

# The Effect of Caffeic Acid on Spermatogonial Stem Cell-type A Cryopreservation

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## Abstract

**Background:** Cancer treatment methods can lead to male infertility. In this regard, cryopreservation of spermatogonial stem cells (SSC) and cell-to-person transplantation after the course of treatment to resolve the problem of infertility is a good one. The cryopreservation of SSC is an important process as it can help on the return of spermatogenesis. However, during this process, the stem cells often become damaged which degrades their value for experiments and treatments. Caffeic acid (CA) is an antioxidant that has been shown to increase the viability of cells under stress. The aim of this study was to investigate the effect of CA has on spermatogonial stem cell (SSC) cryopreservation.

**Methods:** Spermatogonial stem cells isolated from the testes of Balb/c mice pups were cultured in laminin-coated dishes, purified using CD90.1 microbeads, then cryopreserved in vitrification media supplemented with 10  $\mu$ M CA either through a slow or rapid freezing process. After thawing, cell viability was evaluated. Expression of Bax, Fas, Bcl-2 and P53 genes was determined by real-time PCR. Gel electrophoresis was used to confirm the results of the real-time PCR.

**Results:** The viability of the SSCs that were rapidly frozen and treated with CA was observed to be significantly reduced compared to the control group ( $p < 0.003$ ). The viability SSCs that received CA and underwent the slow freezing treatment was significantly reduced compared to controls ( $p < 0.002$ ). The expression levels of BAX, BCL-2, and Fas in the rapid freeze-thaw group didn't significantly change. However, the levels of P53 expression were shown to increase. In the group of SSCs that underwent the slow freezing process, the BAX gene expression levels increased, while the levels of BCL-2 gene expression decreased. No significant changes in the level of Fas and P53 expression were detected. When comparing the groups that received CA treatment, SSCs that were rapidly frozen showed an up-regulation of Fas and P53 expression and a down-regulation of Bcl-2 and Bax expression.

**Conclusions:** Caffeic acid may protect intact SSCs during the cryopreservation process through stimulating the induction of apoptosis in injured SSCs. Supplementing the vitrification media with CA has a superior effect on the preservation of SSCs.

**Keywords:** Apoptosis, Caffeic acid, Cryopreservation, Spermatogonial Stem Cells.

## Introduction

The survival rate of childhood cancer is increasing (1). Chemotherapy and radiotherapy are critical, life-saving procedures involved in combating cancer. However, these treatments can cause the individual to be at risk for infertility (2). The transplantation of

spermatogonial stem cells (SSCs) offers a promising means of restoring male fertility. SSCs are capable of undergoing self-renewal and differentiation into spermatozoa. Spermatogonial stem cells provide a source of mature spermatozoa for infertile male

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patients (3) and also SSCs are not differentiated enough to have acrosomal vesicles, so they have lower metabolic activity and hence they are lower at exposed to abnormalities (4). An important aspect in enabling this transplantation procedure to occur involves the proper storage of these SSCs. The ability to successfully cryopreserved SSCs has provided an avenue for the long-term storage of these cells, without causing visible damage to their function (5). It has been reported that SSC transplantation can lead to the production of spermatozoa (6). The ages of donor SSCs that can lead to the development of spermatogenesis in the testes of infertile males is not limited to a particular age of donors (7) thus, this method can offer a means of restoring the fertility of pediatric male patients that have undergone cancer therapy as well. For these individuals, preserving their fertility is a more difficult and complicated task as prepubescent boys are unable to produce semen or mature spermatozoa (8). As a result of these conditions, there is currently no established option to preserve the fertility of pediatric cancer patients. This is a harsh consequence for those who are longstanding survivors. Spermatogonial stem cells preservation and transplantation therefore provide a treatment to rescue the fertility of males of all ages (9). Currently, methods of freezing and thawing are not completely safe for stem cells (10) as this process often leads to the induction of apoptosis through damaging the cell membrane and DNA. Furthermore, cryopreservation can also lead to the production of reactive oxygen species (ROS) (10-16). Several studies have thus been dedicated to finding methods to prevent this damage from occurring. Gholami *et al.*, have reported that melatonin, an antioxidant, leads to apoptosis in SSCs during the freezing and thawing process (13). Conversely, a separate study has shown that supplementing a glycerol based vitrification media with melatonin was in fact, able to reduce the testicular tissue damage that otherwise occurred during freezing (12). The cryopreservation of human sperm via the addition of Glutathione (GSH) has been shown to lead to improved function of the frozen sperm (17). Oxidative stress is a recognized major factor that affects sperm quality due to reactive species (ROS) generation and lipid peroxidation during semen cryopreservation (18). Supplementing the freezing and thawing media with

