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ORIGINAL ARTICLE

The Selected Zinc Transporters (ZnT and ZIP) Gene Expression, Zinc, Iron and Glycogen Concentrations in Healthy Rat Testis: Effect of Aqueous Ajwain (*Tracispermum ammi*) Seeds Powder Extraction and High-intensity Treadmill Running

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KEYWORDS

High-intensity exercise; aqueous extract of Ajwain seeds; Zinc transporter; Testicular tissue

ABSTRACT: Zinc and iron as two important essential minerals for testis functions are controlled by Zinc transporters. It has been reported that zinc and its transporters are affected by nutrition and training. The main goals of the current experimentation were to the influences of training in combination with extraction of Ajwain seeds on rat testicular zinc and zinc transporters. Forty male rats were randomly assigned into four groups. Rats were orally received an aqueous Ajwain seed extraction (200 mg kg^{-1}) and the saline groups were treated in the same manner. Results showed that gene expression of Znt5 had meaningfully changed in the ST group in comparison with SC ($p = 0.004$) and AT ($p = 0.001$) groups. Expression of this gene had meaningfully alteration in the AT group in comparison with ST ($p = 0.036$) and AC ($p = 0.001$) groups. Gene expression of Znt8 was also significantly increased in AT group compared to AC group ($P = 0.010$). Expression of Znt9 was also significantly increased in AT group in comparison with AC ($P = 0.008$) and ST ($P = 0.026$) groups. Expression of the other genes (Znt6, Zip7, Zip8, and Zip14) and also the content of Zn, Fe and glycogen did not show significant differences. The concurrent implementation of training and supplementation with the extract of Ajwain seed significantly modulated the expression levels of certain zinc transporters. These discoveries can offer new understandings into the underlying mechanisms of the effects of exercise and nutrition on testicular tissue.

INTRODUCTION

Zinc is considered as the most plentiful trace element after iron in the body of humans. The body of humans is contained of 2 to 3 grams (2000–3000 mg) of zinc. The World Health Organization (WHO) estimates that 1/3 of the world's population is deficient in zinc [1]. Zinc is a seafood like oysters, improved cereal, mushrooms, yoghurt low in fat [3], and proteins obtained from animals like milk, meat, and fish. Zinc is essential for proper physiological functions of the body, such as normal growth, reproduction, DNA synthesis, cell

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of vision, wound healing, bone formation, and immune system strengthening [4-6]. About 90% of zinc is found in muscles, liver, and bones. However, it also accumulates in some tissues and cells, including macrophages. Zinc in men has unique properties, including improving fertility by supporting sexual and prostate health, and the levels of testosterone, increasing sperm quality, and fertilization. Zinc has been shown to play an important role in reproductive function [7-9]. For example, zinc deficiency is associated with decreased testicular volume, decreased testis weightiness and hypogonadism, sexual disfunction, insufficient increase of secondary sexual characteristics in humans, contraction of the sperm ducts, malfunction of spermatogenesis, the development of male sex organ, and hypogonadism [10]. It is well established that changes in the levels of this essential element in different tissues occur through changes in the content and activity of the transport and storage proteins of these elements. Zinc transporters are proteins that transport zinc, as well as other metals such as iron and some other metals, across cellular and intracellular membranes. These proteins interact with iron and zinc balance in cells and may be involved in various physiological processes, including sperm production [11-15]. The regulation and control of zinc levels in testicular tissue, which play an important role in the function of the reproductive system, are carried out by some of the zinc transporters [16].

Zinc transporters are divided into two types: ZnT (SLC30) and ZIP (SLC39A). There are 10 ZnT transporters that facilitate the zinc movement from the cytosol to the both spaces of extracellular and intracellular sections. This action contributes to a reduction in cytosolic zinc levels. On the other hand, ZIP transporters, numbering 14, play a role in transporting zinc into the cytosol from both spaces of the extracellular and the organelle of intracellular sections. Consequently, this process leads to an elevation of cytosolic zinc levels [17]. Some of the zinc transporters in testicular tissue include Znt5, Znt6, Znt8, Znt9, Zip7, Zip8, and Zip14. Each of these transporters mediates a wide range of physiological roles, independently or in collaboration with each other. ZnT5 is located in the primary secretory path, together with the Golgi apparatus and COPII-coated vesicles [18]. In addition, ZnT5 is effective in cellular signaling by transporting PKC to the plasma membrane [18]. ZnT6 is localized to the Golgi apparatus for targeting secretory functions [19]. Although, ZnT6 doesn't involve in transportation of zinc, as two histidine remains from the intramembrane site of zinc-binding within the helix are replaced by phenylalanine and leucine [18]. A serine residue from the COOH-terminal cytosolic percentage of ZnT6 is known as a possible site of phosphorylation [20], which in turn suggests that ZnT6 is a modulating sub-unit of ZnT5-ZnT6 heterodimers activity [18]. In the ZnT5-ZnT6 heterodimer, ZnT6 acts as an auxiliary subunit because it lacks zinc transport activity and may have a modulating function for zinc transport [19]. ZnT8 is known as a pancreatic transporter of zinc which is β-cell-specific that is involved in insulin secretion [21, 22] and was subsequently shown to be expressed in α cells [23, 24]. Znt9 is required as a zinc ion transporter to maintain mitochondrial function in human cells. Results suggest that Znt9 may use the mitochondrial proton gradient for zinc transport. Znt9, as a zinc ion transporter, is required to maintain mitochondrial function in human cells. The loss of Znt9 leads to increased zinc levels in mitochondria, which, by swelling the mitochondrial matrix, reduces membrane potential and the increases production of reactive oxygen species, compromising mitochondrial metabolic function. Znt9 is also essential for mobilizing zinc ions during sperm activation, suggesting that dynamic regulation of zinc concentration is required for its signaling function [25].

