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Original Article

Investigating the effects of vitamin B12 and Myo-inositol on human sperm parameters during the freeze-thaw process

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Abstract

Background and aims: While sperm freezing has proven to be beneficial for addressing clinical needs, it can also have negative impacts on sperm function. However, it has been observed that additives with antioxidant properties can help alleviate the damage caused by reactive oxygen species (ROS) and cold shock. The objective of this research was to examine the effects of incorporating vitamin B12 and Myo-inositol into the freezing medium on human sperm and how they influence sperm parameters.

Methods: The semen samples of 20 people with normal fertility were divided into four equal parts after preparation, including the control group, vitamin B12, Myo-inositol, and both vitamin B12 and Myo-inositol. Sperm parameters were examined after melting and washing the samples. The obtained data were analyzed with SPSS software (version 23) and analysis of variance ($P<0.05$).

Results: The results revealed that the groups receiving antioxidants experienced a significant improvement in sperm motility, viability, morphology, and total antioxidant capacity (TAC) levels compared to the control group. Based on the obtained data, the average sperm motility in the control group was 40.65±4, which was less than that of the B12 group's average (49.5±5.92), the Myo-inositol group (68.70±15.42), and the vitamin B12+Myo-inositol group (71.5±8.63), and this difference was significant ($P<.001$).

Conclusion: According to the findings, the quality of sperm and the rate of recovery of sperm parameters increase with an increase in the TAC concentration and a decrease in the malondialdehyde (MDA) concentration.

Keywords: Vitamin B12, Myo-inositol, Sperm, Male infertility

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Introduction

Infertility is defined as the lack of ability to produce a clinical pregnancy after 12 months of frequent, unprotected sexual activity. Both genders may be affected by infertility caused by diseases, or it could be specific to one gender. Male infertility is caused by 50% of all causes, with varicocele, cryptorchidism, infections, obstructive lesions, cystic fibrosis, trauma, and tumors being the usual causes (1). The imbalance between reactive oxygen species (ROS) and antioxidants in the body leads to oxidative stress (2). Human sperm freezing is considered an effective method in assisted reproductive techniques due to the powerful mechanism of oxidative stress causing sperm damage, deformation, and, ultimately, male infertility. A recent report by the World Health Organization (WHO) indicates that, on average, about 50% of the human sperm cells in frozen samples are damaged or destroyed by freezing and thawing, making the seminal fluid ineffective for reproduction. Proposed reasons for poor sperm quality after thawing include sudden temperature changes, ice formation, and osmotic stress during the freezing process. Defense strategies involve adding various supplements to the freezing medium to prevent sperm cells from being damaged. Antioxidants, antifreeze proteins, animal serum, fatty acids, and nanoparticles are some of the additives that can be used (3). One of the most commonly used examples in research and technology is strengthening the antioxidant system around sperm before freezing, which may improve the sperm after freezing and thawing, thereby increasing the ability of frozen sperm to achieve fertilization (4).

The oxidation of cellular compounds may result in this event. Moreover, it causes disruption and damage to cellular structures such as DNA, the acrosome, and the plasma membrane, which ultimately leads to decreased fertility (5).

Cobalamin, also known as vitamin B12, is a vitamin that is water-soluble and has a molecular weight of 1357 kDa. It contains a core of cobalt and a chorine heterocyclic ring. Through critical enzymatic reactions in mammalian cells, vitamin B12 affects fat, carbohydrate, and protein metabolism. The transfer of methyl groups by enzymes, which is dependent on vitamin B12, is necessary for humans (6). The male reproductive organs in humans are

where vitamin B12 is transported from the blood (7). It has been proven by studies that infertile men have a lower plasma vitamin B12 concentration than fertile men (8). The researchers discovered that vitamin B12 can protect the sperm membrane from lipid peroxidation under stress conditions such as freezing and thawing (9).

The worm (*Caenorhabditis elegans*), under conditions of vitamin B12 deficiency, produces up to five generations of different phenotypes, which include reduced egg-laying capacity (infertility), lengthening of the life cycle (growth retardation), and reduced lifespan. The consequences of vitamin B12 deficiency in mammals can be replicated in *C. elegans* in just 15 days through the formation of these phenotypes (10, 11). Vitamin B12 is primarily found in foods that are derived from animal products. The development of vitamin B12 deficiency is highly likely for strict vegetarians or the elderly (12).

