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Original Article

Effects of 3, 4-Dihydroxyflavone on Cryopreserved Testicular Tissue of Neonatal Mouse

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Abstract

Background and Aim: Infertility is a side effect of cancer treatment because of chemotherapy or radiotherapy. Cryopreservation of testicular tissue or spermatogonial stem cells before cancer treatment and their transplantation may preserve the natural fertility. Cryopreservation is a damaging process due to free radicals and toxic effect of frozen solution. The purpose of this paper is to study the antioxidant and antiapoptotic effects of 3',4'-dihydroxyflavone on the structure of frozen-thawed testicular tissue of neonatal mouse.

Materials and Methods: Testes of 6-day-old NMRI mice (N = 20) were isolated. Testicles were randomly divided into four groups: two groups as control groups for quick and slow freezing-melting process, and two others as treatment groups that underwent quick and slow freezing-melting with addition of 3',4'-dihydroxyflavone (10µM) in frozen solution. For the assessment of structural changes and apoptosis in the frozen-thawed testes, the hematoxylin-eosin staining and tunnel pod kits were used respectively. In order to analyze the data, we used Kruskal-Wallis Test and Mann-Whitney Test. The statistical analysis was entirely done by SPSS software.

Results: Histopathological changes and apoptosis rate were significantly decreased after thawing in both treatment groups in comparison with control groups.

Conclusion: Adding 3',4'-dihydroxyflavone (10µM) to freezing-melting environment and the use of quick freezing-melting method can reduce the histopathological and apoptotic changes.

Keywords: Neonatal mouse, Testicle, 3,4-dihydroxyflavone, Freezing-melting, oxidative stress

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Introduction

Despite the effectiveness of methods used in cancer treatment in recent years, which were able to increase the survival rate of five-year-old children suffering from cancer in many cases up to 85% (1), patients still suffer from many side effects resulting from these treatments. One of these risks is fertility reduction among them. Most of the assisted fertility methods for men are based on obtaining sperm and keeping or

using it (2, 3). One of the methods which may lead to the permanent loss of natural fertility after cancer treatment in prepubertal children who do not have the natural ability to produce sperm, is freezing the testicular tissue and spermatogonial stem cells (SSCs) before treatment and returning this tissue or cells to testicle after treatment (3). In animal samples (dogs, goats, pigs and rodents), linking the SSCs to testicles of infertile males has led to the revitalization of spermatogenesis among them (4-11). Freezing the SSCs before cancer treatment and autologous transplantation of SSCs is possible after cancer treatment which leads to the maintenance and recovery of natural fertility as well as having children with parents' genes. Slow and fast freezing-thawing methods are two prevalent procedures in cell and tissue cryopreservation. In slow method, it is important to decrease the damage of ice crystals using freeze solutions or using the quick freezing-melting (12-14). Another method which is quickly exposed to cold is recognized as vitrification. In this method, cells or tissues are exposed to high contractions of cryoprotectants and then are quickly frozen at -196°C. The toxic effects of cryoprotectants and osmotic effects on cells are important in this method. To solve these problems, researchers proposed the utilization of a mixture including some cryoprotectants and also the use of antioxidants. Appropriate solutions should include disaccharides (sucrose), proteins and polymers (15, 16). One of the most important threats for cells in freezing-melting process is the production of active species including reactive oxygen species (ROS) which seriously affect the testicular tissue. Sperm is a cell that is sensitive to the oxygen of lipid peroxidation. Oxidative stress can induce the destruction of membranes and reduce mobility as well as sperm fertility potential. Therefore, in order to reduce the damages induced by freezing-melting method, not only the optimization of environment and conditions in a way that the production of free radicals is minimized, but also the help of fighting systems including subsidiary antioxidants to destroy free radicals is required (17-20). Flavonoids are the biggest group of natural polyphenolic compounds which are almost found in all floral plants (21). A number of effects associated with this group of substances have been discovered. The most important effects include analgesic effects (22), anti-inflammatory (23), antioxidant (24), anti-hypertension (25) and hypoallergenic effects (26). Also, antiplatelet, antifungal, antimicrobial and anti-cancer effects have been discovered (27, 28). Dihydroxyflavone compounds are the most important derivatives of flavonoids. Several groups have indicated that dihydroxyflavone acts as an antioxidant and antiapoptotic agent to support oocytes maturation and embryo's development in vitro (29, 30). The purpose of this paper is to study the antioxidant and antiapoptotic effects of 3',4'-dihydroxyflavone on the structure of frozen-thawed testicular tissue of neonatal mice.