antioxidants is a good option to reduce damage during cryopreservation (19). Antioxidants are emerging as prophylactic and therapeutic agents. These are the agents, which scavenge free radicals otherwise reactive oxygen species and prevent the damage caused by them (20).

Caffeic acid (3, 4 dihydroxycinnamic acid) (CA) is a phenolic compound found in propolis and a wide variety of plants (19). CA has anti-inflammatory, anti-viral, immunomodulatory, anticancer and antioxidant activity. CA has also been shown to decrease the occurrence of breaks and lipid peroxidation in double-stranded DNA (21-23). Previous studies have shown that using 10  $\mu\text{M}$  of CA is able to increase cell viability, while increasing concentrations can lead to a decline in viability. The addition of 10  $\mu\text{M}$  of CA has also been shown to decrease the levels of ROS in L-02 cells (24). It is assumed that CA can reduce the level of p53 expression through preventing DNA damage and reducing the levels of ROS. CA can influence the expression of pro-apoptotic and anti-apoptotic genes through NF- $\kappa\text{B}$  activation, and increase the levels of Nrf2 within the nucleus (19, 25-28). P53 acts as a regulator of the apoptosis and can modulate key points in the intrinsic and extrinsic apoptotic pathway (29). The Bcl-2 family includes two antagonist groups: anti-apoptotic genes such as Bcl-2 and Bcl-XL, and pro-apoptotic genes such as Bax and Bak (30, 31). Interaction with the Fas receptor and ligand (FasL) begins a complex pattern of events inducing intercellular apoptosis (32). The aim of the present study was to evaluate the role of CA as an antioxidant on the SSC cryopreservation.

## Materials and methods

### *Preparation of cell suspension from mice testes*

Eighty, six-day old male Balb/c mice were purchased from Razi Research Centre of Lorestan University of Medical Sciences.

Cell digestion was performed closely adhering to the methods provided by Milazzo *et al.*, (33). Following anesthetising the mice using ketamine HCl (80 mg/kg) and xylazine (10 mg/kg), testes were removed and transferred to Dulbecco's modified Eagle's medium (DMEM; Gibco) containing penicillin (100 IU/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ) (Gibco). The tunica albuginea was carefully removed and the samples were subjected to two-step

digestion. First, testes were incubated in DMEM supplemented with 2 mg/mL of collagenase and 500-200 µg/ml of DNaseI for 15 min at 37 °C, then centrifuged at  $100 \times g$  for 5 min. The enzyme solution (1 mM EDTA, 0.25% trypsin (sigma) and 200 µg/ml DNaseI) was then added to the cell pellets. The cell pellet was resuspended for 5 min at 37 °C and gently pipetted to provide testicular cell suspension. Trypsin was inactivated by adding 10% Fetal Bovine Serum (FBS) to the cell suspension. Finally, the cell suspension was centrifuged at  $100 \times g$  for 5 min, the supernatant was carefully removed and testicular cell suspension incubated in petri dishes (60 mm) overnight with 20 µg/ml laminin

