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Combination of vitamin E with L-carnitine increase CatSper Genes expression in the aging mouse testis

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ABSTRACT

CatSper genes (1-4) are four identified channel-like proteins, which are expressed exclusively in the testis and play a crucial role in sperm motility, and male fertility. In spite of their crucial role in male reproduction, there is little data to evaluate the factors regulating their expression. Hence, the aim of this study was to investigate the effects of vitamin E and L-carnitine therapy on CatSper genes expression and sperm quality in the aging mice. Twenty-four 11 to 12-months and twenty-four 2 to 3-months old male mice were randomly divided into four groups: (a) Young control; (b) Young treated with vitamin E and carnitine (106 mg/kg α-tocopheryl acetate plus 50 mg/kg Lcarnitine) ; (c) Aged control; and (d) Aged treated with vitamin E and carnitine (106 mg/kg α-tocopheryl acetate plus 50 mg/kg L-carnitine). Treatment was administered intra-peritoneally for 5 weeks. The expression of CatSper1 and CatSper 2 were determined by real-time polymerase chain reaction in mouse testis. Cauda epididymis was kept for the sperm analysis. Data were analyzed using SPSS software. Our results indicated that the expression of CatSper 1 and CatSper 2 genes increased in the experimental groups compared to the control ones. Besides, sperm parameters improved especially in terms of sperm count and motility in the experimental groups. Treatment with vitamin E and L-carnitine increases CatSper genes expression in testis as well as sperm parameters.

Key words: CatSper , Sperm, Testis, Mice, Aging , L-carnitine, Vitamin E

INTRODUCTION

Aging in the male reproductive tract is accompanied with degenerative changes in the germinal epithelium, decrease in spermatozoal motility and gene expression suggests that oxidative stress is correlated with the aging process [1, 2]. An extensive generation of reactive oxygen species (ROS) in seminal plasma results in oxidative stress [3]. Sperms are equipped with a number of antioxidant molecules such as glutathione peroxidase, catalase, superoxide dismutase, vitamin E, and carnitine that protect sperms from oxidative damage [3]. Minerals and vitamins are

necessary for function of reproductive system [4]. Vitamin E is an intra-cellular antioxidant that can protect from oxidative damage by breaking lipid soluble antioxidant. Deficiency of vitamin E resulted in the deleterious effects in the seminiferous tubules and a decrease in sperm motility [5,6]. Oral administration of vitamin E reduced malondialdehyde (MDA) concentration in sperm and significantly increased sperm motility in infertile subjects [7]. Supplementation with L- carnitine, a water-soluble antioxidant, could improve sperm motility in infertile men [8,9]. The level of carnitine in tissues decrease with age and administration of carnitine can reverse dysfunctions in the aging subjects [10]. Our previous study showed that selenium affected on CatSper gene expression and sperm quality [11]. CatSper 1-4 are a unique family of sperm specific cation channel that are exclusively expressed in the spermatozoa and required for sperm mobility [12]. In the present study it was hypothesized that a combination of vitamin E and L-carnitine may increase the expression of CatSper genes and sperm motility in aging male mice. Therefore, we investigated changes in CatSper genes expression due to vitamin E plus carnitine treatment in 11-12 months aging and 2-3 months young male mice. Furthermore, we analyzed sperm parameters following vitamin E and L-carnitine administration in the aged and the young male mice.

MATERIALS AND METHODS

Chemical

Vitamin E (α-Tocopherol acetate) and L- Carnitine were purchased from Sigma-Aldrich chemical Co (St. Louis, MO, USA).

Animals

Twenty-four old (11-12 months) male BALB/c mice and twenty -four young old adult male (2-3 months) mice were purchased from the Animal Center of Mashhad University Medical Faculty. The mice were maintained under controlled conditions (12 h light, 12 h dark at 21°C to 23° C) and had free access to the drinking water and standard pellet diet. All animal experiments were approved by the Ethical Committee of Mashhad University of Medical Science.