Materials and Methods

Animals

6-day-old NMRI mice (N = 20) were used in this study. The mice were purchased from Razi Herbal Medicine Research Center of Lorestan University of Medical Sciences. The mice were killed by high doses of ketamine hydrochloride (80mg/kg) and xylazine (10mg/kg). The testicles were dissected by cutting the lower abdomen. The protocols enacted by the Committee of Animals' Care and Use in Lorestan University of Medical Sciences were observed during the research. Moreover, and every attempt was made to minimize the number of animals and their pain and suffering during the research.

Study groups

Testicles were randomly divided into four groups: two control groups, one of them for quick freezing and another for slow freezing, two treatment groups, one of them for quick freezing and another for slow freezing. The method utilized in the preparation of solutions, and freezing-thawing methods are derived from a research conducted by Gholami et al., which previously describe (31, 32). Cryopreservation: For each case of the study groups (control group and treatment group), specialized freezing solution was prepared as described in Table 1.

Cryopreservation

The solutions of treatment groups were prepared similar to those of control groups. All of them have been supplemented with 3',4'-dihydroxyflavone (10µM).

The testicles of each groups underwent the following

stages immediately after exiting from mice's body:
1) They were placed in number 1 specialized solution of the group for 10 minutes.
2) They were placed in number 2 specialized solution of the group for 10 minutes.
3) They were placed in number 3 specialized solution of the group for 10 minutes.
While the testicles were placed in solution number 3, the tunica albuginea of the testicles was perforated by insulin syringe; hence, the solutions penetrated to seminiferous tubules tissue. In order to use the testicles in quick freezing procedure, the testicles of the quick group (control or treatment) from solution number 3 were transferred to cryotube which included appropriate volume of number 4 specialized solution of the group and then the cryotube was directly placed in a liquid nitrogen tank. In order to use testicles in slow freezing procedure, the testicles of slow group (control or treatment) from solution number 3 were transferred to cryotube which included appropriate volume of number 4 specialized solution of the group and the cryotubes were respectively placed in a refrigerator at temperatures of -4 °C for one hour, -20 °C for one hour, and -70 °C for 24 hours. They were subsequently transferred to the liquid nitrogen tank. The cryotubes remained in the liquid nitrogen tank for 2 months.
Four specialized melting solutions were prepared for each of the case study groups (control and treatment groups) before taking the testicles out of the liquid nitrogen tank as follows:

- melting solution number 1: DMEM + 0.5 molar sucrose
- melting solution number 2: DMEM + 0.25 molar sucrose
- melting solution number 3: DMEM + 0.125 molar sucrose
- melting solution number 4: DMEM + FBS (20% of the volume)

The melting solutions of treatment groups were prepared similar to the solutions of control groups. All of them have been supplemented with 3',4'-di hydroxyflavone (10 µM).
The melting method was conducted based on Abrishami et al., method with some changes (33, 34). In order to be melted after two months, the cryotubes of freezing procedure were taken out quickly from liquid nitrogen and then underwent the following stages in order:
1) Cryotubes were kept in room temperature for 30 seconds.
2) Lids of cryotubes were kept opened under the hood, and the vacuity in upper parts of cryotubes was filled with DMEM environment. Afterwards, cryotubes were kept at a temperature of 37 °C and retained until the melting point (about 1 to 2 minutes).
3) The samples were drawn out and immediately transferred to melting solution number 1, melting solution number 2, and melting solution number 3 for five minutes at a temperature of 4°C. Subsequently, the samples were situated in melting solution number 4 for 30 minutes at a temperature of 37°C in an incubator.
In the case of slow melting too, the cryotubes used in slow freezing procedure were taken out of the liquid nitrogen tank after two months and underwent the following stages in order:

- They were placed in a refrigerator at a temperature of -70°C for 24 hours.
- They were placed in a refrigerator at a temperature of -20°C for 1 hour.
- They were placed in a refrigerator at a temperature of -4°C for 1 hour.
- Cryotubes were kept in room temperature for 30 seconds.
- Lids of cryotubes were opened under the hood, and the vacuity of upper of cryotubes was filled with DMEM environment. Afterwards, cryotubes were kept at a temperature of 37°C and retained until the melting point (about 1 to 2 minutes).
- The samples were taken out and immediately transferred respectively to melting solution number 1, melting solution number 2, and melting solution number 3 for five minutes at a temperature of 4°C. subsequently, the samples were situated in melting solution number 4 for 30 minutes at a temperature of 37°C in an incubator.

Assessments: After fixation, the samples were dehydrated and embedded in paraffin for sectioning. Sections stained by hematoxylin-eosin and tunnel pod kit for the assessment of structure and apoptosis were used in the frozen-thawed testes, respectively. Structural changes of the groups were analyzed based on the methods used by Milazzo *et al*. (32). These

methods are described briefly in table 2.