The B vitamin complex includes Myo-inositol, which is a sugar-like molecule. Playing a role in protein synthesis and cell growth, it is one of the most essential elements of the cell membrane (13). Myo-inositol has been shown by evidence to have a physiological and therapeutic impact on the improvement of human reproductive performance (14). Myo-inositol is employed in the treatment of polycystic ovary syndrome and is regarded as a means of inducing reproductive capacity. Ovulation and fertilization are possible with this substance in patients with polycystic ovary syndrome (15). Sertoli cells are primarily responsible for producing Myo-inositol in male reproductive organs in response to follicle-stimulating hormone. It has a role in processes that involve regulating maturation, motility, capacitation, and acrosome reactions in sperm cells (16).

Male genital secretions contain a significant amount of Myo-inositol, which is 30–40 times more than what is found in blood plasma. Myo-inositol can be produced by sperm cells and Sertoli cells. However, seminiferous tubules are responsible for the majority of its production in the testis. Enzymes for Myo-inositol biosynthesis are also expressed in the epididymis, which may be because of the high levels of Myo-inositol in the epididymal ductal fluid (17). MYO activity is found to be concentrated in sperm mitochondria, which has beneficial effects on sperm motility, as evidenced by the results. This study was conducted to examine the impact of vitamin B12 antioxidants and Myo-inositol on the physiological and chemical parameters of sperm during the freezing and thawing process.

Materials and Methods

The seminal fluids of 20 men who had referred to the Alzahra Infertility Treatment Center in Guilan province within three months, according to the standards of the WHO, and had normal sperm parameters, including a volume of 1.5 mL (1.4–1.7) were collected. The seminal fluid included a total of 39 million sperms per ejaculation (33–36); the concentration was 15 million per mL (12–16). Mobility, progressive movement, survival, and morphology were 40% (38–42) 32% (31–34), 58% (55–63), and 4% (3–4), respectively. After 2–5 days, abstinence from sexual intercourse was considered for the study.

Following the initial evaluation of the samples, 2 mL of cryovials were used to freeze all the samples in the molecular laboratories of Guilan University. The samples were 1.5 mL in volume, and the concentration was 22.5 million. They were split into four groups, given sperm-freezing liquid in a 1:1 ratio, and mixed appropriately. The first group was designated as the control group without any antioxidants, only receiving a 1:1 amount of frozen sperm liquid. The second group received 2 mg of vitamin B12, and 2 mg of Myo-inositol was given to the third group. The fourth group was assigned a dose of 2 mg of vitamin B12 and 2 mg of Myo-inositol. The effective dose was obtained by conducting the research with three repetitions of the pilot test.

Subsequently, the samples were moved to an incubator and kept there for an hour. Then, cryovials containing sperm with the desired label were placed for 8–10 minutes at a distance of 5–10 cm from the surface of liquid nitrogen (in the vapor phase of liquid nitrogen) at a temperature of -80 ˚C and then were dropped into liquid nitrogen with a temperature of -196 ˚C. In this study, sperm samples were frozen using a quick freezing method. The nitrogen tank was removed after two weeks, and the samples were heated in a 37-degree bath. Next, the samples were washed with the phosphate-buffered saline solution in a centrifuge for 10 minutes at 3000 rpm after being frozen and then evaluated microscopically.

Several microscopic fields were used to examine at least 200 sperm in order to evaluate their motility. Therefore, after the liquefaction of the sperm, 10 mL of the homogenous sample was placed on a glass slide covered with a 22×22 mm coverslip and examined using a light microscope with a 40×magnification in several microscopic fields.

Sperm viability was assessed using Trypan blue staining, and 20 mL of seminal fluid was mixed with 20 mL of trypan blue (1:1) in this manner. Subsequently, the microtube was put in a 37-degree incubator for 15 minutes. A slide was utilized to prepare a smear after 10 mL of the sample was placed on it. A light microscope and a magnification of 500x were employed to examine it. The live sperms were displayed without color, and the dead sperms were displayed in a dark blue color. Each slide had 100 examined sperm.

A detailed investigation of sperm morphology was performed by preparing a uniform smear of semen on a slide and staining it. As a result, Giemsa solution was used to stain 10 mL of semen. Then, the characteristics of a normal sperm, including an oval head with dimensions of 5×3 µm, 70% of which is covered by a clear and uniform acrosome and without vacuoles, and a healthy neck without cytoplasm, and an elongated tail with no fracture and about 50 µm in length, were considered for evaluation.

Two hundred sperm cells were counted on each slide using × 500 magnification. Subsequently, a large or small head, a whip head, a needle head, a pear-shaped or triangular head or shapeless head, a long head, a round head, two heads, two tails, a long or short tail, no tail or twisted tail, a short or long neck, and the presence of cervical cytoplasm were considered abnormal forms of sperm.