ZIP7 is located in the primary secretory path, containing the Golgi apparatus and endoplasmic reticulum (ER) and [26]. The transportation action of ZIP7 is mainly controlled by phosphorylation by the protein kinase casein kinase 2 (CK2), and by its release. Zinc activates AKT, ERKs, and tyrosine kinases both of them play roles in regulation of migration and cell proliferation [21]. The levels of expression of ZIP7 are in reverse proportional to the levels of cellular zinc and phosphorylated GSK3 [23], while the molecular mechanism triggering their directing associations remains unidentified. ZIP7 plays a crucial role in regulating blood glucose levels in skeletal muscles [27]. ZIP7 expression induced by glucose exposure could facilitate the storage and process in pancreatic β cells

[28]. Dysregulated zinc homeostasis, possibly triggered with abnormal ZIP7 expression, seems to play a role in tumor growth and invasion, leading to the development of an invasive phenotype in breast cancer [29]. Notably, ZIP7 is considered in 10% of genes that are constantly overexpressed in several poorly prognostic breast cancers [20]. ZIP8 is confined to the membrane of plasma and lysosomal membranes, where it plays a role in zinc homeostasis and lysosomal function [30, 31]. ZIP14 has two other alternate intertwining isoforms, ZIP14B and ZIP14A. two types of isoforms are in the membrane of plasma and import zinc [29, 32]. These isoforms, along with ZIP8, contribute to the intricate regulation of zinc homeostasis in various tissues, including breast tissue and skeletal muscles.

It is well known that the serum and tissue content of these elements (iron and zinc) can be affected by conditions such as physical activity, diet, and supplementation with medicinal herbs. This change in content or cellular concentration is actually due to a change in activity and the amount of transporters for these elements. However, cellular sources, including mitochondria and the endoplasmic reticulum, may also be involved. Studies have shown that exercise and supplementation with herbal products, including pumpkin seed oil and white pea extract, increase the gene expression of zinc transporters in the liver [33]. In addition, supplementation with safflower seed extract and oil in rats with metabolic syndrome leads to changes in zinc content in liver tissue [33]. However, the effect of Ajwain plant seed extract on zinc metabolism status and iron content in testicular tissue is unknown.

Ajwain (*Trachyspermum ammi*) is an annual plant from the Umbelliferae (*Apiaceae*) family of plants with compound comb leaves and white petals [30]. which is spread in Iran, Afghanistan, Pakistan, India, and North Africa [34]. This plant has a stalk with a white inflorescence compound called an umbrella and is widely grown in arid and semi-arid areas. It has long been considered in traditional medicine and today plays a vital role in the pharmaceutical and food industries. Traditionally, used as a useful drug for diarrhea, atonic indigestion, abdominal tumors, abdominal pain, loss of appetite, bronchial problems, asthma, and amenorrhea. Pharmacological studies on the biological activities of this plant have shown antifungal, antimicrobial, antioxidant, cytotoxic, abortifacient, anti-inflammatory, analgesic, blood fat-reducing, antihypertensive, antispasmodic, and proven bronchodilator and analgesic effects [35]. In addition to dietary and supplementation changes, a variety of exercise training can play an effective role in the homeostasis of these elements. Changes in the intensity and speed of exercise are key factors that play an important role in this process.

Since transporters play physiological roles in cellular processes, the upregulation or downregulation of transporter expression must be precisely timed for proper transfer. The regulation of ZnT and ZIP transporter expression involves both transcriptional and post-transcriptional mechanisms. These mechanisms include transcription activation, mRNA stabilization, protein modifications, organelle targeting, or degradation, and responding to various stimuli such as hormones, cytokines, stress conditions, and hypoxia, among others [36, 37]. For example, the regulation of Zip6 by the transcription factor STAT3 leads to epithelial-mesenchymal transition (EMT), which is essential for development [22]. Recent studies have shown that microRNAs control the expression of ZnT and ZIP transporters [24, 38]. These gene expression controls all contribute to cellular zinc homeostasis and, consequently, the normal physiological state and, in some cases, play a role in disease pathogenesis [39].

Glycogen is a type of polysaccharide that is used to store carbohydrates in the cells of animals. Studies have shown that the amount of glycogen stored in animals varies in different tissues, with the highest levels being found in the liver and skeletal muscles [40]. For example, in the liver, glycogen accounts for about 5–6% of the organ's weight [41]. This compound is one of the main polysaccharides for storing energy in humans and animals. In addition, it is the most accessible source of glucose during times of intensive physical activity, making it of great biological importance to organisms [42]. The amount of glycogen stored in different tissues of the body depends on exercise, basal metabolism, and dietary habits [43]. However, there are always about 4 grams of glucose in human blood. In fasting individuals, blood sugar is maintained by glycogen stores in the liver and skeletal muscle. Glycogen stores in skeletal muscle

are a type of energy reserve for the muscle. However, the breakdown of muscle glycogen prevents the muscle from absorbing glucose from the blood, resulting in an increase in the amount of blood glucose available for use in other tissues [44]. Liver glycogen stores are used as a store of glucose for use in the body, especially the central nervous system, because the nervous system relies on glucose for nutrition. In skeletal muscles, long-term exercise leads to glycogen depletion and thus affects muscle performance [45].