Study design

The animals were randomly divided into four groups (control group 1: young adult male treated without supplementation, control group 2: aging male treated without supplementation, Experimental group 1: young adult male treated with vitamin E and carnitine; and Experimental group 2: aging male treated with vitamin E and carnitine. The experimental groups were received intraperitoneally 106 mg/kg all-rac-a tocopheryl acetate plus 50 mg/kg L-carnitine per day for 5 weeks. The mice were quickly sacrificed by cervical dislocation at the 3th, 4th and 5th weeks after the treatment. Left testis and epididymis were collected from each group. The left testis from each mouse stored at -80°C for RNA extraction. Sperms were acquired from epididymis and analyzed according to WHO guidelines.

Sperm quality

Sperm quality was performed according to guidelines of World Health Organization given for human sperm examination [13]. The left cauda epididymis was immediately placed in a petri dish contain 1 ml of phosphate buffer saline. After 15 min incubation in a CO2 incubator (5%), a drop of cell suspension was placed on a Neubauer chamber under a cover slip. The percentage of motile sperm determined at least 10 fields were viewed at 400× magnification. Sperm count was analyzed using a hemocytometer according to WHO protocol and the results were expressed as number of sperm per milliliter. A 50-µl drop of the sperm sample was assessed using an eosin-nigrosine staining mixture to obtain morphological abnormalities. To assess sperm viability, sperm suspension was mixed with 1% Eosin. The viable sperm which appeared red, and the nonviable ones which remained colorless, were examined under the light microscope. One hundred sperms were counted to obtain the percentage of the viable sperm.

RNA isolation and cDNA synthesis

Total RNA from the testis was extracted as described [11]. One micrograms of total RNA were reverse transcribed into first strand complementary DNA using the cDNA Synthesis Kit RevertAid (Fermentas Corporation, Germany) , according to the manufacturer′s instructions [11].

Real time PCR

Real-time PCR was performed to determine gene expression. All samples were examined in duplicate, and each reported data set consisted of at least 4 separate experiments. The primer sets are presented in Table 1. PCR master mix contains 2 µl of cDNA, 12.5 µl SYBR Green PCR Master Mix, 0.6 µM of each gene-specific primer, and 9.3 µl of water. The PCR program was: 95°C for 10 min followed by 40 cycles (95°C for 25 s, 60°C for 30 s and 72°C for 30 s). ß-Actin was used as an internal control for each sample to determine the relative expression quantity of the target genes. Before using the comparative threshold cycle method, standard curves were obtained for each primer pair with serial dilution of cDNA. The efficiency of primers was calculated using the standard curve analysis. Real-time data were calculated using the ratio formula (Ratio= $2^{-\Delta\Delta Ct}$), where ΔΔCt = ΔCT (control sample) - ΔCT (target sample) [14].

Table 1. Primers used for Real-time PCR

Gene	Gene bank	Primer sequences	Product size (bp)	Annealing temperature (°C)	References
CatSper1	AF407332	5'-ACC AGG TTG AGG AAG ATG AAG T-3' (R) 5'-TTT ACC TGC CTC TTC CTC TTC T-3' (F)	227	60	Li et al ,2007
CatSper2	AF411816	5'-ACC AAT GAT CCA AGG TGA AGA-3' (R) 5'-GGG TGC TGA GGT CTC TCA AAC-3'(F)	261	60	Li et al ,2007
B-Actin	NM_007393.1	5'-TCA GGA GGA GCA ATG ATC TTG-3'(R) 5'-GGG AAA TCG TGC GTG ACA T-3'(F)	385	60	Li et al ,2007

F: forward, R: reverse

Statistical analysis

Analyses were carried out using the SPSS version 21.0 software. The significance of differences was determined using one-way analysis of variance (ANOVA) followed by Duncan test. A *P*-value less than 0.05 was considered as the statistical significance.

RESULTS

Effects of Vitamin E and L-carnitine on sperm parameters in the aged and the young male mice

The results of the analysis of variance on sperm parameters are presented in Table 2. There was a significant difference in sperm parameters between the aged and young control group (P <0.05). Our results showed a significant improvement in the sperm quality of mice treated with vitamin and carnitine. Treatment with vitamin E and carnitine caused a significant increase in the percentage of sperm motility and sperm count as compared to those of control group, but only the experimental group 2 did not show a statistically significant increase on the day 35. The morphology rate was increased in the vitamin plus carnitine- treated mice when compared to the control group, but there was a statistically significant difference only for the experimental group 2 on the day 35. Morphologically abnormal spermatozoa were as an example vacuolated head, bent tail, and coiled tail. There were no significant changes between the vitamin plus carnitine-treated mice and the control mice for the sperm viability, but the vitamin plus carnitine-treated mice had significantly a sperm count than the control mice (P < 0.05; Table 2).