Lipid peroxidation is a pathological effect caused by ROS that is linked to polymembrane polyunsaturated fatty acid oxidation. Human sperm cells are more susceptible to plasma membrane oxidation than other body cells because of the high concentration of unsaturated fatty acids in the membrane. To measure oxidative stress and lipid peroxidation in sperm, it is recommended that malondialdehyde (MDA) be used as a suitable index. MDA was measured using the kit instructions of Teb Pazhouhan Razi (TPR) in Tehran, Iran.

The evaluation of total antioxidant capacity (TAC) can aid in infertility diagnosis and treatment. The TAC was measured in this research using the TPR kit in Tehran, Iran.

SPSS software (version 23) was employed to evaluate the data obtained from this research, and the results were expressed as means and standard deviations (SD). The average results were utilized to compare the studied groups using a one-way analysis of variance statistical method, and the difference was considered statistically significant at $P < 0.05$.

Results

The freezing process can be improved by vitamin B12 and Myo-inositol, both separately and in combination, as was found in this research. These results were not compared against normal sperm samples before freezing but rather against the control group that was frozen but not given any antioxidants. The findings showed that the addition of antioxidants to the freezing environment improves parameters such as total mobility, viability, normal morphology, and the TAC concentration and subsequently causes a decrease in the concentration of MDA. This statistic demonstrated a more vital role in the combination group of these two antioxidants (Figure 1).

The obtained data revealed that the average sperm motility in the control group was 40.65 ± 4, which was less than the B12 group's average (49.5 ± 5.92), and this difference was significant. In addition, the average total mobility in the control group (40.65 ± 4) was lower than that in the Myo-inositol group (68.70 ± 15.42) and the combined vitamin + myo-inositol B12 group (71.5 ± 8.63). Further, the average sperm motility in the B12 group was significantly lower than in the Myo-inositol group and the vitamin B12 + Myo-inositol combination group. However, the difference in the average sperm motility between the Myo-inositol group and the vitamin B12 + Myo-inositol combination group was not significant ($P < 0.001$, Table 1).

According to the obtained information, there was a significant difference between the viability of the studied groups ($P < 0.05$). The findings indicated that the average viability in the control group was 38.65 ± 12.81, which was significantly lower than in the B12, Myo-inositol, and vitamin B12 + Myo-inositol combination groups, with averages of 49.35 ± 11.65, 59.75 ± 10.46, and 70.55 ± 8.49. Furthermore, the average viability in the B12 group was significantly lower than in the Myo-inositol group. The combined vitamin B12 + Myo-inositol group and the average viability in the Myo-inositol group were considerably lower than the combined vitamin B12 + Myo-inositol group (Table 2).

The results revealed that the average number of spermatozoa with normal morphology in the control group was 16.90 ± 5.8, which was significantly lower than the average of that in the B12 group (21.60 ± 5.14) and in the combined vitamin B12 + Myo-inositol group (25.20 ± 1.51) but this difference was not significant in the control group or the Myo-inositol

Table 1. Comparing the total mobility with the separation of the studied groups using an analysis of variance test

Groups	Mean ± SD	F	*P*
Control	40.65 ± 4.00		
Vitamin B12	49.05 ± 5.92		
MYO	68.70 ± 15.42	48.73	0.001
Vitamin B12 + MYO	71.05 ± 8.63		

Note. MYO: Myo-inositol; SD: Standard deviation.

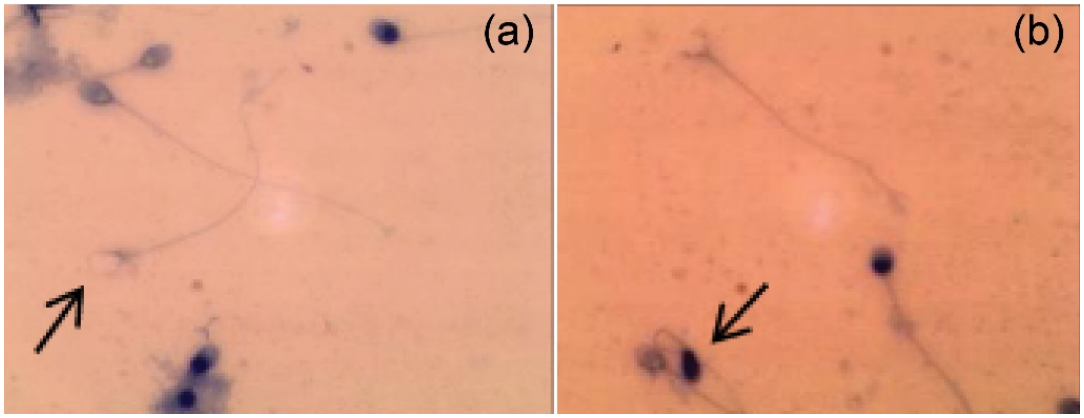

Figure 1. Using Trypan blue staining (Magnification 500×) to determine the viability of squamous cells. The Trypan blue dye cannot be absorbed by live sperm (a), but dead sperm can be observed as dark blue cells when the dye penetrates the cell (b)