Based on these definitions and considering the use of zinc in metabolic pathways and the possibility of zinc depletion in athletes, the need for zinc supplementation is essential given the sensitive role of zinc. Considering that Ajwain is a medicinal plant that contains a significant amount of minerals, the study attempted to answer the research question probing whether supplementation with aqueous an extract of Ajwain with exercise can affect the expression of genes Slc30a5, Slc30a6, Slc30a8, Slc30a9, Slc39a7, Slc39a8, and Slc39a14 and the content of zinc, iron, and glycogen in testicular tissue.

MATERIALS AND METHODS

Animals

Forty male rats were purchased from the Pasteur Institute of Amol and housed in standard cages (5 rats per cage) at the animal laboratory of Mazandaran University, Iran, at a temperature of 22–24°C with a 12/12 light/dark cycle. After one week of acclimatization to the laboratory environment, they were divided into four groups of 10 rats: 10 rats in the saline-control (S-C) group, 10 rats in the saline-training (S-T) group, 10 rats in the Ajwain-control (A-C) group, and 10 rats in the Ajwain-training (A-T) group based on weight homogeneity. The training groups were familiarized with a rodent treadmill for one week. The exercise protocol was performed for 8 weeks [46]. Also, rats had free access to water and a standard diet during the experiment. Rats in the A-C and A-T groups were ***gavaged*** with an aqueous extract of Ajwain seeds, and rats in the S-C and S-T groups were gavaged with saline. The gavage interval for the training groups was 30 minutes after the end of the exercise. All phases of the current research paper were consistent with the Ethics Committee of Mazandaran University of Medical Sciences with the ethics code (IR.UMZ.REC.1401.071).

Extract preparation

To prepare the aqueous extract of Ajwain seeds, 10 mL of boiled water was added up for each gram of Ajwain powder. The mix then had been placed at a temperature of 45-55 °C for 3 days, and then the solution was boiled for one hour with a gentle flame and after cooling, the mixture was filtered three times, first through a single layer of filter cloth, then through a double layer, and finally through a triple layer. The filtrate was then filtered with Whatman No. 1 filter paper. Then, the obtained solution, the obtained solution was concentrated in an oven until it was one-third its original volume. The extract was then stored in a dark, opaque glass container in the laboratory refrigerator [47]. The Ajwain groups of rats received 200 mg kg^{-1} of BW/Day by gavage [48]. The saline group was also gavaged with an equal volume of saline under similar conditions.

Exercise Protocol

The exercise groups underwent an 8-week training program with 5 sessions per week, each lasting 60 minutes. The training program was divided into three phases. The first phase lasted for one week; during this phase, animals underwent 10-15-minute treadmill walking sessions at a speed of 10 meters per minute. This phase was designed to familiarize the animals with the treadmill and to gradually increase their intensity. In the second phase, rats initially started with 20 minutes of running at a speed of 15 meters per minute. The duration and intensity of running were gradually increased over two weeks until they reached the target duration and speed of 60 minutes of running at a speed of 32 meters per minute. This phase was designed to challenge the animals and promote further adaptations. The Last phase was the stabilization phase. In this phase, the exercise groups continued running at a constant speed and duration for the remaining weeks. This phase allowed the animals to consolidate their gains and achieve a stable level of fitness. It is important to note that the first and last 5 minutes of each 60-minute session were dedicated

to warm-up and cool-down at a speed of 15 meters per minute. This was to prevent injury and ensure a smooth transition between exercise and rest.

Sample collection

Rats were intraperitoneally anesthetized with a ketamine/xylazine combination 36 hours later the final exercise period and fasting 12 hours to minimize the acute exercise effect. Blood was collected from the inferior vena cava. Testicular tissue sampling was performed immediately after blood collection, washed with saline, placed in sterile microtubes, and ice-covered in liquid nitrogen and then transferred to a -70°C deep freezer until measurement.

Gene Expression

To measure gene expression, testicular tissue was minced into very small pieces less than 1 mm in diameter using a scalpel on a cold, sterile plate. The tissue was then homogenized in a mortar with liquid nitrogen, and the resulting powder was homogenized until a uniform paste was obtained. RNA had been extracted by RNA extraction kit from DNA Zist Asia (Mashhad, Iran) according to the manufacturer's instructions. Next, the quality and quantity of the extracted RNA were evaluated using agarose gel electrophoresis and UV spectrophotometry (Nanodrop Muba Iranian, Iran). To ensure that the extracted RNA was free of DNA contamination, the extracted RNA was treated with DNase I, an RNase-free enzyme, based on instructions of the manufacturer (Cinaclone, Iran). cDNA was synthesized using a kit (Yakhtehtejazhazma, Iran) according to the manufacturer's instructions. It is worth mentioning that oligo-dT primers were used in cDNA synthesis. In this study, specific primers were designed for the amplification of the genes Slc30a5, Slc30a6, Slc30a8, Slc30a9, Slc39a7, Slc39a8, and Slc39a14 and the reference gene β-actin using Primer Premier 5 software. The sequences of the primers were then synthesized by Bioneer (South Korea). The sequences of the forward and reverse primers for the above genes are shown in Table 1.

