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# The Effect of Ginseng Treatment on Melted and Frozen Human Sperm Quality

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**Abstract**

**Introduction**: Today, freezing method is one of the most common approaches in the treatment of infertility. This is while oxidative stress is a major destructing factor during sperm freezing and thawing. However, antioxidant compounds can play a key role in sperm freezing techniques.
**Materials and Methods:** In this study, 5 groups of sperms were evaluated with and without Ginseng extract and then some parameters were evaluated such as mobility, activity of mitochondrial, amount of reactive oxygen species (ROS) and DNA fragmentation.
**Results:** Findings revealed that the mobility and mitochondrial activity in sperms significantly increased (P≤0.05) in frozen and thawed sperms, which had been treated with 1 mg/mL Ginseng extract compared to the freezing and thawing sperms without Ginseng extract. In addition, the results showed that Ginseng treatment significantly decreased the amount of ROS and DNA fragmentation compared to freezing and thawing in frozen and thawed treatment without Ginseng ($P \leq 0.05$). By preventing the increase of oxidative stress levels, Ginseng prevented the reduction of mobility and mitochondria activity of the sperms after freezing and thawing. It also reduced sperm DNA defeat and reduced the production of free radicals.
**Conclusions:** The results of the present study support the hypothesis that Ginseng has positive effects on the sperm quality in cryopreservation process.
**Keywords:** Ginseng, Free Radicals, Freezing and Thawing, Active Mitochondria of Sperm, Infertility
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## Introduction

Cryopreservation of human sperms has been introduced as an efficient method for increasing the success rate of assisted fertility technology and the sperm bank for artificial insemination (in case of requiring chemotherapy prior to treatment, spinal cord injuries and to prevent the spread of infections such as AIDS and hepatitiss).[1] Cryopreservation is a common method in infertility treatment centers. However, any possible changes occuring in the structure of sperms eventually lead to a reduction in fertility.[2-6] Freezing and thawing lead to mobility reduction, calcium deposit reduction, disruption of living sperms, sperm membrane disruption of infrastructure and the acrosome, reduce sperm quality, produce reactive oxygen species (ROS) and oxidative damages induced by it.[7,8] Freezing process causes excessive production of ROS by inducing chemical and physical pressures on the sperm membrane, and as a result, causes oxidative stress.[2,9]

In the freezing process, the temperature of cellular water decreases until it becomes ice. Actually, without making any damage to the cells, molecule movements halt effectively. The biochemical processes are delayed or stopped in cells, and as a result cell survival increases.[10] The most important principle in the freezing process is reducing the damages caused by intracellular ice crystal formation and toxic salts, which must be decreased with slow cooling and by removing intracellular water with a suitable alternative. Rapid cooling of spermatozoa at above zero temperature can cause irreversible damage to the sperms. This phenomenon is called cold shock.[11] High levels of ROS can affect the sperm structural and functional integrity such as motility, morphology, count and viability.[8]

One of the critical steps in the freezing-thawing process is thawing (bringing the temperature of frozen samples to room temperature). In this step, two events can destroy cells; the first issue is intracellular ice crystal formation, when freezing rate is extremely high, dewatering is not done completely and some water remains intracellular, but if thawing is done slowly, cells will have the opportunity to convert the remained water to ice crystals while passing on the freezing point.[12,13] However, when the melting rate is high, this prevents the formation of intracellular ice crystals. The second issue is osmotic shock which is caused by slow freezing and rapid thawing. This is because imported antifreeze cannot develop fastly into the cells, and at the same time water enters faster, as a result cells stat swelling.[14] Due to the toxicity of antifreeze,

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it should be eliminated from the surroundings and inside of the sperms immediately after thawing. Any disruption in this process leads to the development of free radicals in sperms which causes malicious changes on the structure and the sperm motility.[11,14]

