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FERTILITY PRESERVATION

Does prepubertal testicular tissue vitrification influence spermatogonial stem cells (SSCs) viability?

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Abstract
Purpose Testicular cryopreservation prior to chemotherapy or radiotherapy in children with cancer is one of the ways to preserve fertility. However, cryopreservation may cause damage to the testicular parenchyma cells. The objective of this study was to investigate effects of vitrification on the intracellular LDH leakage, cell cycle/apoptotic responses and apoptosis-related gene expression patterns in the spermatogonial stem cells (SSCs) obtained from the vitrified testis.
Methods The testes of the mice pups (6-day-old, BALB/c) both vitrified and fresh groups were digested with enzymes (collagenase, DNaseI, trypsin-EDTA) to disperse the cells. The SSCs, type A, were isolated from the rest of testicular cells by MACS. The amount of damage to the SSCs immediately was evaluated by Cytotoxicity assay, Flow cytometry assay and Real-time PCR.
Results The intracellular LDH leakage in the SSCs,harvested from the vitrified testes, was less reported compared with the fresh ones. Moreover, the percentage of apoptotic and necrotic SSCs obtained from the vitrified testes was lower than that of yielded from the fresh samples. Also, the apoptosis-related genes of the SSCs,collected from the vitrified testes, changed their expression profile as increasing P53 and BCL-2 expression levels and decreasing Bax and Fas expression levels.
Conclusions The study indicates that vitrification of prepubertal testicular tissue does not increase the expression profile of apoptosis-related genes such as Bax and Fas in the testicular SSCs consistent with diminished cell apoptotic/necrotic responses and no increasing intracellular LDH leakage.

Keywords Vitrification · SSCs · Intracellular LDH leakage · Cell cycle/apoptotic responses · Gene expression profiles

Capsule Vitrification may not create severe apoptosis conditions and a necrosis process in the SSCs obtained from the vitrified testes.

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Introduction

During puberty, the testis is a dynamic tissue and its spermatogonial stem cells (SSCs) constantly proliferate and differentiate to become mature spermatozoa [10]. SSCs proliferation and differentiation is shown in various stages of development [10]. However, this process in the subjects with cancer treated with radiotherapy and chemotherapy usually is impaired and consequently may lead to sterility of the treated cases [8].

In recent years, the cryopreservation has been introduced as a suitable procedure to preserve fertility in patients with cancer that should be treated with chemotherapy or radiotherapy agents [12, 47, 48]. For instance, cryopreservation of sperms with subsequent in vitro fertilization (IVF) and making embryos is an effective technique to help the cancer

subjects to overcome their infertility [25–27]. However, this method is only for adult patients who have the ability to produce spermatozoa.

One relevant method for preservation of prepubertal male fertility is the use of a testicular parenchyma (or SSC) maintenance approach such as the rapid-freezing (vitrification) method or slow programmable freezing (SPF) set [1, 11, 14].

Numerous research reports show that the results of freezing SSCs, testicular tissue and as well as mature sperms by using SPF method are not satisfactory [1, 11].

Moreover, despite the fact that the vitrification method avoids ice crystal formation and cytogenetic abnormalities in the testicular cells [1], it was declared that this cryopreservation procedure could induce damages to the cells such as reducing their viability, inducing apoptosis, loss of DNA integrity and breakdown of cell membrane [1, 6, 7, 13, 36, 37].

It is outstanding to note that SSCs do not have acrosmal vesicle and also their metabolic action,in spite of its large size, is relatively negligible [21–24, 41]. Therefore, it seems that the cytogenetic abnormalities of SSCs after cryopreservation are not considerable and they are more resistant to the cryopreservation injuries in comparison with mature spermatozoa [41].

Nevertheless, detailed examination of the damage to SSCs during cryopreservation process before induction of the in vitro differentiation or transplantation may be important. Although to our knowledge did not study exist about of apoptosis-related gene expression profiles and subsequent intracellular LDH leakage and flow cytometry assay simultaneously. Therefore,in this study, apoptotic genes expression, LDH leakage and cell cycle/apoptotic responses during vitrification have been examined. Properly, determining the molecular and genetic events involved in vitrification will help us to design the most effective vitrification environments for testicular parenchyma (or SSC) preservation.