group (17.95 ± 2.44). The average number of sperms with normal morphology was significantly higher in the B12 group than in the Myo-inositol group. It was less than the combined vitamin B12 + Myo-inositol group. Moreover, the average number of sperms with normal morphology in the Myo-inositol group was significantly lower than in the vitamin B12 + Myo-inositol combined group (Table 3).

According to the results obtained by Giemsa staining, the average number of sperms with normal morphology in the combination group of two antioxidants, vitamin B12 + Myo-inositol, was significantly higher than that in other groups ($P = 0.001$). The control group recorded the lowest value, but it was not statistically significant for the Myo-inositol-treated group ($P = 0.999$) (Figure 2).

Discussion

An antioxidant compound is helpful for infertile men if it can produce a positive change in the basic parameters of semen, such as sperm concentration, complete sperm motility, progressive motility, and normal sperm morphology, according to WHO standards. The level of peri-sperm antioxidant activity decreases after freezing and thawing human sperm, which may have a negative impact on sperm motility improvement following freezing and thawing. To prevent the loss of harmful antioxidant activity, specific antioxidants are included in current freezing media. Sperm cells may experience the destruction of organelles due to cold shock and the formation of ice crystals in the absence of freezing protective factors. This event may appear in the oxidation of cellular compounds as well as causing disruption and damage to cellular structures such as DNA, the acrosome, and the plasma membrane, which ultimately reduces fertility (18).

While there are advantages to sperm freezing, it can also cause detrimental changes in sperm structure and function. The effects of freezing on cells have been well documented. Various factors during the freezing process, including sudden changes in temperature, the formation of ice crystals inside the cell, and osmotic stress, have been presented as reasons for the low quality of sperm after thawing (19).

When the balance between the level of ROS and the antioxidant defense system is lost, free radicals can overcome the antioxidant, leading to oxidative stress. The production of ROS during semen freezing can lead to changes in the morphology, function, and fertility of men. Maintaining the structural and functional integrity of spermatozoa requires maintaining the level of ROS at physiological levels. Apoptosis and cell death are the result of a high concentration of ROS and the loss of antioxidant enzymes. Increasing recognition of the role of oxidative stress in male infertility has led to the recommendation of antioxidant supplementation as a treatment option for idiopathic infertile men (20-22).

The present study was performed to increase our understanding of the influential role of antioxidants in the mechanisms of sperm freezing. In this study, the

Table 2. Comparing the mortality with the separation of the studied groups using an analysis of variance test

Groups	Mean ± SD	F	*P*
Control	38.65 ± 12.81		
Vitamin B12	49.35 ± 11.65		
MYO	59.75 ± 10.46	31.19	0.001
Vitamin B12 + MYO	70.55 ± 8.49		

Note. MYO: Myo-inositol; SD: Standard deviation.

Table 3. Comparing the morphology with the separation of the studied groups using an analysis of variance test

Groups	Mean ± SD	F	*P*
Control	16.90 ± 5.08		
Vitamin B12	21.60 ± 5.14		
MYO	17.95 ± 2.44	18.85	0.001
Vitamin B12 + MYO	25.20 ± 1.51		

Note. MYO: Myo-inositol; SD: Standard deviation.