Effects of Vitamin E and L-carnitine on CatSper genes expression in the aged and the young male mice

The values of CatSper genes expression in testis of mice in all groups are presented in Figure 1, 2. A significant up-regulation of CatSper 1 was detected in the experimental group 1 compared with the control group 1 on the days 21 and 28. The expression of CatSper 1 decreased on the day 35. Similar results were obtained for CatSper 1 expression in the experimental group 2 (Figure 1). The relative expression of CatSper 2 was 3.13- fold higher in the experimental group 1 than in control group 1 (3.13 ± 0.48 vs. control group, p < 0.01) , and then reduced on the day 28 and again increased on the day 35. The relative intensity of CatSper 2 increased on the 21 day (3.50- fold), and then decreased gradually till the day 35 (Figure 2).

Table 2- Results of analysis variance on sperm parameters of control and experimental mice

Parameters	Aging mice		Young mice		P value*
	Control group1	Exp group1	Control group1	Exp group2	
Day 21					
Sperm Count (10^6/mL)	3.80±0.11^{a}	4.45±0.12^{bc}	4.40±0.18^{b}	4.87±0.06^{d}	<0.001
Motile sperm (%)	42.00±3.16^{a}	51.75±3.09^{b}	77.75±2.06^{c}	88.00±2.94^{d}	<0.001
Viable sperm (%)	70.25±4.50^{a}	70.00±7.07^{a}	83.75±6.40^{a}	84.00±3.83^{a}	<0.001
Normal sperm (%)	70.50±3.32^{a}	73.75±5.50^{ab}	81.00±2.71^{bc}	84.25±3.77^{c}	0.001
Day 28					
Sperm Count (10^6/mL)	3.87±0.14^{a}	4.53±0.07^{c}	4.35±0.23^{c}	4.93±0.08^{d}	<0.001
Motile sperm (%)	40.50±4.12^{a}	48.00±1.41^{b}	79.00±2.94^{c}	89.00±1.82^{d}	<0.001
Viable sperm (%)	71.50±5.07^{a}	71.50±5.97^{a}	82.75±5.50^{b}	82.25±2.06^{b}	<0.001
Normal sperm (%)	70.00±4.08^{a}	75.00±5.60^{ab}	79.25±0.96^{bc}	86.00±3.64^{c}	<0.001
Day 35					
Sperm Count (10^6/mL)	3.75±0.17^{a}	4.50±0.08^{abc}	4.30±0.29^{b}	5.02±0.15^{c}	0.002
Motile sperm (%)	39.50±0.58^{a}	49.75±3.30^{b}	78.25±3.95^{c}	81.75±2.06^{c}	<0.001
Viable sperm (%)	71.75±3.59^{a}	71.50±3.79^{a}	84.00±5.10^{b}	83.00±2.58^{b}	<0.001
Normal sperm (%)	72.25±4.50^{ab}	73.75±5.50^{bc}	78.00±6.48^{bc}	86.50±1.29^{d}	<0.001

Values are expressed as Mean± SD n=12 mice per group. Data were analyzed by ANOVA test (p>0.05). Mean with different superscripts are significantly different within row at P<0.05 by Duncan post hoc test. Exp: experimental