#### ***Spermatogonial stem cell (SSC) purification***

Supernatants was carefully removed, then the cells were washed with PBS buffer and incubated with 0.5 mg/mL BSA in PBS for one hour at 37 °C to prevent nonspecific binding. Cells were washed a final time with PBS buffer (34). Spermatogonial stem cells were purified using magnetic-activated cell sorting (MACS). A specific marker for SSCs, Thy-1 (CD90.1 MicroBeads, mouse-Miltenyi Biotec, 130-094-523), was used as a positive control, while c-kit (CD117 MicroBeads, mouse-Miltenyi Biotec, 130-091-224) was used as a negative control (Columns: MS, LS, XS, auto MACS Columns). In brief, a total of  $10^7$  cells were centrifuged at  $300 \times g$  for 10 min and the cell pellet was resuspended in 90 µl of buffer solution. The buffer solution was prepared through combining PBS, pH 7.2, 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). Also, 10 µl of CD90.1 microbeads was added. The cells were mixed well and incubated for 15 min at 4 °C (35), washed by adding 1-2 ml of the buffer solution, then centrifuged at 300 g for 10 min. Up to  $10^8$  cells were then resuspended in 500 µl of buffer solution and loaded onto a MACS column which was placed in the magnetic field of a MACS separator (13).

#### ***Spermatogonial stem cell (SSC) cryopreservation***

The purified SSCs ( $10^6$  cells per cryotubes) were added to cryotubes (Sigma-Aldrich) containing the vitrification solution (10% FBS, 90% DMSO and 10 µM CA). Cryotubes were divided into two

groups, a rapid-freezing group, or a slow-freezing group. For each group, to act as a control, cryopreservation media without CA was added to the cells. The SSCs that underwent rapid freezing were transferred into the liquid nitrogen tank immediately. The cells that underwent a slow freezing process were first left at -4 °C for one hour, kept at -20 °C for two hours, then left overnight at -70 °C and finally, moved into the liquid nitrogen tank. After two months, the cryotubes were thawed and the SSCs were examined for viability and gene expression levels. The viability of isolated cells was determined via trypan blue staining (0.4%). Gene expression levels were measured via real-time PCR.

#### ***RNA isolation, cDNA synthesis and Real-time PCR***

RNA was extracted using a standard RNA extraction kit (Total RNA Purification Kit, PP-210S; Jena Bioscience, Germany) according the manufacture's instruction. Five hundred µl of lysing buffer and 300 µl of isopropanol was added to the cell plates. Cells were centrifuged for 30 sec at  $10000 \times g$ . The liquid that accumulated inside the rotating column was discarded. The primary buffer was added to the rotating column, then the cells were centrifuged for 30 sec at  $10000 \times g$ . Fluid in the rotating column was removed, the secondary buffer was added and the sample was centrifuged for 30 seconds at  $10000 \times g$ , then for two minutes at  $10000 \times g$ . At this stage, the rotating column was placed in a vial (40-50 ul) RNase and DNase free. Aleutian buffer was added for one minute, then the sample was immediately centrifuged for one minute at  $10000 \times g$ .

cDNA synthesis was performed by using the cDNA synthesis kit (Accu Power Cycle Script RT PreMix [dN6], BIONEER, Korea) according to the manufacturer's instruction. Fifteen µl of extracted-RNA and 5 µl of distilled-water was poured into the vials of the extraction kit. Vials were then placed in the PCR machine.

Genes were designed using the previously described mouse sequences (GenBank) and Gene Runner software (version 3.02; Hastings Software Inc., USA) as shown in Table 1. GAPDH, a house keeping gene, was included as an internal control to normalize the PCR. Real-time PCR (RT-PCR) was performed by Qiagene rotor gene 6000. RT-PCR was performed using the prepared cDNA,

the primers and the PCR Master Mix 2X kit (Jena Bioscience) were used under the following conditions: Forty cycles were considered, each cycle

was performed for 15 sec at 95 °C and 45 sec at 60 °C. RT- PCR results were analyzed by the REST 2009 software and the charts were drawn.