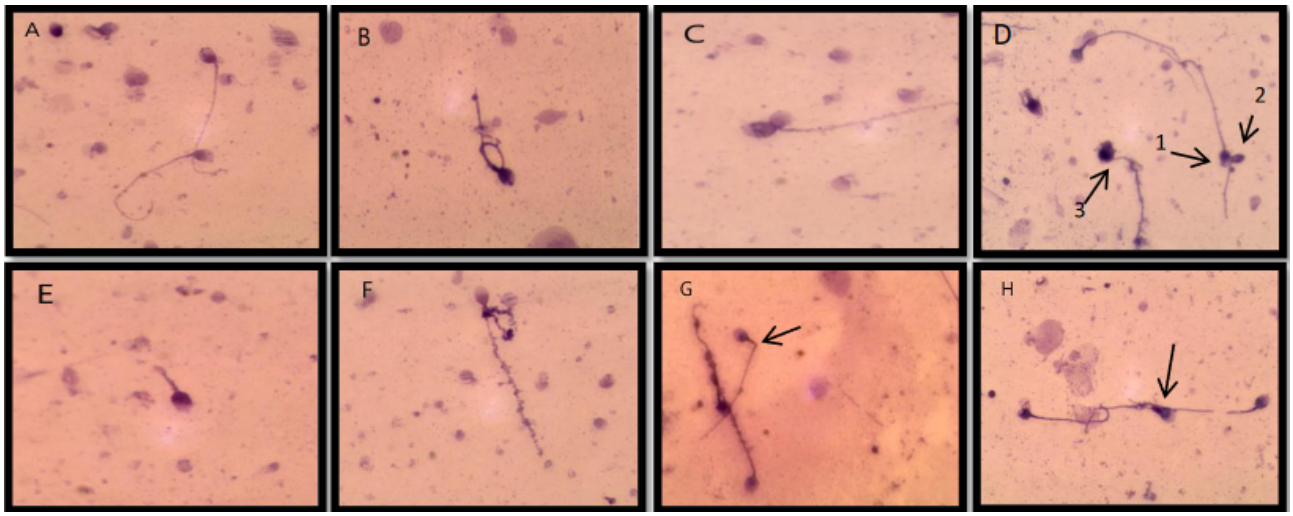

Figure 2. Morphological measurement of sperm using giemsa staining (magnification 500x): (A)Normal sperm, (B) Coiled tail, (C) Multiple heads, (D) Normal and abnormal sperm in the head area: (1) normal head, (2) microcephalous head, (3) macrocephalous head, (E) Short tail, (F) Irregularly shaped tail, (G) Bent tail, and (H) Abnormal acrosome region

antioxidants vitamin B12 and Myo-inositol were used in a human sperm-freezing medium, and the biological and biochemical parameters of the groups treated with antioxidants were compared with those of the control group. The findings of the present study showed that the addition of 2 mg of vitamin B12 and 2 mg of Myo-inositol and a combination of these two improved parameters such as viability, total mobility, progressive mobility, normal morphology, increased TAC, and finally, decreased MDA. The present study's results can be validated by reviewing previous research.

The vitamin-strengthening supplement was found to be studied after thawing in bulls; B12 enhances the quality of sperm. In some previous studies, researchers added different concentrations of vitamin B12 (0 and 5.00 mg/mL) to the freezing medium of cow sperm and evaluated the quality of the sperm in the stage before freezing and after thawing. Their results showed that the effect of vitamin B12 on survival, DNA fragmentation, and sperm motility was significant in both conditions, before and after freezing (23-25). In this study, the survival rate in the group treated with vitamin B12 was higher than that in the control group, which was lower than that in the combination group of two antioxidants, vitamin B12 and Myo-inositol.

In an experiment regarding the role of vitamin B12 in the freezing process, the researchers concluded that this nutrient was also tested to protect sperm from cold damage during the freeze-thaw process that occurs in assisted reproductive treatment. Its addition to the freezing medium enhances the viability and motility of sperm. It lessens the DNA fragmentation that happens during the freeze-thaw process (26). In our study, the total mobility and progressive mobility in the vitamin B12 group were significantly higher than in the control group. However, they were lower compared to the combination of B12 with Myo-inositol and the group treated with Myo-inositol.

This study sought to enhance our understanding of how antioxidants prevent sperm freezing mechanisms. In this study, antioxidants, vitamin B12, and Myo-inositol were used in a human sperm freezing medium, and the biological and biochemical parameters of groups treated with antioxidants were compared with those of the control group. The findings of the present study revealed that the addition of 2 mg of vitamin B12 and 2 mg of Myo-inositol and a combination of these two improved parameters such as viability, total mobility, and progressive mobility, increased normal morphology, and increased TAC. According to this research, the concentration of MDA was lower in groups treated with antioxidants than in the control group.

Conclusion

Based on the findings, sperm freezing could trigger oxidative stress, leading to lipid peroxidation and apoptosis of sperm cells. Low fertility and possibly infertility were also one of its consequences. The performance of biological and biochemical parameters of sperm can be significantly improved by adding antioxidants to the human sperm freezing environment. In this study, it was well observed that each of the antioxidants, both alone and in combination, causes a significant increase in sperm parameters such as motility, viability, morphology, and TAC. In this research, the amount of lipid peroxidation and MDA was found to decrease with antioxidant treatment.

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Authors' Contribution

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Competing Interests

The authors declare that there is no conflict of interests.

Ethical Approval

Ethical considerations in this study included obtaining permission from the Ethics Committee of Guilan University (Ethical No. IR.GUMS.REC.1399.666) and obtaining written consent from the participants to participate in the study.

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