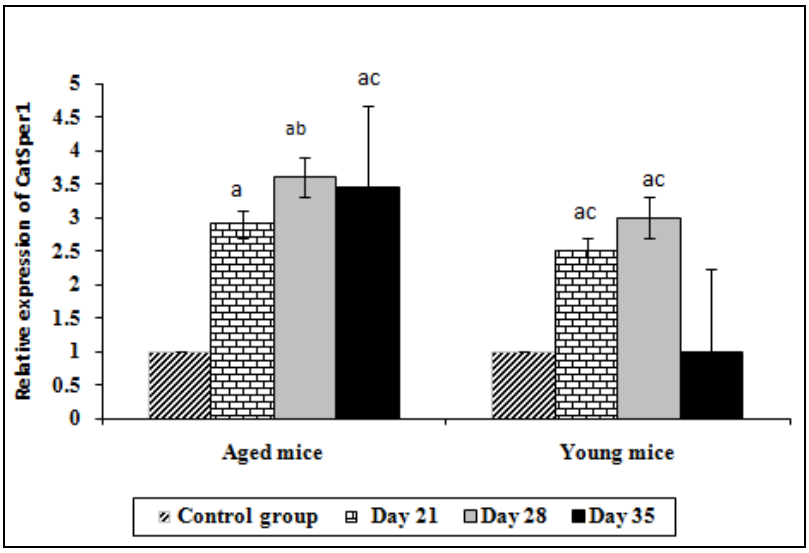

Figure 1. Histogram comparing the relative expression of CatSper 1 gene to ß-actin gene expression in aged and young mice
values are presented as the mean± SD. (n=12 mice every group)
[a] P<0.05 compared to control group
[b] P<0.05 compared to experimental group 2 (28 st day)
[c] P<0.05 compared to experimental group 2 (35 st day)

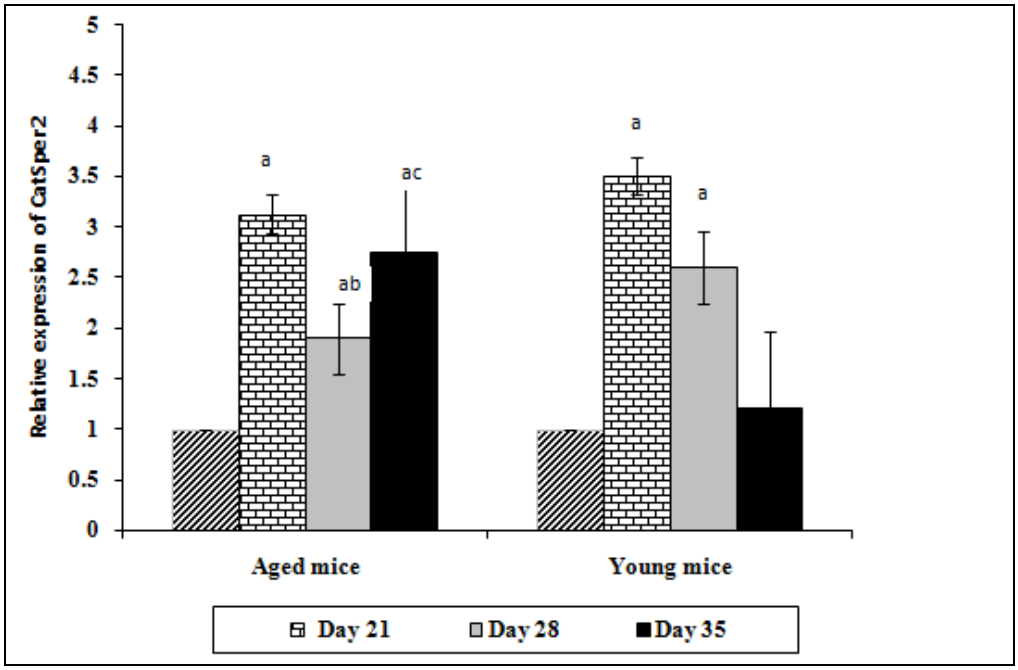

Figure 2. comparing the relative expression of CatSper 2 gene to ß-actin gene expression in aged and young mice
values are presented as the mean± SD. (n=12 mice every group)
[a] P<0.05 compared to control group
[b] P<0.05 compared to experimental group 2 (28 st day)
[c] P<0.05 compared to experimental group 2 (35 st day)

DISCUSSION

The present study used Real-time PCR and sperm analysis to evaluate the effects of combined vitamin E and L-carnitine on the expression of CatSper mRNA and sperm quality in the testis of the young and aged mice.

In a double- blind crossover trial by Lenzi et al. [5] infertile males received 3 g carnitine for 4 months. Carnitine supplementation significantly improved sperm count and sperm motility in these patients. Cavallini and coworkers also showed that supplementation with 2g/day carnitine remarkably improved sexual dysfunction in men aged 60-74 years [15]. In another study, Carnitine deficiency caused an obstructive azoospermia, which restored after L-carnitine treatment [16].