Table 1. Primers used in the Real-Time PCR process

Gene	Forward and Reverse primer	Accession number	Product length(bp)
Slc30a5(Znt5)	F-5'-TGACACAAACATGCTGACACC-3' R-5'-CATGACTGTGGGCGTGACT-3'	NM_001106404.1	89
Slc30a6(Znt6)	F-5'-TGCTAAACTTCTCAGACCACCA-3' R-5'-TCACATTTTTCCCAGGCGTATT-3'	NM_001277279	141
Slc30a8(Znt8)	F-5'-TTCTTTATGGTGGCAGAGGTG-3' R-5'-GCAGGAAACTAGTCAGGTCAATTA-3'	NM_001130538.1	100
Slc30a9(Znt9)	F-5'-ACCAATGGAATCCCTGCTATG-3' R-5'-ATTGCCTGTTATGGAGGTAAGG-3'	NM_001109088.1	247
Slc39a7(Zip7)	F-5'-CTCATTCGCACGATACTCCG-3' R-5'-TGTCTCACAAACTTCTCCACCAC-3'	NM_001164744.1	133
Slc39a8(Zip8)	F-5'-ATAAAGAAGTCGTATTTCCCCAAGA-3' R-5'-GTAAAATCCACCAAACACAGCAAC-3'	NM_001011952.1	165
Slc39a14(Zip14)	F-5'-CAATTATGTCTCCAAGTCTGCTGT-3' R-5'-GTCCGTGATGGTGCTCGTT-3'	NM_001107275.1	110
β-Actin	F-5'-GTGTGACGTTGACATCCGTAAAGAC-3' R-5'-TGCTAGGAGCCAGGGCAGTAAT-3'	NM_031144.3	119

Quantification of gene expression by real-time PCR was performed by measuring the increase in fluorescence intensity due to the binding of the SYBR Green dye. In this step, the polymerase chain reaction (PCR) reaction was performed for cDNA samples of the target genes and the reference gene β-actin using the SYBR Green kit from Ampliqon (Denmark) in the Rotor gene Corbett 6000 instrument. After reviewing the cycle threshold (Ct) values obtained from the biological and technical replicates of each treatment, the mean Ct for the

technical replicates of the target genes and β-actin was calculated. The data were then expressed as the fold change in expression of each of the above-specific genes relative to the reference gene, β-actin.

Measurement of Zinc and Iron

To measure zinc and iron in testicular tissue, the following sample preparation procedure was performed: First, 50–100 mg of tissue was cut with a ceramic blade knife and placed on sterile aluminium foil sheets. The tissue was then placed in an oven at 90-80 degrees Celsius for 24 hours to dry completely. The dried tissue was then transferred to a glass flask, and 3 mL of nitric acid, 1 mL of hydrogen peroxide, and 0.5 mL of perchloric acid were added, respectively. The samples were heated in an oven in four steps at 90, 120, 140, and 150 degrees Celsius for 15, 15, 60, and 60 minutes, respectively. After cooling, the solution was diluted to a volume of 10 mL [49]. After this step, the tissue content of iron and zinc was measured by atomic absorption spectrometry using an ICP-EOS device. It is worth mentioning that the amount of some minerals in Ajwain seeds was also measured using the same method.

Measurement of Glycogen

To measure glycogen, the phenol-sulfuric acid method was used according to the instructions [50]. The sample was then read using the BioTek Elx808 ELISA reader from the United States in duplicate at a wavelength of 490 nm.

Statistical analysis

All statistical analyses were performed using SPSS version 27 software. Data were presented as mean ± standard deviation and were analyzed using descriptive and inferential statistics. The normality of the data distribution was assessed using the Kolmogorov-Smirnov test, and one-way analysis of variance (ANOVA) with Tukey's post hoc test was used to determine the difference between groups. The significance level was set at $P < 0.05$.

RESULTS

The results of gene expression for Znt5, Znt6, Znt8, and Znt9 are shown in Figure 1. One-way analysis of variance (ANOVA) of the results for testicular tissue showed a significant alteration in the expression of the Znt5 gene ($P = 0.001$). Tukey's post hoc test showed that there was a significant difference between the saline-control and Ajwain-control groups ($P = 0.004$), among the saline-training and Ajwain-training groups ($P = 0.036$), and among the Ajwain-control and Ajwain-training groups ($P = 0.001$). A statistically significant difference was not observed in the expression of the Znt6 gene ($P = 0.423$). Additionally, the results of the statistical analysis showed that the expressions of the Znt8 ($P = 0.008$) and Znt9 ($P = 0.008$) genes were significantly different. Further investigation with Tukey's post hoc test showed that in Znt8, there was a significant difference between the Ajwain-control and Ajwain-training groups ($P = 0.010$), and in the expression of the Znt9 gene, a significant difference exists among the saline-training and Ajwain-control groups ($P = 0.026$) and the Ajwain-control and Ajwain-training groups ($P = 0.008$).