Materials and methods

Prepubertal testicular tissue vitrification

Healthy six-day-old male inbred BALB/c mice (n=80) were obtained from the Physiology Research Center. The mice were euthanized by excessive doses of ketamine HCl (80 mg/kg) and xylazine (10 mg/kg) (Pharmacia and Upiohn, Erlangen, Germany) in accordance with the protocol approved by the Animal Care and Use Committee. Every effort was made to minimize the number of animals used and their suffering. Then, the paired testes of the mice pups were collected and divided into two groups of the vitrified testes and fresh testes. Vitrification has confirmed to be an effective approach versus slow programmable freezing (SPF) method to cryopreserve tissue [3, 11]. Therefore,in the current study, the whole testes were vitrified by the use of a modified Abrishami et al. and Gholami et al. protocol [1, 14]. In brief,at the first step, the intact neonate testes of mice were transferred to the vitrification solution (VS) 1 that containing 0.5 ml sucrose, 7.5 % ethylene glycol and 7.5 % DMSO at room temperature for 10 min. Then testes were exposed to VS2 that has 0.5 ml sucrose, 15 % ethylene glycol and 15 % DMSO at room temperature for 10 min. Subsequently, the testes were put into VS3 containing 0.5 ml sucrose, 15 % ethylene glycol, 15 % DMSO and 20 % FBS at room temperature for 10 min. Finally, the testes were placed into cryogenic vials (Nunc, Roskilde, Denmark) that contain VS3 and plunged directly into the liquid nitrogen tank. The cryogenic vials were stored in the liquid nitrogen for at least 2 months. After removal of the samples from liquid nitrogen, they were maintained at room temperature for 30 s. Then, the samples were kept in a warm water bath at 37 °C agitating gently until completely thawed. After that, they were placed into thawing solution (TS) 1 that contains 0.5 ml sucrose for 5 min at 4 °C. Then, the testes were transferred to TS2 containing 0.25 ml sucrose for 5 min at 4 °C. Finally, the samples were put into TS3 that has 0.125 ml sucrose for 5 min at 4 °C.

Separation and purification of spermatogonial stem cells (SSCs)

The tissue digestion was done according to Milazzo et al.'s study with some modifications [33]. Briefly,after removing tunica albuginea, the thawed or fresh testes were digested in two steps. In the first step, testes were incubated in 1 mg/ml collagenase type IV (Sigma, St. Louis, MO, USA) and 200–700 μg/ml DNaseI (Sigma, St. Louis, MO, USA) for 15 min at 37 °C with slow pipetting. In the second step, after being centrifuged at 100×g for 5 min the supernatant was discarded and the cells were resuspended in media containing 0.25 % trypsin/1 mM EDTA (Invitrogen, Carlsbad, CA) and 200-μg/ml DNaseI for 5 min at 37 °C. The enzymatic reaction of trypsin was inactivated by adding 10 % FBS to the cell suspension.

The SSCs separation with laminin was done according to Shinohara et al.'s study with some modifications [42]. Briefly,before plating the cells, the 60 mm petri dishes were coated overnight with 20 μg/ml laminin and incubated with 0.5 mg/ml bovine serum albumin (BSA),1 h at 37 °C, to prevent nonspecific bands and then were washed with phosphate-buffered saline (PBS) buffer. Then the cells,to attach to laminin, were plated in the petri dishes for 1 h at 32 °C and then,after washing by PBS, were isolated by using trypsin-EDTA.

In the next step, the cells were incubated with CD90.1 (Thy1.1^{+}) antibody to isolate the SSCs type A from other cells. The procedure was performed as suggested by the manufacturer (Miltenyi Biotec, order no. 130-094-523). In

brief, 10^7 total cells were centrifuged at 300×*g* for 10 min and the cells pellet was resuspended in 90 μL of specific buffer solution that contains PBS, 0.5 % BSA and 2 mM EDTA by diluting MACS BSA Stock Solution (# 130-91-376) 1:20 with auto MACS Rinsing Solution (# 130-091-222). Ten μL CD90.1 Microbeads was added as well. It was mixed well and incubated for 15 min in the refrigerator (2–8 °C). The cells,after washing with PBS, were centrifuged at 300×*g* for 10 min. Up to 10^8 cells were resuspended in 500 μL of buffer solution. Then, the cell suspension was loaded onto a MACS Column which was placed in the magnetic field of a MACS Separator.