**Table 1.** Sequences of primers

Primer name	Primer sequence	Ref./GenBank
GAPDH	F: GTGAAGGTCGGTGTGAACGG R: GATGCAGGGATGATGTTCTG	NM008084
Fas	F: GAGAATTGCTGAAGACATGACAATCC R: GTAGTTTTCACTCCAGACATTGTCC	(36)
BAX	F: CGAGCTGATCAGAACCATCA R: GAAAAATGCCTTTCCCTTC	NM007527
Bcl-2	F: TAAGCTGTCACAGAGGGGCT R: TGAAGAGTTCCTCCACCACC	NM009741
P53	F: GGAGTATTTGGACGACCG R: TCAGTCTGAGTCAGGCC	NM011640

RT-PCR products for each gene was loaded on the electrophoresis gel. Gel electrophoresis was performed using 1.2% agarose in Tris–Borate–EDTA (TBE). One µl of each sample was loaded onto the gel, with 1× loading buffer (Sigma-Aldrich) at a voltage of 85 for 45 min. The gels were stained with 0.1 µg/ml DNA. Bands are shown in Fig 2.

### Statistical analysis

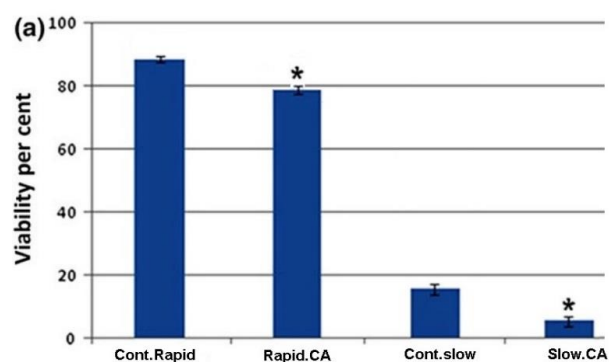
Mann-Whitney test was used to detect significant differences between groups. All statistical computations were performed using SPSS 17.0 (SPSS, USA).

## Results

### Viability

Viability of the rapid and slow freezing controls was 87% and 15%, respectively. Viability of the rapid and slow freezing groups treated with 10 µM CA were 79 % and 5%, respectively. There was a significant reduction in the viability of the SSCs in the rapid freezing group treated with CA compared to the control group ( $p < 0.003$ ). Furthermore, the viability of the SSCs in the slow freezing group treated with CA was significantly reduced compared with the control group ( $p < 0.002$ ). The differences observed between the slow and rapid freezing SSCs without CA and the differences between the slow

and rapid freezing SSCs treated with CA were statistically significant ( $p < 0.001$ , Fig. 1).



**Fig. 1.** SSC viability determined via trypan blue staining. (Cont. Rapid: Control group with rapid-freezing, rapid. CA: rapid-freezing with Caffeic acid. Cont. Slow: Control group with slow-freezing, Slow. CA: Slow-freezing with Caffeic acid).

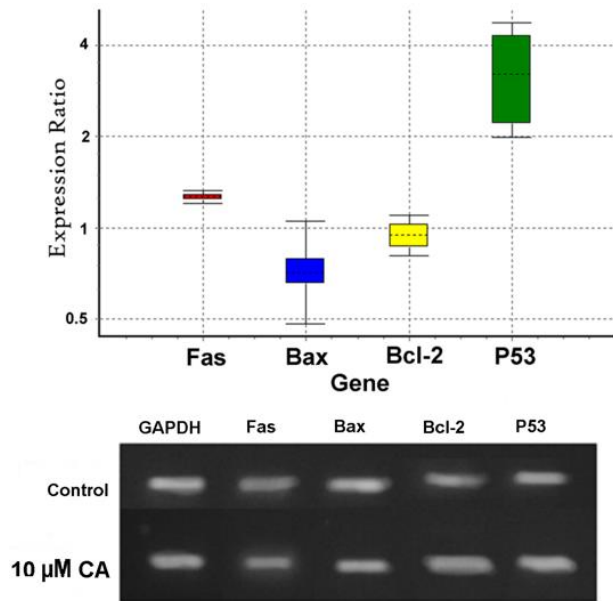
### Gene expression

Expression levels of BAX, BCL-2, and Fas in the rapid freezing SSC group did not show any significant changes compared to controls. However, expression levels of P53 increased in the rapid freezing SSC group (Fig. 2).