Therefore, the total number for each seminiferous tubule is between 0 to 10. Finally, the ultimate number of each group is the average number of 30 seminiferous tubules of the samples of that group. The more the total number of tubules is close to 10, the more and more serious the textural damages of that tubule will be; and the more this number is close to 0, the less damages will be induced. To study the apoptosis, the tunnel kit was used according to the instruction of manufacturer. In the final stage, apoptotic cells were calculated randomly in 10 microscopic fields. In order to analyze the data, we used Kruskal-Wallis Test and Mann-Whitney Test. The statistical analysis was entirely done by SPSS software.

Results and Discussion

Histopathologic changes in treatment groups declined in comparison with control groups in slow freezing-melting method, and this difference is statistically significant ($p < 0.001$) (Table 3). The apoptosis rate in treatment groups declined in comparison with control groups in slow freezing-melting method, and this difference is statistically significant ($p < 0.001$) (Table 4). In the comparison that was done between the treatment and control groups in quick freezing-melting method, it was determined that histopathologic changes of testicular tissue in the treatment groups significantly declined ($p < 0.001$) in comparison with control groups (Table 3). Also, the apoptosis rate of frozen-melted testicular tissue in a quick method in treatment groups significantly declined ($p < 0.001$) in comparison with control groups (Table 4).

Histopathologic changes of testicular tissue and its apoptosis rate were compared in both slow and quick methods in treatment groups. The average numbers of histopathologic changes for quick freezing-melting method and slow freezing-melting method were 3 and 4 respectively, which were statistically significant ($p < 0.001$). The average of apoptosis rate in quick freezing-melting method was 1.87 ± 1.24, but it was 3 ± 0.75 in slow freezing-melting method. This difference was not statistically significant ($p = 0.083$). All of the 4 study groups were compared regarding histopathologic changes of testicular tissue and its apoptosis rate. The results of this comparison are indicated in tables 3 and 4 and charts1 and 2.

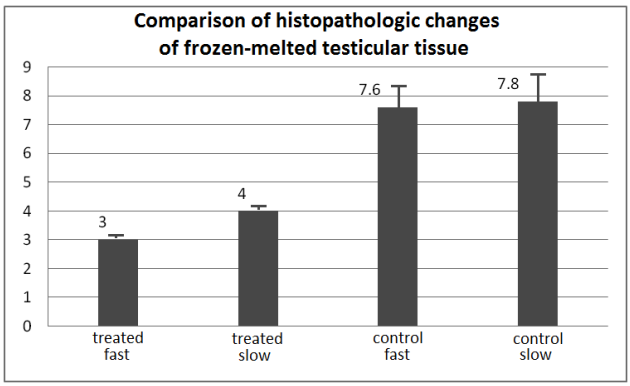

Figure 1. The comparison of histopathologic changes of frozen-melted testicular tissue in case study groups.

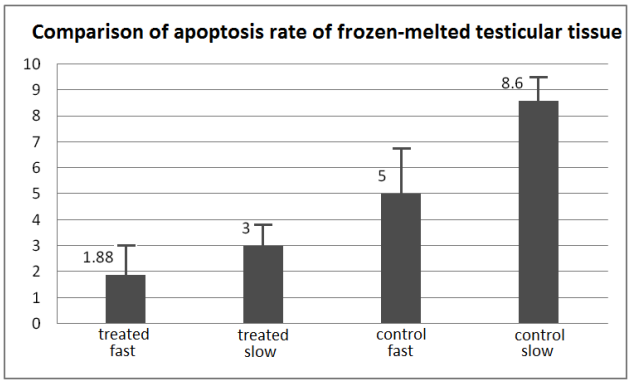

Figure 2. The comparison of apoptosis rate of frozen-melted testicular tissue shows that the apoptosis rate decreased in both treated groups in comparison with control groups.

In freezing-melting process, the cells are exposed to a number of risks including ice crystals, dehydration, and oxidative stress. Most of researchers believe that the protection of cells against the effects of freezing solutions and oxidative stress in this process necessitates the optimization of freezing-melting solutions (14, 33-35). In a study conducted by Kushki, which dealt with studying the use of antioxidant in the freezing-melting process of testicular tissue of neonatal mouse, it was determined that existence of vitamin E and C and selenium during both quick freezing-melting method and slow freezing-melting method caused the reduction of damages in seminiferous tubules of testicular tissue (36). One of the flavonoids is that in

Rui Zhang *et al*., examined in their research the antioxidant effects as well as protective effects of 7,8-dihydroxyflavone and compared them to DNA changes induced by oxidative stress. In this study, it

Table 1: Specialized freezing solution of control groups.