Cytotoxicity assay

The commercially available Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Mannheim, Germany) shows that LDH enzyme of the damaged plasma membranes of vitrified or fresh testicular parenchyma cells was released into the extracellular fluid (ECF). Each experiment was repeated three different times. Calorimetric assay for the quantification of LDH activity was performed according to the supplier's instruction (Roche Applied Science, Sandhofer, Mannheim, Germany). Absorbance was measured with an ELISA reader at 492 nm.

The APO™-BrdU TUNEL assay and flow cytometry analysis

Apo-Brdu Tunnel Assay Kit was used to detect cell cycle/apoptotic responses [4]. The procedure was performed according to the production user's catalog (Invitrogen, Catalog Number A23210). The SSCs evaluation was done immediately after cells collection. Cells suspension analyses were performed using the Partec Flow Max. Fluorchrome was excited with 488 lasers. Green and red fluorescences were detected using FL1 and FL3 detectors, respectively. Red fluorescence also was detected using FL2 detector. For viability, Brdu-FITC/PI 10000 events were analyzed. FL1 and FL2 fluorescence signals were recorded with logarithmic amplification and FL3 fluorescence signals were recorded with logarithmic linear amplification. The percentage of apoptotic cells was determined by the dot plot of FL1 (in the X axis) to FL3 (in the Y axis).

RNA isolation, cDNA synthesis and Real-time PCR

Extraction of RNA was performed by RNeasy Mini Kit according to the manufacturer's catalog (Qiagen, Cat. no. 74104). The purity and concentration of RNA were performed by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. The cDNA synthesis was carried out by the unique QuantiTect Reverse Transcription Kit according to the manufacturer's catalog (Qiagen, Cat. no. 205311). Real-time PCR was done using Applied Bioscience 7500 fast with SYBR Green detection for analyzing gene expression. Twenty seven reaction amplification cycles were performed. Each reaction cycle consisted of: 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. Control mixture consisted of PCR mixture without cDNA. Primers list are shown in Table 1.

Statistical analysis

The percentage (average each iteration) of viable, apoptotic and necrotic SSCs,harvested from the vitrified testes and fresh testes, was interpreted by using Mann-Whitney *U*-test via the SPSS 16 software. Statistical analyses were carried out considering the significance level of $P=0.008$. The results of Real-time PCR were analyzed with LinRegPCR and REST-RG softwares. These results were obtained via REST-RG software by comparing the gene efficiency, crossing points and cycle threshold values of the sample with the control in the presence of housekeeping gene (GAPDH) [38].

Results

Cell cytotoxicity

The basal level of LDH release in the positive control samples was 0.446. The level of LDH leakage in the SSCs

Table 1 Primer feature used for Real-time PCR

Primer name	Primer sequence	Ref./GenBank
P53	F: GGAGTATTTGGACGACCG	NM011640
	R: TCAGTCTGAGTCAGGCCC	
Bcl-2	F: TAAGCTGTCACAGAGGGGCT	NM009741
	R: TGAAGAGTTCCTCCACCACC	
Bax	F: CGAGCTGATCAGAACCATCA	NM007527
	R: GAAAAATGCCTTTCCCCTTC	
Fas	F: GAGAATTGCTGAAGACATGACAATCC	[31]
	R: GTAGTTTTCACTCCAGACATTGTCC	
GAPDH	F: GTGAAGGTCGGTGTGAACGG	NM008084
	R: GATGCAGGGATGATGTTCTG	

harvested from the vitrified testes was observed to be significantly lower than in SSCs yielded from the fresh testes (0.315 vs. 0.356). Indeed, the assay did not indicate toxic effect of the vitrification procedure on the SSCs collected from the vitrified testes. High control (positive control) is assay medium and cells plus lysis buffer.