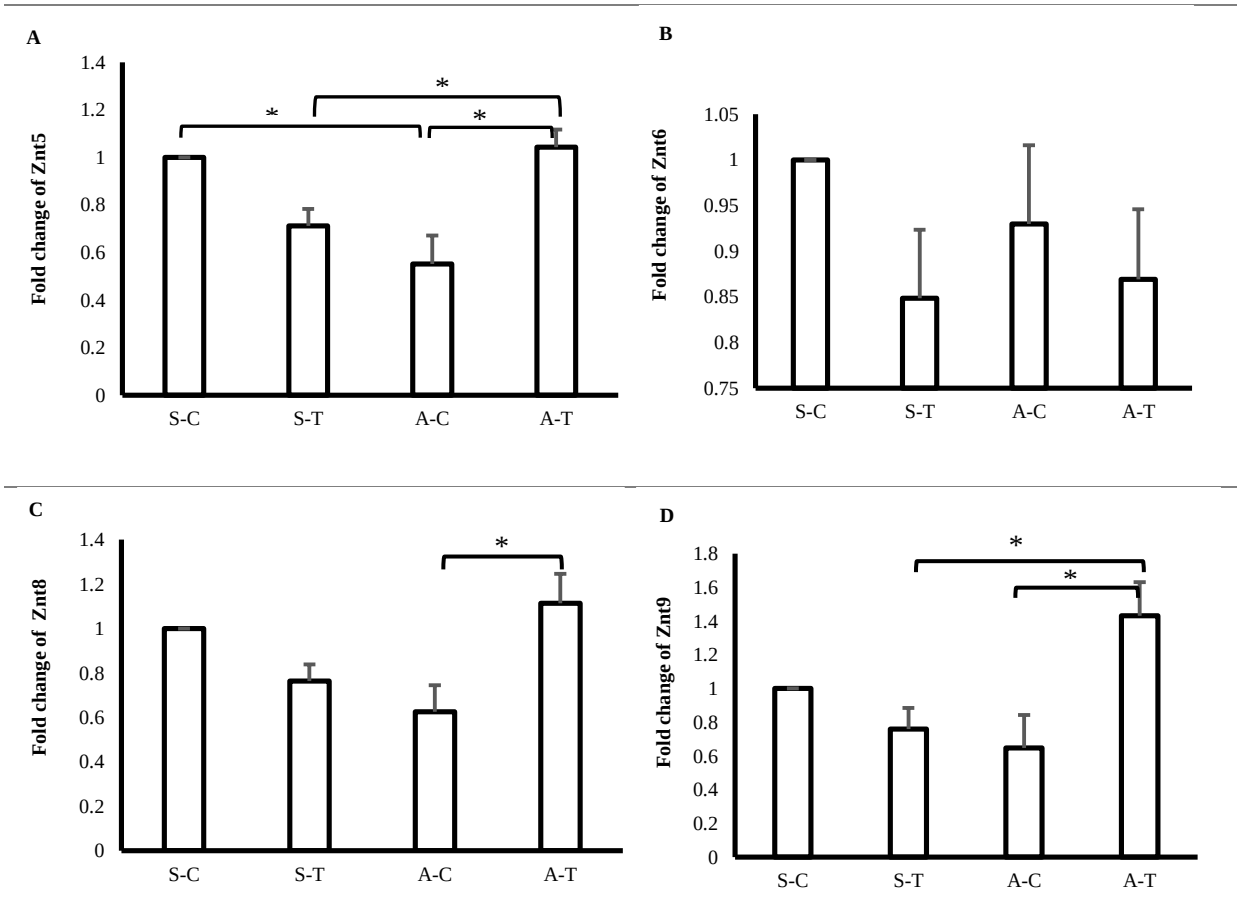
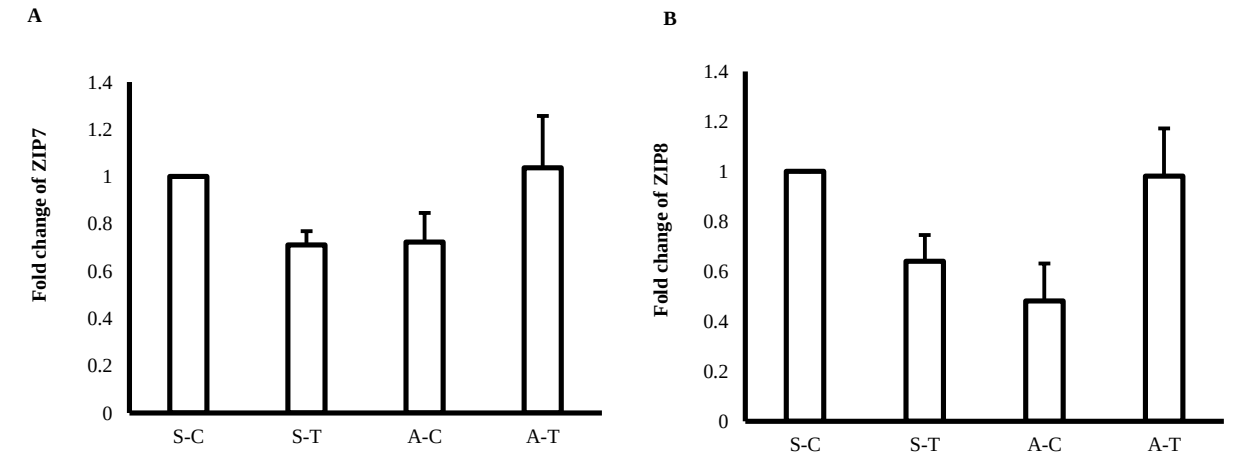


Figure 1. Mean ± SD of changes in gene expression of A: Znt5, B: Znt6, C: Znt8, and D: Znt9 in testicular tissue. *: P<0.05.

In Figure 2, the expression of the Zip7, Zip8, and Zip14 genes and the amount of testicular glycogen are shown. The analysis with a one-way ANOVA showed that the expression of the Zip8 gene was significantly different (p = 0.026). However, the expression of the Zip7 gene (P = 0.172) and the Zip14 gene (P = 0.125) did not show a significant difference. Additionally, the analysis of glycogen content (P = 0.158) also a significant difference was not approved.



