Heat Stroke Induces Pyroptosis in Spermatogonia via the cGAS-STING Signaling Pathway

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Summary
To explore the mechanism whereby cGAS-STING pathway regulates the pyroptosis of cryptorchidism cells, with a view to finding a new strategy for clinically treating cryptorchidism-induced infertility. Spermatogonial GC-1 cells were heat stimulated to simulate the heat hurt microenvironment of cryptorchidism. The cell viability was assayed by CCK-8, and cellular DNA damage was detected by γ-H2AX immunofluorescence assay. Flow cytometry was employed to assess pyroptosis index, while western blot, ELISA and PCR were used to examine the expressions of pyroptosis-related proteins (Caspase-1, IL-1β, NLRP3) and cGAS-STING pathway proteins (cGAS, STING). After STING silencing by siRNA, the expressions of pyroptosis-related proteins were determined. Pyroptosis occurred after heat stimulation of cells. Morphological detection found cell swelling and karyopyknosis. According to the γ-H2AX immunofluorescence (IFA) assay, the endonuclear green fluorescence was significantly enhanced, the γ-H2AX content markedly increased, and the endonuclear DNA was damaged. Flow cytometry revealed a significant increase in pyroptosis index. Western blot and PCR assays showed that the expressions of intracellular pyrogenic proteins like Caspase-1, NLRP3 and GSDMD were elevated. The increased STING protein and gene expressions in cGAS-STING pathway suggested that the pathway was intracellularly activated. Silencing STING protein in cGAS-STING pathway led to significantly inhibited pyroptosis. These results indicate that cGAS-STING pathway plays an important role in heat stress-induced pyroptosis of spermatogonial cells. After heat stimulation of spermatogonial GC-1 cells, pyroptosis was induced and cGAS-STING pathway was activated. This study can further enrich and improve the molecular mechanism of cryptorchidism.

Key words
Cryptorchidism • Spermatogonial GC-1 cells • cGAS-STING pathway • Pyroptosis • Heat stress

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Introduction
Cryptorchidism is a common condition in pediatric urology, with prevalence rates reaching 1-9% in neonates, which increase year by year [1]. Infertility is one of the common complications in adults who developed cryptorchidism in their childhood [2]. Studying the mechanism of cryptorchidism-induced infertility has become a hot current topic.

Pyroptosis is a newly discovered mode of proinflammatory programmed cell death that differs from apoptosis, which is characterized by dependence on inflammatory cysteine protease, cell swelling and rupture, as well as inflammatory cascade caused by inflammatory cytokine release [3]. It is an excessive response to stimuli, whose ultimate result is cell damage [4]. Pyroptosis can be triggered by a variety of endogenous and exogenous risk factors, including Caspase-1-dependent classical pathway and non-Caspase-1-dependent pathway. Studies
Deng et al. have found that the occurrence of pyroptosis comes from the generation of initiating signals, which are derived from the expressions of proinflammatory cytokines pro-IL-β and pro-IL-18, forming inflammasomes [5-6]. A growing number of studies have shown that inflammation is a critical factor for sustained injury of cryptorchidism [7-8]. For example, Fraczek study showed that IL-1β was in a highly expressed inflammatory state in cryptorchid testes [7]. We could not help pondering whether cryptorchidism-induced infertility is correlated with the pyroptosis of gonocytes induced by intratesticular inflammatory microenvironment. The pyroptosis phenomenon of testicular tissues in cryptorchid children and its molecular mechanism remain unclear.

Cyclic GMP-AMP synthase (cGAS), also known as C6orf150 or MB21D1, belongs to the nucleotidyltransferase family and is a recently discovered DNA receptor in mammalian cytoplasm [9]. After being stimulated externally, cGAS can provide energy through magnesium ion mediation by adenosine triphosphate (ATP) and guanosine triphosphate (GTP), and undergo intramolecular structural adjustment to catalyze 2’3’-cyclic GMP-AMP (cGAMP) synthesis. As a second intracellular messenger, cGAMP has a strong binding ability with cytoplasmic sensors, mainly binding to STING proteins, IRF3-activated mitochondrial mediator (MITA), N-terminal methionine-proline-tyrosine-serine plasma membrane tetraspanner (MPYS), or endoplasmic reticulum IFN stimulator [10]. STING protein in resting state aggregates in the endoplasmic reticulum as homologous dimers, specifically located on the surfaces of mitochondria and endoplasmic reticulum. When stimulated by external stimuli, cGAMP binds to STING protein, while STING protein transfers from the endoplasmic reticulum surface to the nuclear periphery and activates the downstream pathways [11-12].