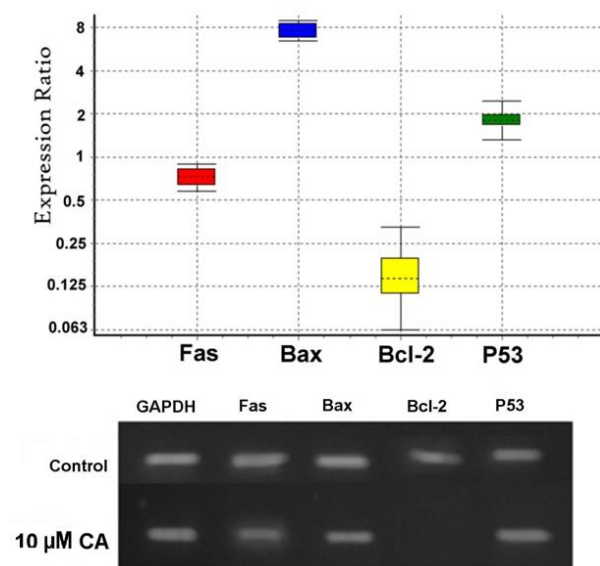
In slow freezing SSC group, Bax gene expression levels increased and BCL-2 gene expression levels decreased in comparison to the control group. Fas and P53 did not show any significant changes between groups (Fig. 3).

SSCs of the rapid freezing group treated with CA showed up-regulation of Fas and P53, and a down-regulation of Bcl-2 and Bax in comparison

to the group of SSCs treated with CA and underwent the slow freezing process (Fig. 4).



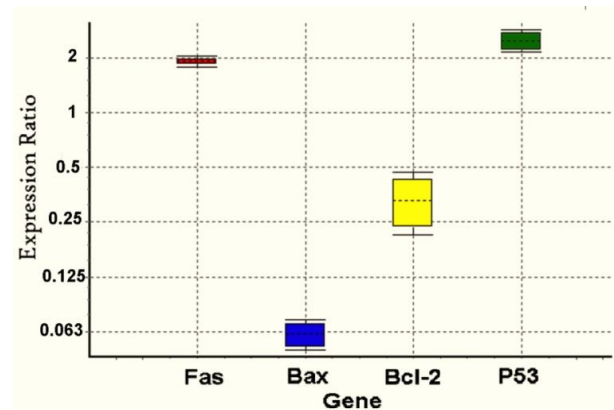
**Fig. 2.** Gene expression levels in the rapid freezing SSC group. Expression of apoptotic genes in the CA treated SSCs that underwent rapid freezing compared with the control group in the presence of the gene reference, GAPDH.REST 2009 software was used for analyses.



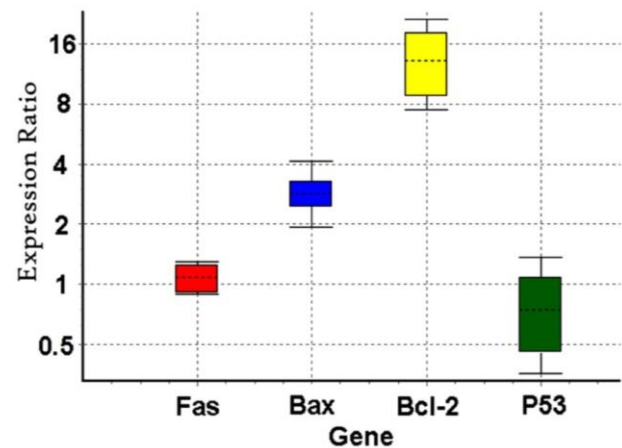
**Fig. 3.** Chart gene expression levels in slow freezing SSC group. Expression of apoptotic genes in the slow freezing SSC group treated with CA compared with the slow freezing controls in the presence of the gene reference, GAPDH.REST 2009 software was used for analyses. CA increased Bax and decreased Bcl-2.

When examining the differences between the SSCs that underwent either slow or rapid SSCs, without CA treatment, the levels of BAX and BCL-2 gene expression was observed to

increase, while Fas and P53 gene expression did not show any significant changes (Fig. 5).