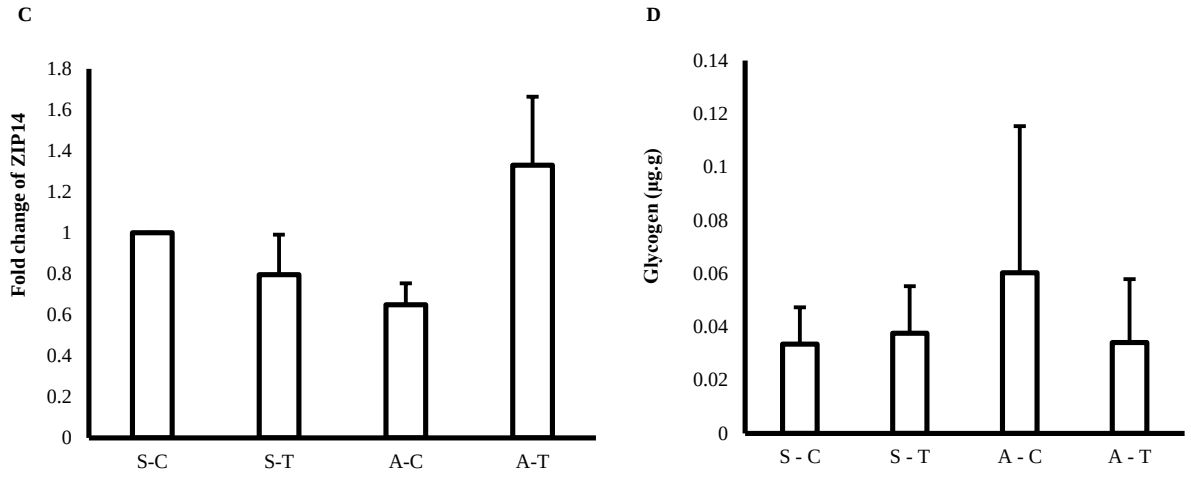


Figure 2. Mean ± SD of changes in gene expression of A: Zip7, B: Zip8, C: Zip14 and D: content of glycogen in testicular tissue. *: P<0.05.

In Figure 3, the amounts of zinc and iron in testicular tissue are shown. One-way ANOVA analysis disclosed that there wasn't any significant difference statistically in the content of Zn (P = 0.303) and Fe (P = 0.437). The results of amount of some minerals in Ajwain seeds are shown in Table 2.

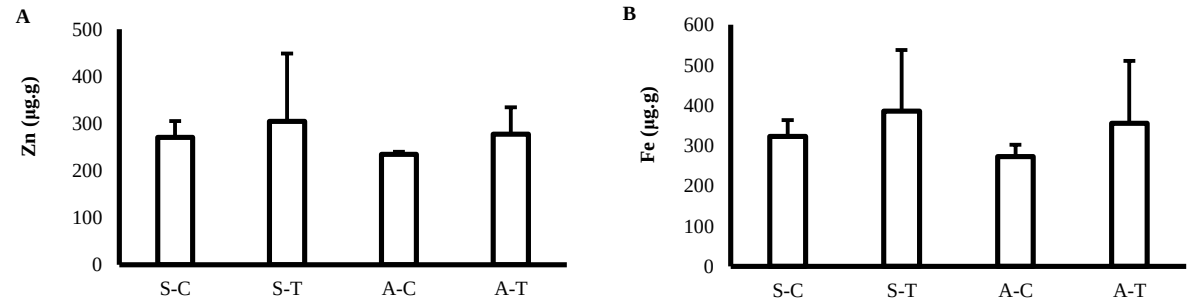


Figure 3. Mean ± SD of changes in content of A: Zn and B: Fe in testicular tissue. *: P<0.05.

Table 2. Minerals of Ajwain seeds based on µg/gr

Ca	Cu	Fe	K	Mg	Mn	Na	P	Se	Zn
14829.3	7.3	72.2	5531.5	3906	25.39	1621.3	2876.1	1	53.4

DISCUSSION

Our results showed that the expression levels of Znt5, Znt8, and Znt9 transporters increased in the A-T group and significantly decreased in the A-C group. However, other variables in this study, including tissue levels of zinc, iron, and glycogen, did not show significant changes. In addition, changes in gene expression of Znt6, Zip7, Zip8, and Zip14 were not significant. Detailed examination of the pattern of changes in the gene expression of zinc transporters Znt5, Znt8, Znt9, Zip7, Zip8, and Zip14, showed that although their values were not equal, the pattern of their changes was similar. There are few studies and reports regarding zinc and iron metabolism and zinc transporters in the testes. Studies have shown the gene expression of Znt6 and Znt5 in prostate tissue [51], Znt5 in stomach, small intestine, and duodenum, and Znt6 in epithelial absorptive cells, jejunum, rectum, colon, and rectum of rats [52], as well as the expression of Zip7, Zip8, Zip14, Znt5, Znt6, Znt8, and Znt9 genes in jejunum, large intestine, liver, and kidney tissues [53]. In addition to the difference in expression in the tissue, it seems that the use of some toxins and therapeutic supplements with zinc itself can

change the expression of these transporters, especially Znt5, in the femur tissue [54]. In another study, researchers investigated that the effect of exercise on cognitive performance and its relationship with the gene expression of Znt6 and Znt5 in healthy male Sprague-Dawley rats. For this purpose, they divided the rats into two groups of six, the training and control groups, gave the sports group forced running exercise for eight weeks, used the Morris water maze for cognitive performance, and concluded that the exercise group, measured up to the control group, had better cognitive performance. Also, the gene expression of Znt6 and Znt5 was increased in the hippocampus of the training group compared to the control group [55]. The results of this study are consistent with our research on increasing the gene expression of Znt5, but the result is opposite regarding the gene expression of Znt6, with the difference that we examined the testicular tissue and they examined the hippocampal tissue. In addition, in our study, the level of zinc in the S-T group showed an increase compared to the control group, although it was not significant. This can be due to the anti-inflammatory effects of exercise, which cause the recruitment and migration of white cells, including macrophages. In this situation, macrophages are one of the most important and great sources of zinc. The results of our study also showed that the lowest level of expression of these transporters was observed in testicular tissue in the A-C group. The findings of this study disclosed a similar pattern for changes in the expression of zinc transporters in the testicular tissue in the S-T group compared to the control group. The expression levels of Zip7, Zip8, and Zip14 transporters were decreased in the S-T group compared to the S-C group and inversely increased in the A-T group compared to the A-C group. In fact, the comparison of the expression results of the three groups of Ajwain—the exercise group and the A-T group—shows that exercise can effectively influence the expression of these transporters to maintain zinc homeostasis. Conversely, the comparison of these results indicates that exercise training in two different nutritional conditions can produce different effects. When exercise is accompanied by Ajwain's supplementation, the expression of these transporters increases, and without supplementation, their expression decreases compared to the control group. This distinctive behavior actually showed the effects of complementary aid.