As a broad-spectrum DNA receptor in cytoplasm, cGAS-STING signaling pathway can activate its downstream pathways upon release of external stimuli like bacteria, viruses or DNAs. This pathway is associated chiefly with cellular immunity and autoimmunity, which can also regulate the release of inflammatory cytokines and antitumor properties [12]. After activation, it can enhance the autoimmunity of human body, but in pathological state, its activation may cause hyperinflammatory response [14]. Relevant research has found that cGAS-STING pathway is activated and pyroptosis occurs in stressed cells in liver, nervous and heart tissues: acute liver injury can activate the signaling pathway to cause cellular pyroptosis [15]. Ding et al. found that inhibition of cGAS-STING pathway could relieve the inflammatory burden of neurocytes, reduce pyroptosis, and better promote the neurocyte development [16]. In diabetic cardiomyopathy, activation of cGAS-STING pathway leads to pyroptosis of cells, thereby promoting the disease development [17]. The role of cGAS-STING pathway in pyroptosis of testicular cells remains unknown.

By simulating the internal environment of cryptorchid testes, this study explores the role of cGAS-STING pathway in the occurrence of pyroptosis after heat stress treatment of the spermatogonial GC-1 cells, with a view to providing new etiological molecular clues for cryptorchidism, thus offering innovative ideas and targets for its clinical prevention and treatment.

**Materials and Methods**

**Cell line and reagents**

The GC-1 cell lines were kindly provided by Chinese Academy of Sciences, and the passages of these cell lines used for the experiments were approximately 17-25. DMEM/F12 medium and fetal bovine serum were purchased by Gibco (USA). Primary antibody Cleaved Caspase-1, Pro-Caspase-1, N-GSDMD, NLRP3, STING, β-actin and cGAS were purchased from Cell Signaling Technology. γ-H2AX immunofluorescence assay kit was purchased from Beyotime (C2035S). The Cell Counting Kit 8 (CCK-8) solution is from the Nanjing, Jiancheng Bioengineering Institute (Nanjing, Jiancheng, China). Flow cytometry assay kit purchased from BD company. ELISA kit purchased from Wuhan Huamei. STING-siRNA was constructed by Gima Pharmaceutical Technology Co. LTD (Shanghai, China). The Trizol reagent and Prime Script reverse transcriptase reagent kit are from TaKaRa Biotechnology.

**Cell culture and heat-shock treatment**

GC-1 cells were cultured routinely in DMEM/F12 (Gibco, USA) supplemented with 10 % (v/v) FBS, 100 u/ml penicillin and 100 mg/ml streptomycin in a humid atmosphere of 37 °C, 5 % CO₂ and 95 % air. For the heat stress treatment, the Petri dishes were placed inside a 42 °C incubator for 90 min [18]. Following treatment, all cells were recovered at 37 °C for 6 h.
Cell viability detection

The viability of GC-1 cells was evaluated via CCK-8 assay (G021-1-3, Jiancheng Bioengineering, Manjing, China). The cells were seeded onto 96-well microplates (3650; Corning, NY, USA), and different experiments were conducted as per the design protocol. CCK-8 solution (10 μl) was added to each well and the cells were further incubated at 37 °C for 2 h, followed by measurement of absorbance at 450 nm. Cell viability was calculated by comparing the optical density (OD) values of treated vs. untreated cells.

Western blotting

Proteins extracted from GC-1 cells were separated by SDS-PAGE and electrotransferred onto the nitrocellulose membranes. The Pierce BCA Protein Assay Kit was used for protein quantitative analysis (23225, Thermo Scientific, USA). Next, the membranes were blocked with 5 % skim milk for 2 h, and then incubated with antibodies against Cleaved Caspase-1 (#89332, rabbit), Pro-Caspase-1 (#24232, rabbit), N-GSDMD (#39754, rabbit), NL RP3 (#15101, rabbit), STING (#50494, rabbit), cGAS (#31659, rabbit), β-Actin (#3700, mouse) (Cell Signaling Technology, Boston, MA, USA). Secondary antibodies were selected from sheep antibodies.

