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Effects of L-carnitine treatment on expression of CatSper proteins in the aging mouse model

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ABSTRACT

The aim of this study was to investigate the effect of L-carnitine therapy on CatSper 1 and CatSper 2 proteins expression in the aged male mice. In spite of the important role of CatSper genes in male fertility, little data is available about the factors regulating their expression. CatSper1-4, four unique sperm cation channels, were identified to be exclusively expressed in the sperm and required for sperm motility and male fertility. A total of 48 aged (11-12 months) and young (2-3 months) mice were randomly divided into four groups: (1) aging control; (2) aging treated; (3) Young control; and (4) Young treated. Control groups did not receive any injection. The treated groups were received intra-peritoneally 50 mg/kg L-carnitine daily for 35 days. Immunohistochemistry staining was performed on days 21, 28 and 35 after carnitine treatment. Sperms were analyzed based on the WHO guidelines given for human sperm examination. Data analysis was carried out using the SPSS software version 21. Carnitine treatment caused a remarkable increase in CatSper1 and CatSper2 proteins expression. Besides, immunohistochemistry staining detected CatSper proteins in the head and the sperm flagellum. Carnitine therapy increased CatSper proteins expression in the old and young mice.

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

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INTRODUCTION

Calcium channels play an important role in the regulation of sperm cells functions [1]. Many calcium channels have been found in spermatozoa, including voltage-sensitive Ca^{+2}- selective channels, cyclic nucleotide-gated channels and transient receptor potential channels [2].

CatSper1-4 and two additional subunits, CatSper γ and Cat-Sper ß are a novel family of sperm calcium channels that are merely expressed in the spermatozoa [3-5]. CatSper1 was first discovered by Ren and colleagues [6] that are required for sperm motility and male fertility [2, 7]. A high degree of conservation was observed in CatSper amino

sequences in both human and mice [8]. Two further members of CatSper genes family, CatSper3 and CatSpr4, were discovered using in silico gene idenification [3]. Deletion of the CatSper3 and CatSper4 in mouse revealed their essential role in sperm hyperactivation motility and fertility [2, 9].

Degenerative changes in the somniferous tubules, the decrease in gene expression and sperm motility suggest that oxidative stress is related to the aging process [10]. Besides, it has been proposed that an imbalance between oxidant and antioxidant lead to aging [10]. Semen contains antioxidants, such as vitamin E, vitamin C, catalase, glutathione peroxidase, superoxide dismutase, pyruvate, and carnitine that protect sperm cells from oxidative stress [11]. Minerals and vitamins are necessary for function of reproductive system [12]. Carnitine is well known as a water-soluble antioxidant that highly concentrated in the testis. L-carnitine plays a critical role in sperm motility, maturation and protecting spermatozoa against free radicals [13, 14]. Carnitine deficiency let to epididymal dysfunctions and infertility [15]. However, an obstructive azoospermia and infertility was restored after treatment with L-carnitine. Besides, infertile men have lower level of L-carnitine in semen than fertile subjects and L-carnitine treatment could increase sperm motility [16, 17].

Several studies have been showed a direct relationship between sperm motility and male fertility as well as a reduction in sperm quality with increasing age [18]. To our knowledge we evaluate the effects of carnitine treatment on CatSper proteins expression for the first time; hence, we designed this study to investigate the effects of L-carnitine supplementation on expression of CatSper proteins in the aged and young male mice.

MATERIALS AND METHODS

Chemicals

L- Carnitine was purchased from Sigma-Aldrich Company.

Study design

The experimental protocol was approved by the Animal Ethics Committee of Mashhad University of Medical Science. A total of forty-eight 11-12 months old aging male BALB/c mice and 2-3 months old young adult male mice were used from experiments. The mice were maintained under standard conditions. The animal were randomly divided into four groups of twelve each: an aged control group (group 1), an aged group with carnitine treatment (group 2), a young control (group 3), and a young with carnitine treatment (group 4). Control groups did not receive any supplementation. The group3 and group 4 were received intra-peritoneally 50 mg/kg/day L-Carnitine for 35 days. Mice were rapidly sacrificed by cervical dislocation, and the sperm suspension was acquired from the left cauda epididymis.