Antioxidants are the molecules, which convert free radicals to other molecules (H2O, O2), prevent the overproduction of free radicals, and somehow remove them. They are the most important elements against free radicals.[15] An imbalance manner between free radicals production and antioxidants leads to damaging biological molecules by free radicals.[16] Seminal plasma is an important source of antioxidants but it discards during the sperm freezing process. Thus, the sensitivity of sperms to oxidants increases. Oxidative stress in spermatozoa leads to overproduction of free radicals that can cause infertility in men. As mentioned above, it seems necessary to add antioxidant compounds to the sperm media. The presence of antioxidant compounds (such as vitamins E and C, selenium, methyl gzantyn, taurine, L-carnitine and others) reduce the severity of damages caused by ROS.[17] There are some studies about the effect of antioxidants on sperm parameters after freezing and the main topic of most studies is identifying healthy antioxidants in natural available resources.[18]

Ginseng is one of the performance-enhancing herbs that has a great reputation in the world. In addition, it has been proven that this herb has minimal side effects. This component improves immunity,[19] prevents skin damage from ultraviolet rays,[20] reduces the risk of cancer incidence,[21] inhibits tumor growth and metastasis,[22] increases antioxidant effects,[23] and improves the percentage of sperm with normal morphology, total motility and progressive motility.[24]

In this study, the effect of treatment with Ginseng extract on freezing-thawing human sperms is investigate, by assaying the motility, viability and DNA fragmentation of human sperms.

## Materials and Methods

### Sample Preparation and Study Design

Semen samples were collected from twenty-five healthy volunteers with an age range of 25-40 years old (informed consent was obtained from all cases) according to the World Health Organization (WHO) standards. Each sample was divided into two 1.5 mL samples and each one was separately poured into a 15 mL falcon. The content of each falcon was separately placed on a track gradient. Finally, supernatant was evacuated. During this study two falcons were used for each semen sample which 1 ml complete media was added to one of the falcons and 1 ml complete media containing 1 mg/mL Ginseng was added to the other one (American Ginseng (Panax quinquefolium) Sigma). In this study, 5 groups were evaluated, including the gradient group (control), 45 minutes incubated of the gradient group(test 1), 45 minutes incubated of the gradient group with the Ginseng environment (test 2), freeze-thawed sperm without Ginseng (test 3) and freeze-thawed sperm treated with Ginseng extract (test 4). Therefore, each semen sample was divided into 5 groups. Sperm morphology with color Diff-Quik (Ral Diagnostic, France), free radicals through DCF-DA color (Abcam, USA) and active mitochondria with dye Rhodamine 123, DNA fragmentation by Halo sperm (Halotech®, Spain), and ultimately sperm parameters were evaluated by CASA (Video Test Sperm1.2, Russia). All assays were repeated 3 times.

### The Freezing and Thawing Method

The sperm freezing medium (Vitco, Germany) contains HEPES- Buffer, 10 mg/mL of human albumin, glycerol (as an antifreeze element) and gentamycin. The sperm cryopreservation was done in the following steps: 500 µL of the sperm freezing medium (about 20-25°C) was slowly added to 500 µL semen (1:1 ratio), after pipetting cryopreserved sperm medium was combined with sperm cells. Cryovials were maintained for 10 minutes at 4°C and then moved to the freezer and maintained for 20 minutes. Afterwards, cryovials were immediately placed in the steam of liquid nitrogen for 30 minutes (10 to 20 cm above the liquid nitrogen) then cryovials were transferred to the liquid nitrogen.

For thawing, cryovials were transferred to 37°C water and each melted cryovial was moved to a 15 mL falcon and then sperm washing medium was gently added to the melted sperm suspension. For washing, the ratio of 1 to 4 with the Ham's F-10 medium (Sigma-Aldrich, USA) were mixed and centrifuged in 400 g for 3 minutes. The supernatant was removed and the mobile spermatozoa came out of the melted mode.[25] After centrifugation, the supernatant was collected. According to the Swim up method, 0.5 mL HTF medium without HEPES + 10% serum albumin was slowly added to the settled sperms and the tube was incubated for 40 minutes at 37 °C and 6% CO2 to sperm cells moved to medium. Then, the supernatant was gently removed and transferred to another pipe for running the analysis.