After thrice rinsing with TBST, the membranes were incubated with secondary antibody at room temperature for 2 h. β-actin was used as an endogenous control. ImageJ software 1.49v (National Institutes of Health, Bethesda, MD, USA) was used for semi-quantitative analysis of the gray value of bands. The ratio of the target protein to actin gray value was calculated for quantification, and the statistical results from multiple measurements were analyzed.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was reverse-transcribed using the Easy RT-PCR kit (LS1040, Promega, Madison, WI, USA) according to the manufacturer's instructions. β-actin was analyzed in parallel as an internal control. Real-time PCR assays were performed using Power SYBR Green Master Mix and an ABI 7300 real-time PCR detection system (both from Applied Biosystems, USA). The primer sequences used for the RT-PCR were: IL-1β forward, 5′-GACTCTTGCCTCACTTCAAGG-3′ and reverse, 5′-CAGGCGCTGTTTCTGGTCAACGA-3′; NLRP3 forward 5′-AACATGCTCAAGGAGGAAGA-3′ and reverse, 5′-GGCTGTTTCCAATCCATGA-3′; cGAS forward 5′-GTTCAAACACAAAGATAAGCTACTG-3′; reverse 5′-GCTGACGGAGTACACAATCCT-3′; STING forward 5′-CATTGGATGTGGCCCTC-3′, reverse 5′-GCCACGTTGAAATTCCTTTT-3′; Actin forward 5′-CTACAATGAGCCTCTGTCGCC-3′; reverse 5′-CAGGTCCAGACGCAAGATGC-3′. All of the primers were synthesized by RiboBio (Guangzhou, China). Fold changes in expression of each gene were calculated by a comparative threshold cycle (Ct) method using the formula 2^(-ΔΔCt).

ELISA

IL-18 and IL-1β levels in the culture medium were measured using enzyme-linked immune sorbent assay (Elisa) kits (Boster, China) according to the manufacturer’s instructions.

Flow cytometry

The cellular pyroptosis was detected by flow cytometry (556547, BD, New Jersey, USA). Fluorochrome-labeled inhibitors of caspases (FLICA) and propidium iodide (PI) were added to GC-1 cells. After FLICA/PI staining, flow cytometry was employed to detect pyroptosis, which was defined as PI (+) and FLICA (+).

γ-H2AX IFA assay

GC-1 cells from different experimental groups were placed on culture microplates, immobilized in 4 % paraformaldehyde for 30 min, washed with phosphate buffer saline (PBS), permeabilized with 0.3 % Triton X-100 for 30 min, washed with PBS for 5 min thrice and sealed with 5 % bovine serum albumin for 1 h. After overnight incubation with rabbit anti-γ-H2Ax (C2035S; Beyotime Biotechnology, China) antibody (1:200) at 4 °C, the cells were washed with PBS, incubated further with sheep anti-rabbit fluorescent secondary antibody (1:200) at room temperature for 1 h, and then washed with PBS for 5 min thrice. The 4′,6-diamidino-2-phenylindole (DAPI) solution was added to accomplish a 10-min incubation in the dark. After washing with PBS thrice, appropriate amount of anti-fluorescence quencher was added for image observation and photography by fluorescence microscopy.
Statistical analysis

All the data were presented as the means ± SD. The GraphPad Prism 8 (GraphPad Prism; La Jolla, CA, USA) was used for statistical analysis. Statistical differences between two independent groups were tested using a t-test or one-way or two-way ANOVA (Analysis of Variance). Correlation analysis was performed using Pearson’s linear correlation. Data are expressed as mean ± SD of three independent experiments. Differences were considered statistically significant at p<0.05.

Results

HS induces pyroptosis in GC-1 cells

GC-1 cells cultured under HS conditions were used to develop the cryptorchidism cell model (Fig. 1A). Cell viability was significantly lower in the HS than in the control groups (Fig. 1B). According to the γ-H2AX immunofluorescence (IFA) assay results, after heat stimulation, the endonuclear green fluorescence was significantly enhanced, the γ-H2AX content markedly increased (Fig. 1C); the pyroptosis rate in the HG group was markedly higher by flow cytometry (Fig. 1D). To investigate the role of pyroptosis in cryptorchidism cell model, we detected changes in caspase-1, N-GSDMD and NLRP3 in GC-1 cells. As shown in Figures 2A and B, Western blotting analyses showed that the expression of N-GSDMD and NLRP3 protein was markedly increased, the expression of pro-caspase-1 was obviously reduced after HS treatment. In addition, as shown in Figure 2C, qRT-PCR showed that the NLRP3 and IL-1β mRNA level were increased. ELISA results were consistent with previous results (Fig. 2D).