Immunohistochemistry study

Sperms acquired from the Cauda epididymis were placed on slides, air dried and fixed with ice-cold methanol [19, 20] . The slides were antigen retrieved in Tris-EDTA at 95° C for 17 minutes and then incubated with 3% H_2O_2 to block the endogenous peroxidase activity. After being rinsed twice in PBS, the slides were blocked with 1% BSA (Gibco U.K) and incubated (overnight) at 4° C with mouse polyclonal CatSper1 and CatSper 2 antibodies (diluted 1:100; Santa Cruz, USA) and then with a secondary antibody (2 hour, 37° C, 1:100, horse-radish peroxidase anti-goat, Serotec, Germany). After being washed twice in PBS, the immunoreactivity was visualized by DAB (3',3' diaminobenzidine) staining. Then the slides were gradually dehydrated through the graded ethanol, immersed in xylene and mounted with Entellan (Merck, Germany). The control slides, except the primary antibody, which was replaced by PBS, were under similar conditions. The immunohistochemical results were observed by a light microscope. The intensity staining was graded as weak, +; moderate, ++; strong, +++; highly strong, ++++ [21].

Statistical analysis

Statistics analysis was performed using SPSS version 21.0 and ANOVA. Post-hoc, Duncan test were used after using analysis of variance. $P<0.05$ was considered significant.

RESULTS

Effects of L-carnitine treatment on expression of CatSper 1 protein in the aged and the young male mice

To investigate the effect of carnitine treatment on the distribution of CatSper in the mouse sperm, immunohistochemistry was performed. CatSper1 was detectable throughout the middle piece, the principal piece,

and head of mouse sperm (Figure 1). Similar results were observed for CatSper2 protein. The intensity of CatSper staining in the mouse sperm was quantified as demonstrate in method. As carnitine treatment progressed from days 21 to days 28, intensity of signal for CatSper1 in the ACT group increased in the middle piece but was low on the day 35. At Day 21 of carnitine treatment, staining intensity for CatSper1 in the YCT group increased in the middle piece but staining was weak on the day 28 and thereafter (Figure 2).