### Evaluation of Mitochondria

Rhodamine 123 was used to consider mitochondria activity as a living marker for sperms. Freeze-melted sperms ($5\times10^6$ cells) were washed by phosphate buffered saline (PBS). Then 5 µg/M Rhodamine was added, and incubated for 10 minutes at 37°C. Thawed cells were twicly washed with PBS. Finally, PI dye was added and active mitochondria were assayed (live sperms have activated mitochondria) by flow-cytometry (Partec, Germany).

### Evaluation of Reactive Oxygen Species

The DCF-DA was used for the measurement of ROS in our study. Thus, melted sperm cells were washed with PBS. Every one million sperm was put in a dark place of 10 µl DCF-DA (20 mM) and were after on incubated at 37°C. After 45 minutes, the sperms were washed by PBS and were prepared for the study by flow cytometry.

### Sperm Counting

A drop of semen was put on a neubauer chamber under a light microscope with a magnification of ×400 and sperms were counted then multiplied in $10^6$. The obtained number indicated the approximate number of sperm per mL. After counting the sperms in squares, the number of them in 1 mL

sample volume was calculated using the following formula:

$N = a \times b \times 10000$

$N$ is the number of spermatozoa in 1 mL of sample,
$a$ is the number of spermatozoa in 5 squares,
$b$ is the dilution factor (in case of the dilution of the semen samples).

### Statistical Analyses

The statistical analyses were performed using Prism software (GraphPad 6). Paired Student's $t$ test was used with a confidence interval of 95%. $P$ values less than 0.05 were considered to be statistically significant.

## Results

### Demography of the Semen Samples

At first, the sperms were analyzed according to the WHO for determining the demography of the semen samples. The measurement of the various parameters in all five groups is presented in Table 1.

### Evaluation of Sperm Parameters After Treatment With Ginseng Extract

According to Table 2, the average number of sperms in the freezing and thawing media (1 mL) group was around 14.45 ± 2.19. There was no significant differencein this group compared to the control group. The morphology of normal sperms in the control group was 35.31 ± 2.12%. After incubation it became 29 ± 2.11% and in the incubation group treated with Ginseng it reached to 30 ± 2.03%. The percentage of normal sperms in the freezing and thawing group without antioxidant significantly reduced to 24.85 ± 1.67, while this parameter was reduced to 26.1 ± 1.63 in the freezing and thawing group with antioxidants. Generally, there was no significant difference between the groups without freezing.

As shown in Table 2, the percentage of sperm progressive motility in the control group was 64.83 ± 3.88. After 45 minutes of incubation it increased to 70.17 ± 3.42 and in the Ginseng group, it increased to 72.08 ± 3.72. No significant change was observed among groups. However, after freezing the progressive motility became 39.22 ± 2.31 and after freezing in the case of treating with Ginseng it increased to 51.30 ± 1.37, which represents a significant reduction ($P \leq 0.05$) in the progressive motility between the groups before and after freezing. In addition, a significant increase was observed between freezing with and without Ginseng. As shown in Table 2, the sperm motility percentage in the control group was 80.72 ± 2.51. After 45 minutes incubation, it became 82.91 ± 2.53 and this parameter in the group with Ginseng and with the same time of incubation raised up to 87.07 ± 2.10. As a result, no significant change was observed in these groups. This is while the total motility percentage after freezing without Ginseng was 50.98 ± 1.34 and after treating with Ginseng reached to 64.43 ± 1.97, which showed a significant increase ($P \leq 0.05$).