Suleiman et al. demonstrated that vitamin E treatment caused improvement in sperm motility and decline in MDA level in the a steno or oligoasthenospermic patients [7]. Besides, our previous results also indicated that selenium treatment could improve viability, morphology rates and total antioxidant capacity of the spermatozoa in the aging mice [17,18]. On the other hand, dietary deficiency of vitamin E leads to deleterious changes on the male reproductive tract, such as histological alternations in seminiferous tubules and degenerative spermatogonium [5]. In agreement with these reports, in the present study sperm parameters increased in mice treated with vitamin E and carnitine. With regard to low levels of antioxidants in aging mice, it can be postulated that the to xidative stress can cause a reduction in antioxidant levels. Hence antioxidant therapy could be considered as a method for improvement of the sperm parameters of aging subjects.

There is little knowledge available to investigate the effects of antioxidant on the expression of CatSper genes family and the regulation of CatSper genes factors. Previous studies have demonstrated that antioxidants could alter gene expression [11]. The results of our previous study showed a significant up-regulation of CatSper genes of mice treated with selenium. However, administration of 0.2 mg/kg selenium in the 11-13 months old aging mice showed more significantly increase than that in the young male mice. Selenium treatment increased sperm quality in the aged mice by up-regulating CatSper genes family. In line with this study, we have found that treatment with a combination of vitamin E and L-carnitine can improve sperm parameters in the aging subjects. Treatment with

vitamin E and carnitine caused more profound effect on the expression of CatSper 1 and CatSper 2 genes in the aged mice. Besides, a significant up-regulation was detected in both the aged and the young mice treated with L-carnitine on the days 21 and 28, which were inconsistent with the results of our previous study. Inconsistently, the relative expression of CatSper genes peaked in both of the aged and young mice treated with vitamin plus carnitine on the days 21 and 28 of experimental period, whereas in the aged mice treated with selenium the highest expression detected on the days 21 and 28, in the young mice peak expression occurred on the days 35 and 42 .

Mutation of CatSper1 and CatSper 2 were found in the patients with male infertility [19, 20]. Wang et al. also reported down-regulation of CatSper 1, which is related to an impairment of sperm motility in a mouse- model of asthenospermia [21]. However, a reduction in CatSper 1 gene expression as well as decrease in sperm motility was restored after administration of 6o mg/kg Sheng-Jing-San. Sheng-Jing-San, a Chinese medicine recipe, contains a variety of trace elements such as Se, Cu, Ca, Zn, Fe, and Mn [21]. Similar to this result, we found that the expressions of CatSper genes and sperm parameters dramatically were affected by dietary treatment.

Lee et al. showed that in vitamin-A- deficient rats, the arrest of spermatogenesis resumed after injection of 7.5 mg retinol/head over period of 70 days [22]. Besides, retinol treatment caused up-regulation of cytochrome P450, and sulfated glycoprotein 2 on days 9-15. The expression level of protein kinase inhibitor was increased on days 0, 20-25, and 35-43, whereas a decrease in expression of Cath L was found on days 20-43 in the rat testis [22]. In another study, Gan et al. demonstrated that intraperitoneal injection of 0.02 mg /kg selenium significantly increased thioredoxin reductase (TR) and gluthatione peroxidase (GSH-Px) expression in the testis and liver of selenium-deficient model [23]. However, TR and GSH-Px mRNA level in the testis and liver decreased for rats received 40 and 80 mg/kg selenium over period of 15 days. Therefore, Gan and colleagues was recommended that appropriate dose of selenium, as an antioxidant, can up-regulate antioxidant defense by raising TR and GSH-px activities. These results are agreement with our study that an adequate amount of the antioxidants can increase gene expression in the mouse testis. However, we need to do more molecular studies to confirm our results.

CONCLUSION

Our finding indicated that administration of 106 mg/kg vitamin E and 50 mg/kg L-carnitine increase sperm parameters and CatSper expression in the aged mice. It seems a combination of antioxidants may be useful for improvement of sperm parameters in aging subjects; and hence may contribute to the treatment of the aging men.

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