Freezing solution	Components				
	DMEM	**DMSO**	**Ethylene Glycol**	**Sucrose**	**FBS**
Number 1	85%	7.5%	7.5%	0.5M	_
Number 2	70%	15%	15%	0.5M	_
Number 3	50%	15%	15%	0.5M	20%
Number 4	50%	15%	15%	0.5M	20%

Table 2: Methods used to study histopathologic changes.

Histological assessment	**Parameters**	**Scores**
Nuclear changes	distinction of Spermatogonia cells of Sertoli cells	Easy: 0 Difficult: 1 Impossible: 2
	observing nucleoli	Easy (visible >40% of cells): 0 Unrecognizable: 1
	condensation of nuclei	Maximum in one nucleus: 0 In Less than 40% of nuclei: 1 In more than 40% of nuclei: 2
Changes of epithelium	Separation of cells of base membrane	Not separation: 0 Slight separation: 1 Complete separation (or>75%): 2
	formation of gap and wrinkles	Lack of gap and wrinkle: 0 Slightly: 1 Observing more: 2

was determined that 7,8-dihydroxyflavone in its optimized contraction can inactivate 81% of free radicals ROS. H_2O_2 has the ability to damage DNA. A treatment in which 7,8-dihydroxyflavone plays a role, can reduce the such a damage. Any damage to H_2O_2 causes the cell death, especially in the form of apoptosis. Another point referred to as a finding in this study, is the ability of 7,8-dihydroxyflavone to reduced the cell death induced by H_2O_2 (37). The results of our study coincide with those of this paper. That is to say, in our study, histopathologic changes and apoptosis rate in treatment groups that were

Table 3: The comparison of histopathologic changes of frozen-melted testicular tissue in case study groups.

case study groups	Histopathologic changes of testicular tissue		
	Average±standard deviation	average of rating	p value
quick freezing method in treatment groups	3±0	4.5	
slow freezing method in treatment groups	4±0	12.5	
quick freezing method in control groups	7.6±0.69	25.6	
slow freezing method in control groups	7.8±0.91	27.40	<0.001

*type of statistical test: Kruskal- Wallis Test

Table 4: The comparison of apoptosis rate of frozen-melted testicular tissue in case study groups.

case study groups	apoptosis rate of testicular tissue		
	average ±standard deviation	average of rating	p value
quick freezing method in treatment groups	1.87±1.24	6.75	
slow freezing method in treatment groups	3±0.75	11.88	
quick freezing method in control groups	5±1.77	19.31	
slow freezing method in control groups	8.6±1.17	29.15	0.001

treated with 3',4'-dihydroxyflavone were meaningfully less than control groups. Also 3',4'-dihydroxyflavone is one of the flavonoid compounds many effects of which have been explored in large numbers of studies that have concentrated on it. Jiang et al., concluded that 3',4'-dihydroxyflavone has strong antioxidant effects, and significantly prevents the accumulation of superoxides in cells. This substance also prevents the oxidation of NADPH in phagocytes that finally leads to cytoprotection in stages of cardiac, cerebral ischemia and probably in other organs (38). In this regard, various studies have been conducted about using the flavonoids to protect the evolution of cells and also protect them against oxidative stress. A study indicated in 2013 that 7, 8-dihydroxyflavone protects the maturation of oocytes in laboratory environment as an antioxidant. The role of 7,8-dihydroxyflavone in the maturation of cytoplasm is greater than its role in the maturation of nucleus. Moreover, it contributes to the development of embryonic tissue in laboratory environment (39). A complete halt in the development of embryonic tissue in laboratory environment usually occurs due to high contractions of free radicals, reduction of antioxidant defense and oxidative stress (40). In another study which was carried out on the effects of 3',4'-dihydroxyflavone on fetal bovine tissue, it was determined the reduction of free radicals' rate by this substance contributes to the increase of antioxidant levels, activation of antiapoptotic mechanism as well as activation of genes associated with the growth from the development of fetal bovine tissue in laboratory

environment (29). In our study, histopathologic changes in control groups in slow freezing-melting method were the same as changes in quick freezing-melting method. Nevertheless, due to the damages of free radicals, apoptosis rate of cells was greater in slow freezing-melting method. In slow freezing-melting method, both histopathologic changes and apoptosis rate were less than control groups, so this difference was statistically significant. In quick freezing-melting method, differences in both histopathologic changes and apoptosis rate between treatment groups and control groups were the same as slow freezing-melting method.

Conclusion

These findings indicated positive the effects of 3',4'-dihydroxyflavone on the protection of cells against oxidative stress in slow freezing-melting method and quick freezing-melting method. In aforementioned studies, the results were similar to our study. All in all, after comparing all groups we concluded that the least histopathologic changes and the least apoptosis rate were related to the group which was treated with 3',4'-dihydroxyflavone. Moreover, the freezing-melting method was quick.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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