Flow cytometry

Dot plot results of the spermatogonial stem cells (SSCs) obtained from the vitrified and fresh testes and as well as the positive and negative controls supplied with Apo-Brdu Alexa flour kit are shown in Fig. 1. Testing for the SSCs,harvested from the vitrified and fresh testes, were repeated five times. During each iteration of the experiment, the SSCs yielded from the vitrified and fresh testes several times were analyzed by flow cytometry. The results of flow cytometry were evaluated to determine the percentage of viable, apoptotic and necrotic SSCs harvested from the vitrified and fresh testes. The rate and percentage of apoptotic SSCs yielded from the vitrified testes were significantly lower than that of obtained from the fresh testes (P=0.008, Figs. 1 and 2). Also the percentage of necrotic SSCs obtained from the vitrified testes was significantly less than that of isolated from the fresh testes (P=0.008, Fig. 1). However, the percentage of viable SSCs harvested from the vitrified testes was significantly higher than that of collected from the fresh testes (P=0.008, Fig. 1).

Real-time PCR

Expression levels of apoptosis-related genes Fas (CD95), P53 (a tumor suppressor gene), BCL-2 (B-cell lymphoma 2) and Bax (Bcl-2–associated X protein) were determined using the method of Real-time PCR. The results of gene expression profiles are shown in Fig. 3. All apoptosis-related genes that stated above were expressed in SSCs (type A) that isolated from the vitrified and fresh testes. The expression rate of P53 and BCL-2 genes was higher in the SSCs obtained from the vitrified testes than that of harvested from the fresh testes. Conversely, the expression rate of Bax and Fas genes was lower in the SSCs yielded from the vitrified testes than that of collected from the fresh group.

Discussion

Approximately 80 % of children who suffer from childhood cancers are being cured after radiation or chemotherapy, but

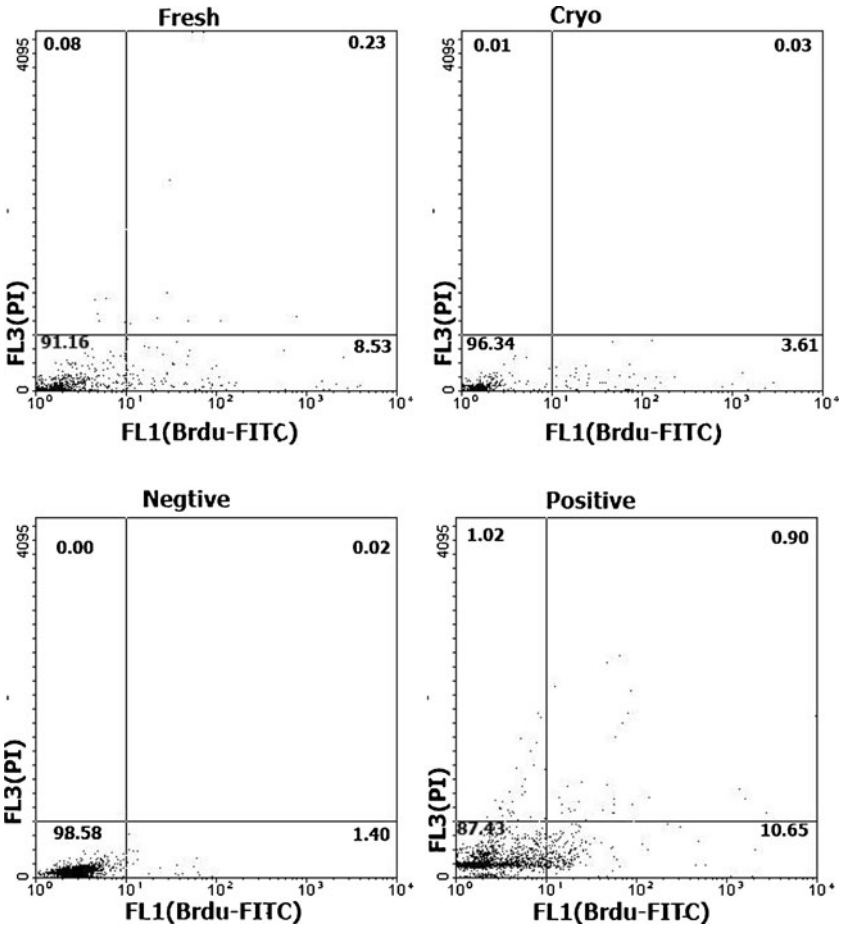

Fig. 1 The flow cytometry analysis using BrdU and PI to show the cell cycle/apoptotic responses in spermatogonial stem cells (SSCs) obtained from the vitrified and fresh testes and as well as in the positive and negative controls. Negative and positive controls were supplied with Apo-Brdu Alexa flour kit. The cells were analyzed for green fluorescence (FITC) and for red fluorescence (PI) by Flow cytometry. Percentages of viable, apoptotic and necrotic cells were determined by the *dot plot* of FL1 (in the X axis) to FL3 (in the Y axis). *Lower left*, *lower right* and *upper right quadrants* showed percentages of viable, apoptotic and necrotic cells respectively