The investigations demonstrated that running downhill (eccentric contraction) resulted in a significant decrease in plasma zinc concentration 24 hours after the exercise session, and remained low at a lower level after 2 weeks of exercise. However, Zinc concentration in gastrocnemius muscle decreased significantly on the third day and did not show significant changes at other measurement times. Additionally, following eccentric contraction, gene expression studies of ZIP7, ZIP8, and ZIP14 revealed different expression patterns. ZIP7 showed a significant increase 12 hours and 1 week after the exercise session, whereas ZIP8 exhibited significant increases after 6 and 12 hours, as well as after 2 weeks. Moreover, gene expression analysis of ZIP14 indicated an immediate significant decrease after exercise, followed by a significant increase at 24 hours and 2 weeks post-exercise. These findings suggest that the expression patterns of these genes differ from each other and may influence cellular signaling pathways, replication, and differentiation processes through various routes [56]. Supplemental therapy with different doses of zinc carbonate showed that the diabetic group rats had almost doubled their food and water consumption, but the food consumption of the diabetic group that received the supplement was reduced compared to the normal diabetic group. The excretion of zinc through urine and feces in diabetic animals was significantly increased compared to the normal control group, but in the group treated with zinc, they were the same in the normal and diabetic groups due to the extra zinc intake, and the amount of zinc absorption in diabetic animals decreased. It was found that it had reached the normal state in the condition of zinc supplementation. The levels of zinc in plasma, liver, kidney, spleen, muscle, pancreas, and bone in the zinc supplement group increased in healthy groups but decreased significantly in the diabetic group. They investigated the gene expression of Znt5 in the heart tissue and observed that in healthy groups, the supplement increased the gene expression of the Znt5. But in the diabetic groups with normal food, it increased the expression of Znt5 gene, and the supplement in the diabetic groups caused a substantial decline in the

expression of Znt5, and they detected that the supplement in the diet in diabetic mice inhibited the excessive expression of Znt5 in the heart tissue [57]. In our research, the amount of Znt5 gene expression and the amount of zinc increased in the training groups, with the difference that we studied healthy rats supplemented with Ajwain aqueous extract and diabetic rats with different doses of zinc supplement.

A study on human samples and rats showed the relationship between zinc, ZnT8, and testosterone in Leydig cells: increasing zinc increased the gene expression of ZnT8, and testosterone. Also, the reduction of ZnT8 decreased testosterone and zinc levels, and they stated that ZnT8 is a protein that transports zinc to the Leydig cells in the testis and may have a role in testosterone production through the PKA signaling pathway [58]. Examining the metabolism of glucose and Zip7 in muscle showed that the decrease in the surface level in muscle cells increased the amount of glucose and caused the cells' sensitivity to insulin to decrease. In addition, increasing the amount of zinc in muscle cells reduced glucose in the cells and increased insulin sensitivity. Deletion or knocking of Zip7 caused an increase in glucose and decreased insulin sensitivity, and on the contrary, an increase in Zip7 gene expression resulted in a reduction in glucose and, as a result, a growth in insulin sensitivity. It is possible that Zip7 helps in glycemic control in muscle cells and a better understanding of glycemic control mechanisms. It means that Zip7 regulates glucose metabolism in skeletal muscles [27].

Researchers studied 35 obese women aged 18–28 years into 2 groups of placebo (n = 18) and supplementation (n = 17), 30 mg of zinc per day, for 8 weeks and observed that serum zinc concentration in the group that supplemented increased [59]. The result of this research is inconsistent with our research about supplementing in the Ajwain control group and congruent with the Ajwain training group. In another study, researchers divided 48 women aged 65 ± 7.8 years into 4 groups: the 1-zinc group (40 mg per day), the 2-ALA group (2000 mg Linum usitatissimum L seed oil per day), the 3-group the combination of zinc with Linum usitatissimum L seed oil, and the 4-placebo groups was divided, and the amount of zinc in the plasma was checked. It was observed that the amount of zinc was significantly increased in the groups of zinc and the group of zinc combination with Linum usitatissimum L seed oil (groups 2 and 3). But there was no difference between the Linum usitatissimum L seed oil group and the placebo group [60]. In our research, the amount of zinc declined in the ajwain-control group, but then it enhanced slightly in the ajwain-training group in comparison to the control group, although this increase was not significant, and these differences may be due to the different types of tissue studied.