As shown in Table 2, the average percentage of sperm viability in the control group, was 85 ± 2.24, which reached to 86.2 ± 2.11 after 45 minutes incubation and it reached to 87.9 ± 1.76 in the group, which was incubated with Ginseng. The sperm survival rate in the freezing group without Ginseng was 64.8 ± 2.05 that significantly declined. The sperm viability improved in the freezing group with Ginseng compared to the freezing group without Ginseng. This is while freezing media and washing media after thawing were treated with Ginseng, but it was not significant. According to Table 2, it was found that the average sperm viability in the freeze

**Table 1.** Demography Studied Semen Samples

| Groups | Maximum Amount | Minimum Amount | Average | Standard Deviation |
|---|---|---|---|---|
| Sperm Count | $93\times10^4$ | $33\times10^4$ | $75.5\times10^4$ | 3.34 |
| Progressive motility | 40.40 | 29.80 | 34.05 | 4.64 |
| Total mobility | 99.50 | 90.00 | 93.35 | 3.36 |
| Survive | 48 | 40 | 43.33 | 2.943 |
| Percentage of normal morphology | 81 | 12 | 30 | 2.136 |

**Table 2.** The Percentage of Sperm Parameters (± Standard Error) Between the Experimental Groups After Treatment With Ginseng Antioxidant

| Group | Control* | Test 1* | Test 2* | Test 3* | Test 4* |
|---|---|---|---|---|---|
| Viability percentage | $85^a \pm 2.24$ | $286^a \pm 2.11$ | $87.9^b \pm 1.76$ | $64.8^b \pm 2.05$ | $73.5^b \pm 1.91$ |
| Abundance ($\times10^4$) | 15.25 ± 1.85 | 14.65 ± 1.71 | 15.95 ± 2.15 | 14.45 ± 2.19 | 15.05 ± 1.99 |
| Percentage of natural form | $31.35^a \pm 2.12$ | $29^a \pm 2.11$ | $30^a \pm 2.03$ | $24.85^b \pm 1.67$ | $26.1^b \pm 1.63$ |
| Progressive motility | $64.83^a \pm 3.88$ | $70.17^a \pm 3.42$ | $73.08^a \pm 3.72$ | $39.22^b \pm 2.31$ | $51.30^c \pm 1.73$ |
| Percent of total mobility | $80.72^a \pm 2.51$ | $82.91^a \pm 2.53$ | $87.07^a \pm 2.10$ | $50.98^b \pm 1.34$ | $64.43^c \pm 1.79$ |

* Average + standard error.
Control: gradient, Test 1: gradient +45 min of incubation, Test 2: gradient Ginseng +45 min of incubation, Test 3: freezing and thawing, Test 4: freezing and thawing + Ginseng.
[a] Significant difference with groups 3 and 4 in all parameters.
[b] Significant differences between the control and tests 1 and 2 in all parameters.
[c] Significant difference with all the groups in the percentage of progressive motility parameters and total mobility.

group significantly declined compared to the control group ($P < 0.05$).

### Evaluation of Free Radical Concentration of Sperms in the Experimental Groups

According to Figure 1 there was no significant difference between test groups 1, 2 and the control group. Free radicals in the test group 3 significantly increased compared to the control group but free radicals decreased in test group 4 (in the presence of Ginseng). Free radicals in test group 4 were significantly higher than control and test groups 1 and 2 ($P \leq 0.05$). In the following diagram, free radical changes are visible in different groups. As shown in Figure 1 free radicals in test 4 significantly decreased in the presence of Ginseng.

### Assessing the Active Mitochondria of Sperm

According to Figure 2, the intensity fluorescence of active mitochondria among the control group and test groups 1 and 2 was not significant. However, after freezing and thawing this activity significantly decreased. In addition, in test group 4 (freezing and thawing with Ginseng) the intensity fluorescence of active mitochondria was more than the freezing group without antioxidant treating and it was significant ($P < 0.05$). This is while the percentage of those activities in comparison with groups before freezing was not significant. As shown in Figure 2, the fluorescence intensity of mitochondria in test group 4 significantly increased in the presence of Ginseng.