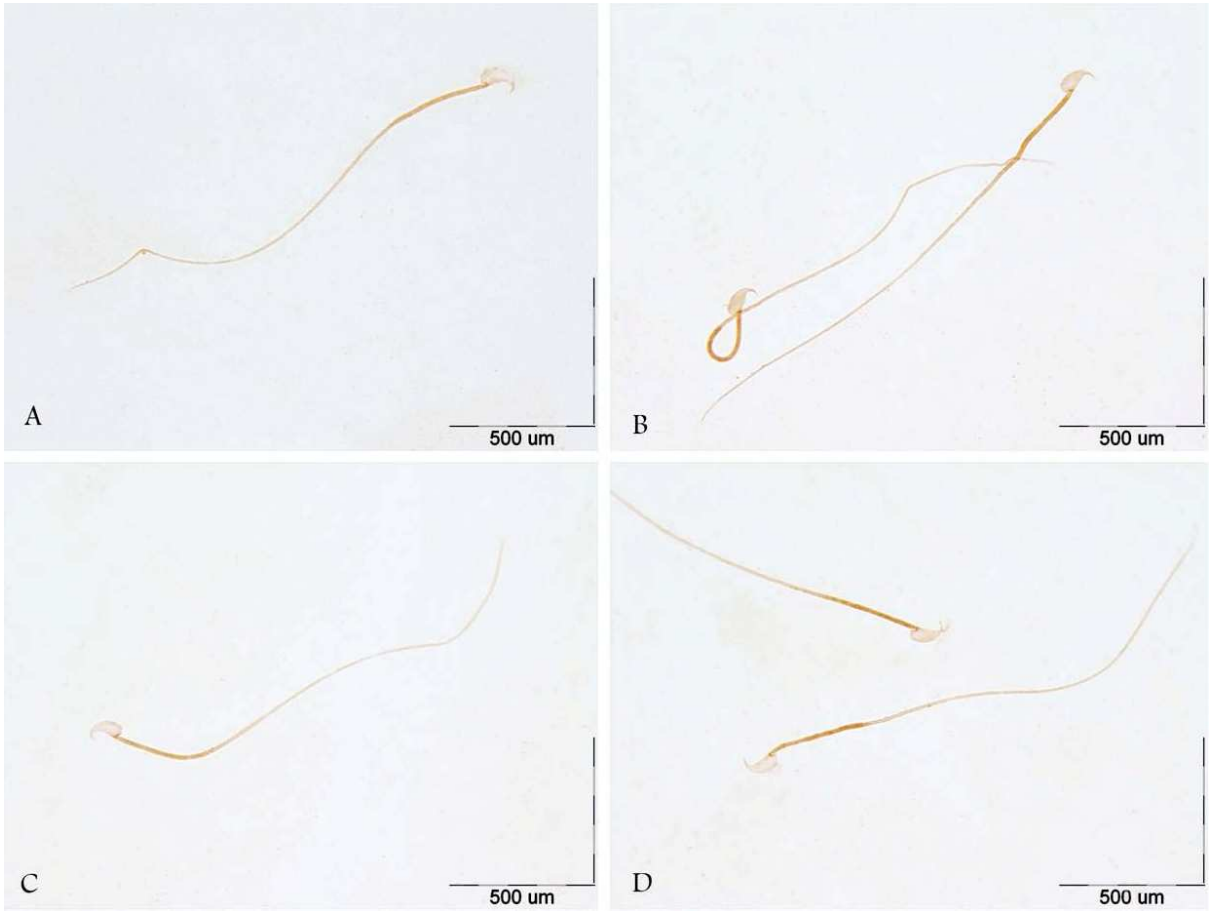

Figure 1. (A-C) Staining intensity of CatSper 1 and CatSper2 proteins by immunohistochemistry in the head, middle piece and principal piece of sperm in the aged and young mice without carnitine treatment (B-D)

Staining intensity of CatSper 1 and CatSper2 proteins in the head, middle piece and principal piece of sperm in the aged and young mice following carnitine treatment. In all slides, the immunoreactivity was visualized by DAB staining.

Effects of L-carnitine treatment on expression of CatSper 2 protein in the aged and the young male mice

A moderate expression of CatSper2 was found in the head, and principal piece of sperm in the ACT group on the day 28 (Figure 3) whereas a strong CatSper2 immunoreactivity was detected in the middle piece of sperm tail of the ACT group on the day 28. A moderate signal was observed in the head, middle piece and principal piece of sperm on the days 21 and 35 of the YCT group as compared to that of the control group (Figure 3). No immunoreactivity was observed in control slides in response to incubation with phosphate buffer saline instead of primary antibody (Figure 1).

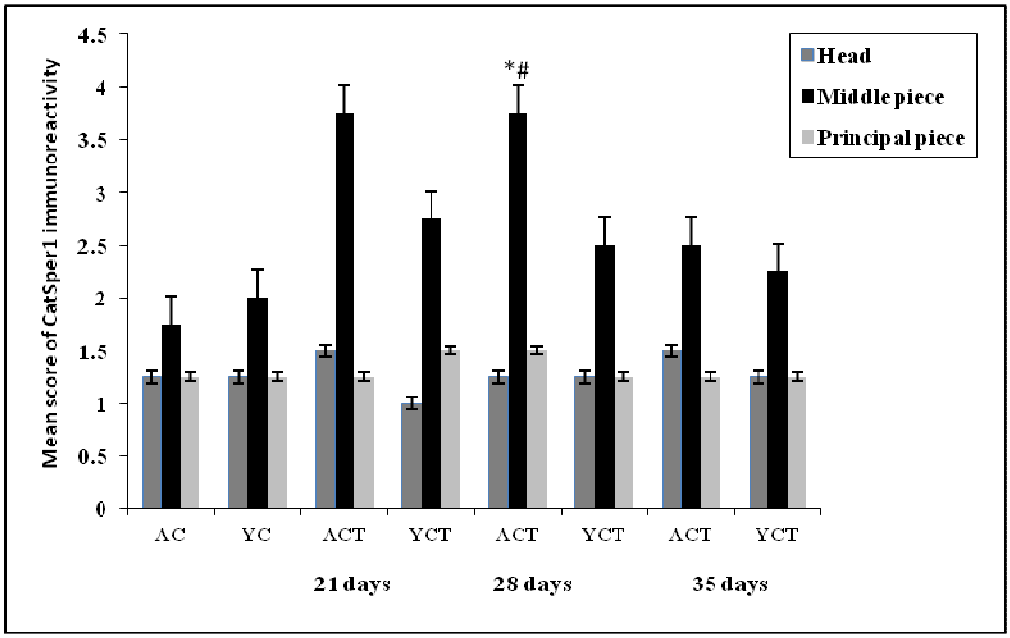

Figure 2. Effect of L-carnitine administration on expression of Catsper1 protein in mice testes measured on the days 21, 28 and 35. Values represent the mean ± SD (n=12 for each group). ACT: Aged with carnitine treatment, YCT: Young with carnitine treatment
**P<0.05 compared to control group*
 # P<0.05 compared to aged carnitine treatment (35 st day)

