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# Comparison of apoptosis pathway following the use of two protocols for vitrification of immature mouse testicular tissue

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ABSTRACT

Our objective was to evaluate the apoptosis incidence in immature mouse testicular tissue after two different protocols of vitrification and short-term culture. Testes of 7-day-old Naval Medical Research Institute mice were isolated and distributed into control and vitrification groups. In vitrification 1 group, testes were vitrified using a combination of ethylene glycol and DMSO in three steps, and in vitrification 2 group, testes were vitrified using a combination of ethylene glycol and sucrose in five steps. Then, fresh and vitrified-warmed testis fragments were cultured for 20 hours. Morphology, cell viability, apoptosis incidence, and apoptosis gene expression (*BAX*, *BCL2*, *Caspase 3*, *Fas*, *Fas ligand*, *p53*) were evaluated at 0, 3, and 20 hours of culture by light microscopy, flow cytometry, and real-time polymerase chain reaction, respectively. Significant decrease of early apoptosis (annexin V+/PI− cells in vitrification 1 and 2 groups at 0 hours of culture, 37.34 ± 0.91 and 30.72 ± 2.2, and at 20 hours of culture, 1.46 ± 0.28 and 0.76 ± 0.11, respectively), increase of late apoptosis (annexin V+/PI+ cells in vitrification 1 group at 0 hours of culture, 14.46 ± 0.86, and at 20 hours of culture, 37.18 ± 2.34), and *BAX*/*BCL-2* ratio (in vitrification 1 and 2 groups at 0 hours of culture, 7.31 ± 0.31 and 6.83 ± 1.38, and at 20 hours of culture, 24.08 ± 4.32 and 9.35 ± 1.91, respectively) were observed in vitrification groups during culture period. *Caspase 3* expression was significantly decreased in all groups after 3 hours of culture (in control, vitrification 1, and vitrification 2 groups at 0 hours of culture, 1.00 ± 0.0, 1.56 ± 0.09, and 0.79 ± 0.06, and at 20 hours of culture, 0.37 ± 0.0, 0.96 ± 0.10, and 0.12 ± 0.03, respectively). Expression of *p53* was significantly lower in vitrification 1 (0.32 ± 0.02) and control (0.50 ± 0.03) groups in 20 hours of culture as compared with vitrification 2 (0.88 ± 0.14) group. *Fas* (in vitrification 1 and 2 groups at 0 hours of culture, 2.29 ± 0.23 and 1.14 ± 0.15, and at 20 hours of culture, 12.43 ± 0.46 and 6.7 ± 0.48, respectively) and *Fas Ligand* (in vitrification 1 and 2 groups at 0 hours of culture, 1.2 ± 0.28 and 5.24 ± 0.32, and at 20 hours of culture, 21.75 ± 2.00 and 25.82 ± 2.15, respectively) expressions significantly increased in vitrification groups after 20 hours of culture. Although both vitrification protocols cause cell death via apoptotic and necrotic pathway, it seems that vitrification 1 protocol induces cell death more via apoptotic pathway than via necrosis. The apoptosis incidence after vitrification may have occurred independent of *p53*.


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## 1. Introduction

Because of the advancements in cancer research, the life expectancies of young boys with cancer have been increasing [1]. Unfortunately, such patients often have to struggle with impaired fertility, caused by the disease itself or by the treatment processes [2]. Cryopreservation of testicular tissue is one of the noteworthy methods that has been used for fertility preservation [3]. Curaba et al. [4] showed that mouse testis tissue could be well preserved by vitrification and slow freezing. Another recent research showed that vitrification of testicular tissue is a time- and cost-efficient strategy to preserve spermatogonial stem cells [5].

Although vitrification of testicular tissue is considered as an appropriate and successful technique for fertility preservation in different species [6], the occurrence of inappropriate molecular and structural alterations during preservation is undeniable [7,8]. Apoptosis that happens after cryopreservation and culture is one of the important molecular alterations which caused the integrity reduction of testis [8].