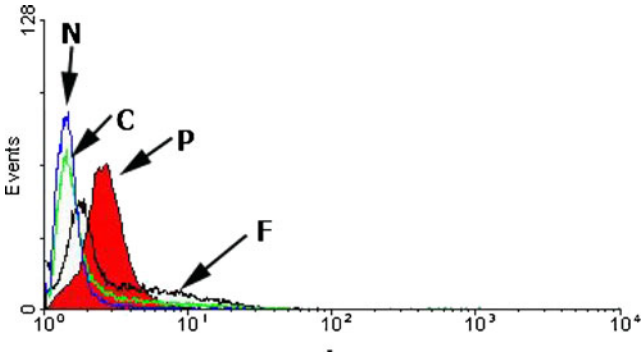

Fig. 2 The flow cytometry analysis using BrdU to show the apoptotic rate in the SSCs obtained from the vitrified (*C*) and fresh testes (*F*) and as well as in the positive (*P*) and negative (*N*) controls. Negative and positive controls were supplied with Apo-Brdu Alexa flour kit

nearly a third of them suffered from severe damage to the reproductive system and it may lead to infertility [40]. In order to preserve fertility in children with cancer, testicular parenchyma (or SSC) cryopreservation with the aim of transplant these tissue (or cells) after cancer treatment is of particular importance [30, 34, 45]. Extensive researches have been done regarding the development of testicular tissue (or SSC) cryopreservation method.

Researchers believe that SSCs should be considered as a good solution that can help the treated cancer patients to prevail over their infertility crisis [34]. Therefore, cryopreservation of the testicular parenchyma (or SSC) is regarded as a useful technique and the assessment of the effects of cryogenic damages of SSCs at molecular level seems crucial in designing molecular strategies against this injuries.

Surprisingly, in the present study, the lactate dehydrogenase (LDH) enzyme release from damaged plasma membranes of the SSCs,obtained from vitrified testicular parenchyma, into the extracellular fluid was dramatically lower when compared with the level of LDH leakage from the fresh ones. It's noteworthy to state that LDH is a stable cytoplasmic enzyme present in the cytoplasm of the cells. It is rapidly released into the cell culture supernatant upon the damage of the plasma membrane [46].

Also, in this study, it has been suggested that vitrification may have no negative influences on the SSCs obtained from the vitrified testis parenchyma by no increasing expression of some apoptosis-related genes such as Fas and Bax. In keeping line with the current results, Poels et al. [39] confirmed that in generally seminiferous tubules exhibited good integrity after cryopreservation. Hemadi et al. [17] reported the less effect of cryopreservation on the apoptotic rate of the germ cells as well.

Additionally, in the present study, the its LDH and Real-time PCR results were confirmed by observation low percentage of apoptotic and necrotic in the SSCs obtained from the vitrified testes.

Although, Gouk et al. [15] did not examine the expression patterns of apoptotic genes, but the results obtained from cell cycle/apoptotic responses and cytotoxicity are consistent with the findings gained in this study. Hermann et al. [20] showed that cryopreservation decreased cell viability and induced damage to testicular parenchyma cells. Meanwhile, the cryopreserved testicular cells produced more colonies compared with fresh cells [20]. Baert et al. [5] showed that,in overall, the cryopreservation can induce damage to the testicular tissue. However, they reported that ultra-structural damages during cryopreservation are relatively negligible and it cannot make major changes in the developmental activities of the cells after transplantation so that the SSCs obtained from the cryopreserved testicular tissue could be led to spermatogenesis [5]. Larman et al. [29] showed that following blastocysts vitrification, expression profiles of the four apoptosis genes (Survivin, Fas, Hsp-70 and Caspase-3) were increased significantly.