cGAS-STING pathway was upregulated in response to HS and triggered pyroptosis in GC-1 cells

We further investigated the potential involvement pathway of cGAS-STING in HS-induced cryptorchidism. The protein levels of cGAS and STING of GC-1 cells were examined. From Figure 3A, B, we found the protein levels of STING were significantly increased. qRT-PCR analysis revealed that cGAS, STING expression increased after exposing GC-1s to HS (Fig. 3C). These results demonstrated that cGAS-STING pathway promoted HS-induced pyroptosis in GC-1 cells.

HS-induced GC-1 cell pyroptosis was reversed by STING siRNA

To further determine the effect of altered cGAS-STING signaling pathway activity on HS-induced pyroptosis in GC-1 cells. We also found that pyroptosis of GC-1 cells was significantly inhibited by pretreatment with STING-targeted siRNA (Fig. 4A). As shown in Figure 4B, C, compared with the control groups, pyroptosis of GC-1cells pretreated with STING-targeted siRNA was significantly attenuated upon exposure to heat stress.

In Figure 4D, cleaved-caspase-1, N-GSDMD and NLRP3 activity were no significantly increased in the inhibition of the cGAS-STING pathway. Moreover, qRT-PCR analysis revealed that NLRP3, IL-1β and IL-18 expression not increased after exposing GC-1s to HS (Fig. 4E), indicating that cGAS-STING pathway overexpression induces pyroptosis.

Discussion

Although it has been recognized that cryptorchid children may develop infertility in adulthood, the molecular mechanism whereby cryptorchidism leads to infertility remains unknown [19]. According to a recent report, the loss of spermatogenic cells is the severest cause of spermatogenesis obstacle in cryptorchidism [20]. Research has found that the testes of cryptorchid children are under chronic thermal injury, and the DNA fragmentation of gonocytes is significantly enhanced when testis temperature is high, leading to the quantitative decline and functional changes of gonocytes [21]. Most studies have speculated that the apoptosis of heat stress-treated testicular spermatogenic cells is the primary cause of testicular cell injury in cryptorchidism, but apoptosis does not involve inflammatory or immune responses [22,23]. In this study, we treated spermatogonial GC-1 cells with heat stress to simulate the sustained heat injury of cryptorchid testes. We found that heat stress could induce pyroptosis in spermatogonial cells. Further mechanism exploration revealed that the cGAS-STING signaling pathway could reverse pyroptosis induced by heat stress. As indicated by these results, the cGAS-STING pathway plays an important role in pyroptosis of GC-1 cells induced by heat stress. This study further clarifies the molecular mechanism underlying sustained testicular injury in cryptorchidism, with a view to providing innovative ideas and targets for its clinical prevention and treatment.

Pyroptosis, as an adaptive immune response of the body upon stimulation of intracellular and extracellular environments, is a unique form of
Fig. 1. HS condition induce morphological changes in GC-1 cells. (A) Morphological detection found cell swelling and karyopyknosis; (B) GC-1 cells were exposed to HS conditions and tested for viability at 90 min using a CCK8 assay. (C) According to the γ-H2AX immunofluorescence (IFA) assay results, after heat stimulation, the endonuclear green fluorescence was significantly enhanced, the γ-H2AX content markedly increased; (D) Flow cytometry revealed a significant increase in pyroptosis index.

Fig. 2. HS condition activates pyroptosis genes in GC-1 cells. (A and B) Protein expression of activated pro-caspase-1, cleaved-caspase-1, N-GSDMD, NLRP3 was detected by Western blot, and band density was quantified relative to β-actin and is graphed on the right. (C) qRT-PCR analysis of NLRP3, IL-1β levels in GC-1 cells in HS condition. (D) ELISA analysis of IL-1β, IL-18 levels in GC-1 cells in HS condition. One representative image of more than three independent experiments is shown. Data are expressed as mean ± SD. * p<0.05, ** p<0.01, compared with the control group.
Fig. 3. cGAS-STING pathway was upregulated in response to HS and triggered pyroptosis in GC-1 cells. (A and B) HS caused cGAS-STING activation in the GC-1 cells. The levels of STING were increased. The active form of cGAS-STING was measured by western blotting following HS treatment. (C) qRT-PCR analysis of cGAS, STING levels in GC-1 cells in HS condition. β-actin was used as loading control. One representative image of more than three independent experiments is shown. Data are expressed as mean ± SD. * p<0.05, ** p<0.01, compared with the control group.

