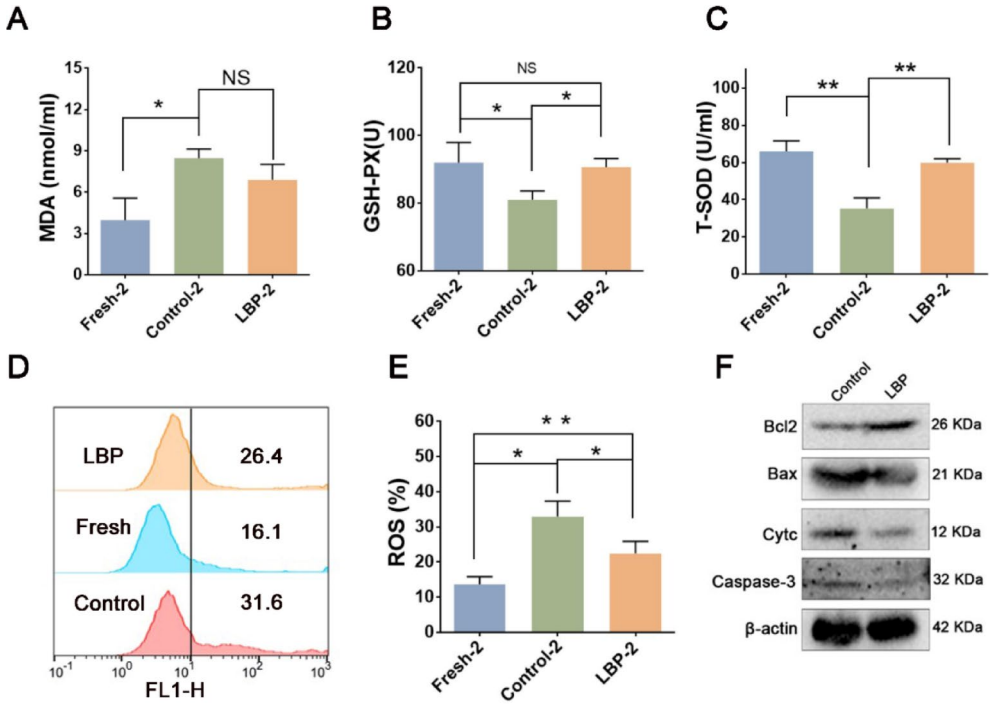

Fig. 6. Detection of ROS-related markers and apoptosis-related proteins in SSCs under heat stress and LBP intervention. (**A**) MDA levels in SSCs. (**B**) GSH-Px levels in SSCs. (**C**) T-SOD Levels in SSCs. (**D**) Flow cytometry results of ROS in SSCs. (**E**). Bar chart showing ROS levels in SSCs. (**F**) Western blot results of apoptosis-related proteins in SSCs after LBP Intervention. Note: Data are presented as mean ± standard deviation ($n = 3$). *$p < 0.05$, ** $p < 0.01$. *SSCs* spermatogonial stem cells, *NS* not significant.

enzyme digestion method, we successfully isolated, cultured, and identified SSCs from mouse testes. The stem cell markers (Id4, CD90, CD49f)[29-31] were confirmed through immunofluorescence and flow cytometry, further confirming them as SSCs. We utilized the TdT enzyme-IV endonuclease-fluorescent probe sensor to detect DNA breaks in stem cells, confirming that the number of breakpoints is proportional to the sample input. This finding demonstrates the feasibility of this new sensor for detecting DNA breakpoints in stem cells.

We further conducted experiments where we co-cultured cells with oxygen-free radicals, specifically using H_2O_2 as an example. We observed that DNA breaks in cells increased over time, emphasizing the role of ROS in causing DNA damage. Additionally, in a heat stress model, we observed that heat stress exacerbated DNA breaks. However, the application of Lycium barbarum polysaccharide (LBP)[32-35], a natural antioxidant, mitigated the heat stress-induced DNA breaks. Similarly, in a cold stress model, we observed an increase in DNA damage in cells, but LBP alleviated this effect.

Moreover, in both the heat stress and cold stress models, we utilized the novel sensor to detect extracellular free DNA fragments released from stem cells into the culture medium. Interestingly, we found that stress not only increased DNA damage within the cells but also led to an increase in the release of extracellular free DNA fragments. However, when a certain concentration of LBP was applied, it effectively reduced both DNA damage and the release of these fragments. Consequently, we successfully achieved a non-invasive assessment of stem cell DNA integrity using this innovative sensor.

In the heat stress model, we conducted further measurements of oxidative stress markers and observed an increase in reactive oxygen species (ROS) levels, along with a concurrent decrease in antioxidant components such as GSH-PX and T-SOD. However, we found that LBP was able to counteract this trend. Moreover, through the detection of key proteins in the signaling pathways associated with cell apoptosis, we discovered that LBP's beneficial effect on DNA integrity could be attributed to its ability to synergistically maintain GSH-PX and T-SOD enzyme activities, thereby reducing the damage caused by reactive oxygen species to cells. Our research findings indicate that LBP possesses antioxidant properties while simultaneously promoting an increase in the anti-apoptotic protein Bcl2 and a reduction in the pro-apoptotic protein BAX. This effectively slows down the release of cytochrome CytC from mitochondria in the cell apoptosis signaling pathway, ultimately leading to a decrease in the activation and release of the Caspase-3 protein (Supplementary Fig. 5). It is important to note that other signaling pathways may also contribute to this protective effect.

Excessive reactive oxygen species (ROS) can cause damage to cell membranes, leading to impaired DNA integrity and an increase in extracellular free DNA fragments. The introduction of LBP as an antioxidant helps reduce ROS levels and enhances the protection of DNA integrity through antioxidant mechanisms. As a result, our research validates the accuracy of the novel sensor in identifying and quantifying extracellular free DNA fragments induced by oxygen-free radicals. This non-invasive assessment method provides valuable insights into

the impact of ROS on DNA integrity in stem cells, laying the groundwork for studying DNA damage mechanisms and protective strategies. It also holds potential clinical applications in stem cell therapy and other areas.

It is noteworthy that the innovative technology utilized in this project holds promise not only for the study of DNA damage in SSCs but also for broader applications in DNA damage analysis across various cell types. This advanced technology offers several advantages[17] over the commonly used TUNEL assay, including enhanced accuracy, reduced costs, and the incorporation of quality control standards. Moreover, its capability to detect fragmented DNA released by cells into the extracellular milieu expands its potential for evaluating cellular responses and environmental influences. As a result, this technology provides new research tools for disciplines such as cell biology, tumor research, and regenerative medicine. Therefore, advocating for and implementing the use of this technology will contribute to a deeper understanding of DNA damage mechanisms in diverse cell types and their significance in biomedical research endeavors.

Conclusion

This method promotes the study of the relationship between environmental factors such as free radicals and the DNA integrity of SSCs, enhancing our understanding of male reproductive capability. This technology can assess the impact of free radicals and ions on DNA integrity, contributing to regulatory decisions and public health policies. By elucidating the link between ROS and stem cell DNA integrity, this groundbreaking technology also holds promise for reproductive health and environmental risk assessment.

Data availability

Data is provided within the manuscript and supplementary information files.

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Author contributions

Methodology, J.W., Y.Z. and L.F.Z.; Formal Analysis, J.W., L.H.M. and B.Y., J.W. wrote the manuscript. All authors reviewed the manuscript. L.H.M. and B.Y. designed the study; Y.Z. and L.F.Z prepared Figs. 1, 2, 3, 4, 5 and 6.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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