It will be possible that the close communication of cryoprotectant (CPA),as a toxic agent, with germ cells in the testicular parenchyma will cause increased the high risk of side effects of CPA. Nevertheless, even in a healthy situation, the apoptosis process occurs permanently for preservation

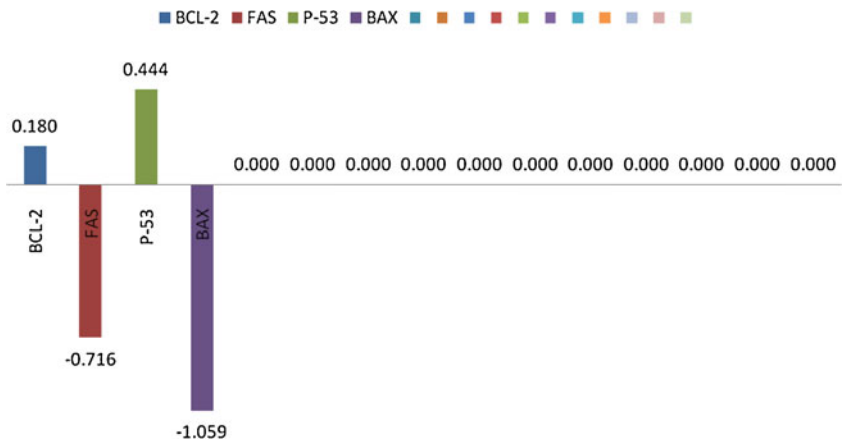

Fig. 3 Quantitative expression analysis of the apoptosis-related genes Fas, P53, BCL-2 and Bax in the SSCs obtained from the vitrified and fresh testes

the testicular tissue homeostasis during the dynamic spermatogenesis cycle [32]. However, the high occurrence of apoptosis can make negative effects in the male genital system [43]. The apoptosis activity is dependent on expression of some apoptosis-related genes and proteins (i.e., caspase, Apaf-1, NF-KB, P53 and death receptors) and also some anti-apoptosis-related genes and proteins (i.e., BCL-2). Indeed, these genes and proteins play a critical role in the apoptosis program [2].

Furthermore, some studies show that there is a relationship between apoptosis-related proteins and genes and male infertility [9]. BCL-2 family proteins play an important role in the process of apoptosis or programmed cell death [28]. BCL-2 family proteins contain two groups of proteins that some of which are pro-apoptotic (such as Bax) or anti-apoptotic (such as BCL-2) [28]. P53 gene expression plays different functions including participating directly in DNA repair, inducing mitochondrial proteins such as Bax (pro-apoptotic protein from BCL-2 family protein) and death receptors expression i.e., Fas [35, 44, 46]. In this study, P53 expression may lead to DNA repair because concurrent with P53 expression, the expression of Bax and Fas decreased. However, the role of these genes is needed to study precise in the future.

Therefore, with considering above information, it can be supposed that vitrification may not create severe apoptosis conditions and a necrosis process in the SSCs obtained from the vitrified testes.

However, although the damage to the SSCs yielded from the vitrified testes is neglected compared to that of obtained from the fresh testes, it should be considered. Hemadi et al. [18, 19] reported that if appropriate dose of antioxidant was added to testes vitrification media, the spermatogenic cells lineages in the vitrified testis grafts were increased. So taking into account the above information and the results of this study it suggest that adding some anti-apoptotic agent in the vitrification medium may be useful for keeping more alive the cells which suffer from several possible deleterious factors such as cryoprotectant toxicity. In order to study the exercise of the mechanisms of apoptosis pathway of cells during vitrification, more studies need to be done. Indeed, since vitrification as reported in our previous studies [16, 17, 19] may have negative effects on graft development, further studies should be relevant with the focus on follow up the continuing effects of cryogenic injury on long-term culture or transplantation of testicular parenchyma (or SSC) to optimize the outcome.

Conclusion

In conclusion, data from this study indicates that vitrification of prepubertal testicular tissue may not increase some of the expression profile of apoptosis-related genes i.e., Bax and Fas in the testicular SSCs with also subsequent no more observing the cell apoptotic/necrotic responses and no increasing intracellular LDH leakage.

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