Extrinsic and intrinsic pathways are involved in testicular germ cell death [9]. In the extrinsic pathway, activation of death receptors (such as Fas, TNF, etc.) causes activation of initiator Caspase 8 [10]. On the other hand, the intrinsic or mitochondrial pathway is activated by BCL2 family members that cause changes in mitochondrial membrane permeability and release of cytochrome c into the cytosol. Cytochrome c is involved in the formation of apoptosome complex with apoptotic protease activating factor 1 and causes activation of initiator Caspase 9 [11]. Both initiator caspases that are involved in intrinsic and extrinsic pathways activate executioner Caspase 3 and advance the apoptosis incidence further [12].

P53, tumor suppressor protein, is a sensor of genotoxic stress that is responsible for regulating the transcription of genes associated with cell cycle arrest, DNA repair, and apoptosis [13]. Research in 2008 showed increase of apoptotic cells in frozen testicular cells after 20 hours of culture [14].

In the present study, we have evaluated the effects of two different protocols of testis vitrification on tissue integrity, cell death, and expression of apoptotic genes during 20 hours of culture.

## 2. Materials and methods

### 2.1. Animals and study design

Forty-five immature male Naval Medical Research Institute mice (7-day-olds) were handled according to the Ethical Guideline of Royan Institute (Tehran, Iran). Their testicular tissues were isolated and randomly distributed into three experimental groups: Fresh control tissue (Cont), vitrification 1 (Vit 1), and vitrification 2 (Vit 2) groups (Fig. 1).

Experiments were replicated six times for cell viability and apoptosis assessment and three times for gene expression and morphology evaluation.

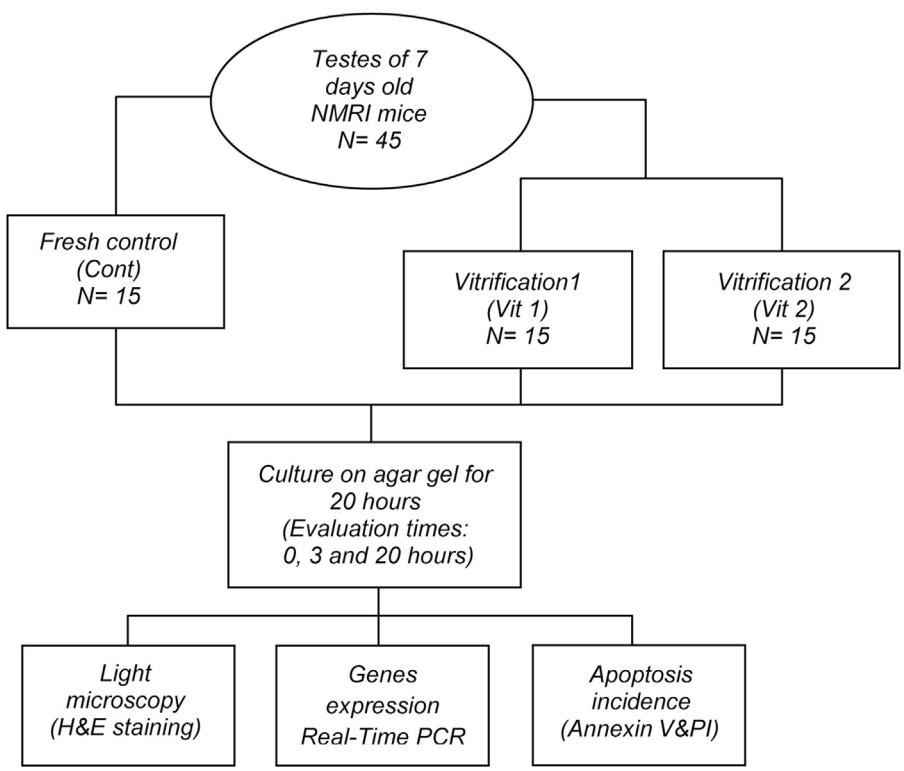


**Fig. 1.** Outline of the experimental procedure.