Fig. 4. siRNAs targeting STING the GC-1 cells pyroptosis were reversed. (A) Western blot indicated that STING expression had been inhibited. (B) γ-H2AX immunofluorescence analysis of nuclear changes in GC-1 cells of vector and si-STING groups. (C) Pyroptosis was detected using flow cytometric analysis. (D) Western blot analysis of pro-caspase-1, cleaved-caspase-1, N-GSDMD, NLRP3 in GC-1 cells of vector and si-STING groups. (E) qRT-PCR analysis of NLRP3, IL-1β, IL-18 levels in GC-1 cells of vector and si-STING groups. Data are expressed as mean ± SD. * p<0.05, ** p<0.01, compared with the control group.
programmed cell death in terms of morphology and mechanism, which plays a regulatory role by affecting the assembly and activation of inflammasomes [24]. When the nod-like receptor protein-3 (NLRP3), an inflammasome family member, binds to proCaspase-1 and adaptor protein ASC, it processes proCaspase-1 into catalytically-active Caspase-1, which promotes the maturation and release of IL-1β and IL-18 and triggers strong inflammatory response, thereby inducing pyroptosis [25,26]. In this study, a model of spermatogonial GC-1 cells damaged by heat stress was established first. IFA assay showed pronouncedly increased nuclear damage and DNA double-strand breaks. Flow cytometry revealed that the pyroptosis index increased obviously. Western blotting and PCR assay were employed to detect the activities of pyroptosis-related proteins like Caspase-1, NLRP3 and GSDMD, finding elevated intracellular expressions of pyroptosis-related molecules. These results suggest that pyroptosis occurred in cells.

When cells are stimulated or damaged, plenty of changes take place in the mitochondrial biological functions, such as changes in membrane permeability and loss of membrane potential [27,28]. As one of the major pattern recognition and effect pathways in the innate immune system, cGAS-STING is mainly responsible for recognizing the cytoplasmic DNA molecules and activating the downstream signaling pathway to produce type I interferon (IFN-I) and other inflammatory cytokines [29]. Upon stimulation by external stimuli, cGAMP binds to STING protein, and the STING protein transfers from the endoplasmic reticulum surface to the nuclear periphery and activates the downstream pathway [30]. In our study, the expressions of cGAS-STING pathway-specific proteins cGAS and STING were detected after heat stimulation of spermatogonial GC-1 cells, finding that the change of cGAS was unobvious, while the STING protein was significantly upregulated.

A growing number of studies have found that STING protein, a cytoplasmic DNA sensor, is closely related to the pyroptotic process of cells. STING protein and TANK-binding kinase 1 (TBK1) form STING-TBK1 complex, which phosphorylates and regulates pyroptosis-related proteins to promote pyroptosis [31]. In human bone marrow cells, pyroptosis occurs when STING is highly expressed [32]. Upon LPS-induced elevation in STING expression, the synthesis of intracellular inflammasomes is enhanced, and intracellular pyroptosis appears. In myocardial infarction, when STING-NLRP3 pathway is inhibited, pyroptosis of cardiomyocytes is weakened and their survival rate is higher [33]. Studies on STING protein and spermatogonial GC-1 cells are rather rare, and the correlation between STING protein and testicular development is even less clear. Our study revealed that the expression of STING protein increased after heat stimulation of GC-1, implying that STIGN protein was involved in the heat stimulation-induced process of pyroptosis. After inhibiting STING protein, the pyroptosis of GC-1 cells was reduced. Thus, presumably, the cGAS-STING pathway mediates the pyroptosis phenomenon caused by cryptorchidism.

Conclusively, we found that pyroptosis occurs in the spermatogonial GC-1 cells after heat stimulation. Further exploration showed that cGAS-STING pathway plays an important role in pyroptosis induced by heat stimulation of GC-1. This discovery is conducive to improving the molecular mechanism of cryptorchid injury, which provides an innovative idea for subsequent recovery of cryptorchidism. Our study had several limitations. For example, our results were from an in vitro study using a cell line. Moreover, mechanisms of the body are complex with many interactions which cannot be replicated by in vitro work. Additionally, in vivo animal experiments should be performed to confirm our in vitro results.

**Conflict of Interest**

There is no conflict of interest.

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