### Evaluation of DNA Fragmentation of Sperm

According to Figure 3, there were no differences between the control group and test groups 1 and 2. After freezing and thawing the sperms, the DNA fragmentation significantly increased ($P < 0.05$). In the freezing group, in the presence of Ginseng (test group 4) the DNA fragmentation significantly reduced compared to test group 3 ($P<0.05$). l The percentage of DNA fragmentation in test group 4 was statistically significant in comparison to the control and experimental groups 1 and 2.

## Discussion

The process of freezing and thawing can be affected by various factors such as cold-shock, osmotic pressure and oxidative stress which can all decrease the quality of sperms.[26] According to our results, it seems that sperm protective mechanisms cannot protect the sperms structure against oxidation stress during freezing and thawing. Therefore, it is essential to compensate sperm deficiency by adding extra antioxidants. This study determined that free radicals in the test groups are not statistically significant as compared to control groups 1 and 2, because of non-intervention among the groups. After the process of freezing and thawing, cold shock caused a sudden production of free radicals. Therefore, suddenly the amount of natural anti-oxidants reduced and free radicals increased. Because of Ginsengs antioxidants, the cells received Ginseng extract before the process. Figure 1 shows that the amount of free radicals significantly reduced in test group 4. Although, the percentage of free radicals reduced in test group 4, but the sperms were not able to return to a normal situation before freezing and thawing. As expected, the percentage difference of progressive and total sperm mobility was not significant among control, test 1 and test 2. It is considerable that test group 2 (which received antioxidants) showed a more progressive motility compared to other groups, but it was not significant. The motility after freezing and thawing process dramatically decreased. This reduction may be related to the increase of free radicals and the creation

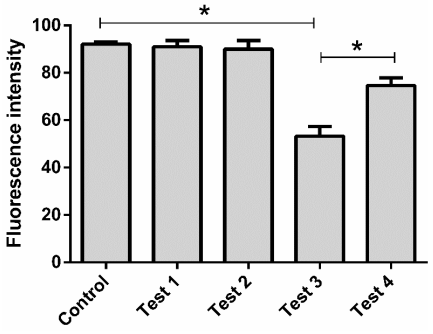


**Figure 2**. Assessing the Active Mitochondria of Sperm in the Experimental Groups. Fluorescence intensity of mitochondria monitored in 5 groups and as seen in the figure Fluorescence intensity of mitochondria in test 4 significantly increased in the present of Ginseng. *$P$ value < 0.05.

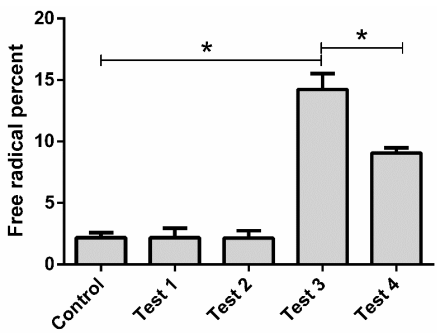


**Figure 1**. Evaluation of free radical concentration of sperm in the experimental groups: amount of free radicals monitored in 5 groups and as seen in the figure free radicals in test 4 significantly decreased in the present of Ginseng. *= $P$ value <0.05.