**Fig. 4.** Expression of apoptotic genes in the rapid and slow freezing SSCs treated with CA. Fas and P53 levels increased, while Bax and Bcl-2 decreased in the group that underwent the rapid freezing process.



**Fig. 5.** Expression of apoptotic genes in the cryopreserved (slow and rapid) SSCs that did not receive CA.

## Discussion

Significantly pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family set by MAP kinases include of P38 (37). It has been proven that the activation of p38- $\alpha$  MAPK (p38) and JNK leads to the activation of Bax and Bak, and decreases Bcl-2, which leads to apoptosis (10, 38). CA has been shown to be able to increase p38 expression and ERK (19, 40). Activation of NRF2 / KEAP1 leads to the presence of NRF2 in the nucleus, as well as the accumulation of a "free" KEAP1 pool which is desirable for anti-apoptotic signaling. On the other hand, KEAP1 mediates ubiquitination of the main anti-apoptotic protein, BCL-2 (41). Therefore, because of CA's ability to increase p38 and ERK expression, the presence of CA can lead to an

increase in Bax and reduction of Bcl-2, activation of KEAP1/NRF2 and, cause the subsequent ubiquitination of the BCL-2 protein. It is reported that Caffeic acid phenyl ester (CAPE) can increase Bak and Bax protein levels and, reduce Bcl-2 in C6 glioma cells (42). The ratio between the anti-apoptotic and pro-apoptotic proteins of the Bcl-2 family members ultimately determines the fate of cells (30). Therefore, the decreased expression of the Bcl-2 gene and increased expression of Bax observed in the slow freezing group (Fig. 2) may be a result of the reduced viability of damaged cells by CA.

It has been reported that CA is able to reduce the expression of phospho-Rel A (28) as well as reduce the levels of NF- $\kappa$ Bp65 protein expression (43). Given NF- $\kappa$ B's ubiquitous nature, the inhibitory effect of CA on NF- $\kappa$ B results in the reduction of several different factors involved in apoptosis control. NF- $\kappa$ B inhibition by CA lead to a decrease in p53 expression therefore reduced levels of pro-apoptotic gene expression, including Fas, and a suppression of anti-apoptotic genes such as BCL XL. Furthermore, the inhibitory effect of NF- $\kappa$ B on p53 by MDM2 is also be reduced by CA. The induction of these pathways will depend on cell type and nature of the stimulus inducing both p53 and NF- $\kappa$ B. Thus, the influence of NF- $\kappa$ B activity depends on the nature and cellular context in which it is induced (26). NF- $\kappa$ B-dependent regulation of the Fas gene has been well established (32). Ettou *et al.*, has reported that in primary bone marrow cells, NF- $\kappa$ B may directly upregulate the Fas gene (44). As mentioned above, the inhibitory activity of CA on NF- $\kappa$ B functioning can thus account for reduction of Fas expression in

cells exposed to CA. As shown in the graph (Fig. 1) in both groups, Slow-freezing and rapid-freezing, due to decrease in viability, at least, Fas did not show significant changes. CA has been shown to inhibit kinase Fyn activity through the suppression of Nrf2 (28, 40) and result in the accumulation of Nrf2 within the nucleus (45). The activity of transcription factors, including temperature shock transcription factor 1 (HSF1), NF- $\kappa$ B and P53, and the induction of pathways such as MAPK, are all related to the nature and duration of stress experienced by the cell, and the cell type undergoing this cellular stress. The induction of apoptosis is highly dependent on context and on the stress signals experienced by the cells (46).

In conclusion, supplementing the freeze-thaw media with CA has a better effect on the rapid freezing process and leads to increased P53 levels of expression. The increased P53 levels may be activating and accelerating apoptosis within the damaged SSCs, therefore the remaining and viable SSCs present offer a good choice for cell transplantation. More studies are required to further understand the mechanisms occurring within the freezing process with CA. Furthermore, future investigation needs to consider the utility of these frozen SSCs for transplantation.

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