In another study, the researchers examined three groups of people: the control group (people who died due to non-infectious reasons such as injury or sudden death, N = 10), the people who died due to COVID-19 (absence of testicular virus infection, N = 15), and the people who died due to COVID-19 (testes infected with virus, N = 9). It was observed that viral infection caused the activation of interferon alpha and gamma pathways and also caused a decrease in the expression of testicular-specific genes that play a role in spermatogenesis. They also observed that the gene expression of Znt9 was significantly decreased in both testes infected with the virus and in testes not infected with the virus, compared to the control group [61]. Previous studies have shown that the expression of zinc transporter genes can be influenced by exercise and nutrition. For example, a study investigated the effects of aerobic exercise with pumpkin seed oil and white pea extract supplementation on the gene expression of zinc transporters in the liver of healthy male rats and observed that the gene expression of Znt5 and Zip14 in the liver tissue of rats did not show any significant alterations, but the gene expression of Znt9 in the pumpkin oil training group was significantly enhanced in comparison with the control and chickpea training groups. They also observed that the content of zinc in the serum was not significantly different, but the amount of zinc in the liver tissue of the training group—pumpkin oil—was significantly increased compared to the training group—pea extract [33]. In our study, both Znt5 and Znt9 were significantly different, but contrary to the results of these researchers, the changes in Zip14 were increased in our study in the Ajwain-training group, while this increase was not considered significant statistically. In our study, the changes in zinc content

also showed that the amount of zinc increased in the exercise groups compared to the control groups, but it was not statistically significant. The difference in results could be due to changes in zinc transport caused by co-transport with ions such as calcium. Zinc ions are able to pass the biologic membrane via several calcium canals; ZnT and ZIP transporter family proteins have an important role as pathways of transportation [62]. Zip family increases the cytoplasmic levels of Zn by bringing Zn into the cell, and ZnT family decreases the cytoplasmic levels of Zn [39].

Various studies have shown that the nutrients and main components of Ajwain extract can have several nutritional and pharmacological effects, including improving metabolic, anti-inflammatory, and antioxidant status. In addition, the results of the atomic absorption analysis of Ajwain extract in this study showed that this plant product is a great source of micronutrients such as iron, zinc, manganese, magnesium, and copper, which can play a important role in the challenge of these elements homeostasis that exercise induces. As we know, the change in the homeostasis of the cell is costly for it, and it is associated with changes in the energy levels and the proteins that are involved in it. These changes are mainly associated with an increase in the need for elements such as zinc and iron. The main cellular sources available for these elements are secretory vesicles connected to the Golgi apparatus, endoplasmic reticulum, and mitochondria. On the other hand, the urgent need for zinc in the secretory system, which is considered the main function of the cytoplasm, increases in this condition. Providing a sufficient amount of zinc in this case is the function of Zips and Znts transporters. This can be a justification for increasing the expression of these transporters in response to doing sports exercises and supplementing Ajwain aid. In addition, as we mentioned, exercise is linked with variations in the state of inflammation, which can cause changes in the content of immune cells in the surrounding tissues. In addition to secretory activity, these cells are rich reservoirs of micronutrients such as zinc.

The changes of Znt6 in the supplement groups of Ajwain and saline groups were similar, so performing sports exercises both in the saline training group compared to the saline control and in the Ajwain-training group compared to the Ajwain-control group caused a decrease in the expression of Znt6. However, this decrease was not statistically significant. It seems that this zinc transporter does not play a significant role in this tissue, or that the necessary and sufficient stimulation through these factors to change their expression might not have been sufficient. In addition, the gene expressions of Znt8 and Znt9 were significantly increased in the Ajwain-training group as opposed to the saline-training group compared to the control group. The patterns of their changes were similar to those of Zip 7, Zip 8, and Zip 14. They were increased in the Ajwain-training groups compared to the Ajwain-control group, as opposed to the saline-training groups, although this increase was not statistically significant. The changes in zinc and iron levels were similar, and in the training groups, they increased compared to the control groups, although this increase was not statistically significant. These results indicate the activation of homeostasis maintenance mechanisms in this tissue. Transferrin is a protein that plays a major role in transporting iron to cells, and ferritin acts as an essential source of iron for its absorption and storage [63]. In the male reproductive system, Sertoli and Leydig cells are important sources of ferritin. Ferritin acts as a source of iron available to growing sperm while providing a protective layer for the testicular tissue [64]. Iron plays an important role in the synthesis of nucleic acids and proteins, electron transport, cellular respiration, proliferation, and differentiation [63]. All of these factors affect the spermatogenesis and sperm metabolism [65]. It seems that exercise and the consumption of Ajwain extract can maintain iron homeostasis through the proteins involved in its absorption and storage. Another important finding of this research was that the amount of glycogen in the saline-training and Ajwain-control groups had increased compared to the saline-control group; although this increase was not statistically significant, in the Ajwain-training group it decreased to baseline levels. The results in the Ajwain-control group showed that glycogen content was increased. This result can be due to the involvement of effective Ajwain substances in glucose metabolism and glycogen overcompensation mechanisms. These results indicated the provision of sufficient glycogen resources for exercise.

CONCLUSIONS

Zinc and iron are essential minerals that play important roles in testicular health and fertility. The expression of zinc transporter genes and the levels of zinc, iron, and glycogen in testicular tissue can be affected by various factors, including exercise and nutrition. Based on these results, we can conclude that physical activity combined with supplementation with an aqueous extract of ajwain seeds can increase the expression of zinc transporter genes in testicular tissue. This may provide an appropriate compensatory response for the availability of glycogen, iron, and zinc. These findings suggest that healthcare professionals, with a better understanding, can prescribe exercise and herbal interventions in a more targeted and effective way.

Conflict of interests

The authors have declared that there is no interest collision.

ETHICAL CONSIDERATION

Compliance with ethical guidelines: This study followed the ethical standards and was approved by the University of Mazandaran Ethics Committee (IR.UMZ.REC.1401.071).

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CRediT authorship contribution statement

All authors contributed to the study design. Araz Nazari and Khadijeh Nasiri collected the data. Abbas Ghanbari Niaki revised the final version of the manuscript. All authors read and approved the final manuscript.

Data availability

Data will be made available on request.

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