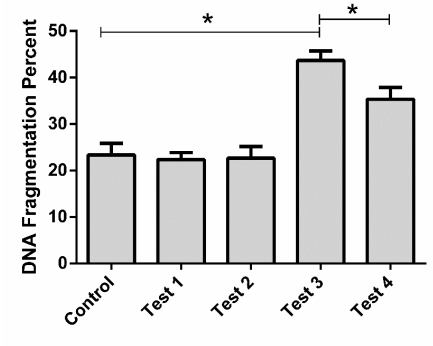


**Figure 3**. Evaluation of DNA Fragmentation of Sperm in the Experimental Groups. DNA fragmentation monitored in 5 groups and as seen in the figure DNA fragmentation in test 4 significantly decreased in the present of Ginseng. *$P$ value < 0.05.

of sperm membrane lipid peroxidation phenomenon.[27] Finally, this phenomenon was compensated by using herbal antioxidants and after that, motility significantly increased. It is noteworthy to mention that increasing mobility could not completely prevent the destructive effects of freezing and thawing. However, this significant increase of mobility after freezing can be relating to the Ginseng extract and it may be helpful in infertility centers in order to achieve better results in the future. Our study was in line with Kim et al in 2013, which showed that ginsenoside Rg1 (50 µg/mL) significantly increased sperm motility and the membrane integrity of sperms after freezing and thawing as compared with fresh and untreated thawed sperms.[28]

Ginseng as a herbal medicine has different protective effects on sperm cells. For example: in 2008, Nazm Bojnordi et al investigated on the effectiveness of the different types of antioxidants on frozen and melted sperms and presented a significant increase in sperm parameters, especially progressive motility, after treating with vitamin E but they showed no significant effect on sperm morphology.[29] Also, Zhang et al reported that Ginseng can reduce the radiation-induced effects on testicular tissue probably by removing free radicals or accelerating the repair of damaged DNA,[30] which are both in line with this research.

In the present study, the morphology of spermatozoa between different groups did not change much. This is while, after the freezing and thawing process the percentage of morphologically normal sperms reduced but this parameter increased in the presence of Ginseng extract. As a result, Ginseng could not prevent the damaging effects of sperm freezing and thawing. In line with our results, Nazm Bojnordi et al have demonstrated that antioxidants (vitamin E) increase the progressive motility after freezing and thawing but their report showed no significant effect of this antioxidant on the morphology of sperms.[29]

Cell membrane and the inner mitochondrial membrane changed after cryopreservation because of oxidative shock.[28] In the prsent study yhe Ginseng extract increased the fluorescence intensity of active mitochondria by reducing the oxidative process. Therefore, it can be said that Ginseng can protect the mitochondrial membrane of damaging of oxidative process . In fact, the antioxidant composition of the Ginseng extract could maintain the active mitochondria. Our results are in agreement with Hwang and Kim's study that reported Ginsenosides can be used as a protective additive for the suppression of intracellular mitochondrial oxidative stress caused by cryopreservation.[28]

According to the present study, Ginseng could protect sperm cells against sudden temperature changes during the cryopreservation by reducing the percentage of DNA fragmentation. In line with our study, Martinez-Soto et al in 2010 showed, adding Genestein (a chemical antioxidant) to frozen human sperms, during the freezing process, reduced the production of free radicals. Also, it improved the range of motion and decreased sperm damage cell membranes and DNA structure.[31] The obtained results also show that the amount of DNA damaging increased significantly after freezing and thawing without Ginseng, but in the presence of Ginseng, DNA damaging reduced. Because of degradation of nuclear DNA in sperms during freezing and thawing process, the sperm viability greatly reduced. However, in the present study, Ginseng extract could compensate the degradation of nuclear DNA, but this improvement was not seen in the sperm viability.

## Conclusions

Results show that the effectiveness of the Ginseng extract can reduce ROS and reduce DNA damaging and protect the sperm motility by protecting the membrane of organelles cell. Therefore, adding Ginseng as a herbal antioxidant to the freezing medium during freezing process can be used as a positive and effective factor to protect sperms during the freezing process.

### Authors' Contributions

AHM contributed to all the experiments and which were supervised by MM. All authors participated in the statistical analysis and the preparation of the manuscript. All authors read and approved the final manuscript.

### Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

### Ethical Approval

Ethics Committee of Tarbiat Modares University approved the study.

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