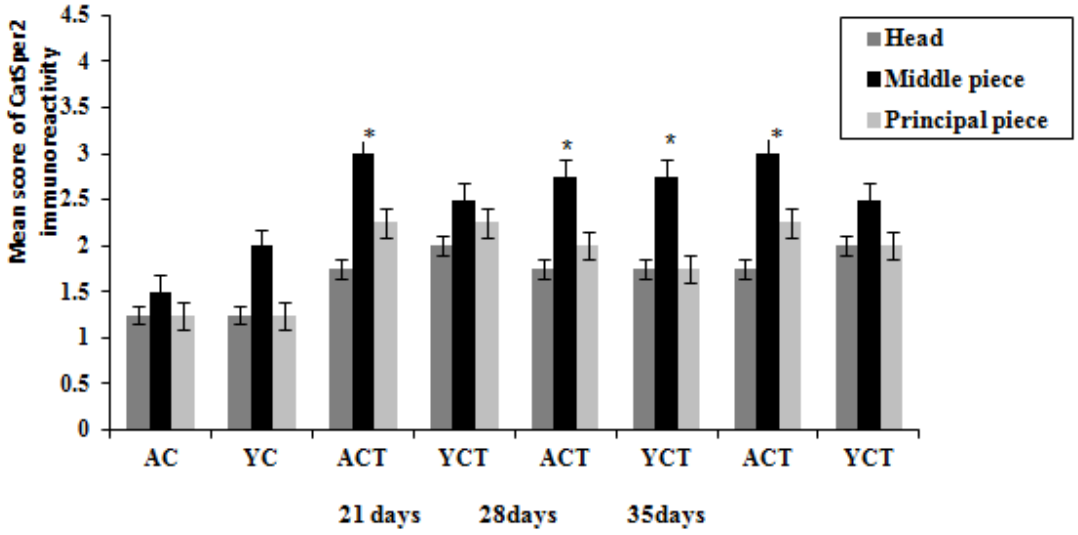

Figure 3. Effect of L-carnitine administration on expression of Catsper1 protein in mice testes measured on the days 21, 28 and 35. Values represent the mean ± SD (n=12 for each group). ACT: Aged with carnitine treatment, YCT: Young with carnitine treatment.
**P<0.05 compared to control group*

DISCUSSION

Our results showed the localization of CatSper proteins to the head and the intermediate piece and principal piece of sperm. Some studies demonstrated that CatSper proteins were localized only in the principal piece of sperm tail [6, 8, 22, 23] whereas, CatSper proteins were also detected in the mid-piece and head of sperm [6, 23]. According to their resembling structure, four CatSper proteins were predicted to constitute a channel in sperm [3] but the different localizations between the CatSper genes family verified the hypothesis that CatSper 1 and CatSper 2 are probably

responsible for the separate channels of CatSper 3 and CatSper 4 [24]. The results of immunohistochemistry in the present study also support this hypothesis.

We also investigated the expression of CatSper 1 and CatSper2 proteins following carnitine treatment. In the aged and young mice, the expression of CatSper1 protein is upregulated on the day 21. While in the aged mice the expression remains high until day 28, it decreases in the young mice and then shows a decreasing trend. In addition, in the aged mice, the expression of CatSper2 reaches to a high level on the days 21 and 35, whereas in the young ones it occurs on the day 28. The expression of CatSper1 and CatSper2 is higher in the aged mice than in the young mice. The immunoreactivity of CatSper 1 and CatSper2 increased until the day 28 and then dropped to a low status.

CONCLUSION

Our results showed that the administration of 50 mg/kg L-carnitine increases CatSper1 and CatSper 2 proteins expression in mice testis; hence, it seems that L-carnitine may be useful for treatment of aging subjects and oligoasthenospermia.

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