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Milk fat globule membrane supplementation with voluntary running exercise attenuates age-related motor dysfunction by suppressing neuromuscular junction abnormalities in mice

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ABSTRACT

Age-related loss of skeletal muscle mass and function attenuates physical performance, and maintaining fine muscle innervation is known to play an important role in its prevention. We had previously shown that consumption of milk fat globule membrane (MFGM) with habitual exercise improves the muscle mass and motor function in humans and mice. Improvement of neuromuscular junction (NMJ) was suggested as one of the mechanisms underlying these effects. In this study, we evaluated the effect of MFGM intake combined with voluntary running (MFGM-VR) on morphological changes of NMJ and motor function in aging mice. Seven months following the intervention, the MFGM-VR group showed a significantly improved motor coordination in the rotarod test and muscle force in the grip strength test compared with the control group at 13 and 14 months of age, respectively. In 14-month old control mice, the extensor digitorum longus muscle showed increased abnormal NMJs, such as fragmentation and denervation, compared with 6-month old young mice. However, such age-related deteriorations of NMJs were significantly suppressed in the MFGM-VR group. Increase in the expression of NMJ formation-related genes, such as agrin and LDL Receptor Related Protein 4 (LRP4), might contribute to this beneficial effect. Rotarod performance and grip strength showed significant negative correlation with the status of denervation and fragmentation of NMJs. These results suggest that MFGM intake with voluntary running exercise effectively suppresses age-related morphological deterioration of NMJ, thus contributing to improvement of motor function.

1. Introduction

Along with the increase in life expectancy, healthy aging has become a major concern for people worldwide. Age-related physical impairments mainly caused by loss of skeletal muscle mass and function (Breen and Phillips, 2011) have a significant impact on the quality of life in the elderly by interfering with performing daily activities and a good mental health. The decrease in physical performance including motor coordination is also closely related to increased risk of fall-related injuries and deaths, thus becoming a major problem worldwide (Tinetti, 2003). Therefore, sustaining motor functions by maintaining muscles and their fine control is one of the key factors for healthy aging.

Age-related loss of muscle mass is attributed to the decrease in muscle fibers number and size. In humans, after the age of 50, decrease in muscle mass is noticeable, and the number of muscle fibers begins to decrease gradually around the age of 50s (Faulkner et al., 2007). These findings indicate that the loss of muscle fiber might be a primary trigger for severe loss of muscle mass and function. Multiple muscle fibers are innervated by a single motor neuron, defined as a motor unit, and decline in the number of motor units is considered to be closely related to muscle fiber loss. The process of denervation and re-innervation of muscle fibers, also called motor unit remodeling, contributes to maintaining the muscle fiber numbers and function (Ansved and Larsson, 1990; Campbell et al., 1973; Kadhiresan et al., 1996). Moreover, the loss of motor units is reported to begin at about 50 years of age, in humans, which is consistent with the initiation of muscle fiber loss (Faulkner et al., 2007). Based on these observations, maintaining well innervated muscle fibers during aging could be the effective countermeasure against the age-related muscle deterioration.

There are two major interventions to maintain muscle mass and function. One is the physical exercise and the other is nutritional supplementation. The beneficial effect of exercise on muscle is not only increased protein synthesis but also improved muscle innervation. Valdez et al. (2010) have reported that 1-month voluntary running wheel exercise effectively reduced the occurrence of age-related

Abbreviations: MFGM, milk fat globule membrane; NMJ, neuromuscular junction; EDL, extensor digitorum longus; AChR, acetylcholine receptors; α-BTX, α-bungarotoxin; LRP4, LDL Receptor Related Protein 4.

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abnormalities in neuromuscular junction (NMJ), a critical structure of a motor unit, in 22-month old mice. Nutritional intervention is effective for improving muscle and physical function particularly when combined with exercise. For instance, moderate-intensity exercise in combination with amino acid (Kim et al., 2012) and tea catechins (Kim et al., 2013) intake showed significant improvement of muscle mass and walking ability in elderly people. Such enhancement of the exercise effect by nutritional supplementation can be quite beneficial especially in older people due to their age-related decline in physical functions.

Based on this, we have searched for an effective nutritional intervention and found that the intake of milk fat globule membrane (MFGM) with habitual exercise has a potent improving effect on muscle mass and physical performance, such as muscle strength (Haramizu et al., 2014a) and endurance capacity in mice (Haramizu et al., 2014b). The MFGM is the structural membrane covering triglyceride globules that are dispersed as emulsified bodies in milk and contains unique polar lipids and membrane specific proteins (Cavaletto et al., 2008). These beneficial effects of MFGM with habitual exercise were also confirmed in several human studies in participants ranging from middle-aged to older adults (Ota et al., 2015; Soga et al., 2015; Minegishi et al., 2016). Our previous study indicated that increased expression of NMJ-related factors in skeletal muscles might contribute to the beneficial effects of MFGM (Haramizu et al., 2014a). However, we have not confirmed the actual alteration in NMJ condition with MFGM and exercise, therefore the importance of its improvement for muscle and physical performance has yet to be clarified.

Therefore, we aimed to investigate the effect of MFGM intake combined with voluntary running exercise on the morphological NMJ changes and to clarify its contribution to muscle and motor function in aging mice. In this study, we performed the rotarod test for measuring motor coordination, which is highly related to neuromuscular function, and the grip strength test for measuring muscle force to evaluate motor function. We found that MFGM intake with voluntary running exercise suppresses the age-related morphological deterioration of NMJ, which significantly correlated with improvement of motor function.

2. Materials and methods

2.1. Materials

The MFGM was prepared from buttermilk by filtration and centrifugation, and its composition was also analyzed at the Japan Food Research Laboratories (Tokyo, Japan). The composition of MFGM was as follows: 53.4% protein, 25.2% fat, 13.4% carbohydrate, 20.4% phospholipids (5.69% phosphatidylcholine, 5.69% phosphatidylethanolamine, 1.76% phosphatidylinositol, 2.03% phosphatidylserine, 3.72% sphingomyelin, and other phospholipids), 5.5% ash, and 2.5% moisture. Protein was determined by the Kjeldahl method, fat by the acid hydrolysis method, carbohydrate by calculation, and phospholipids by colorimetric assay and thin-layer chromatography. These procedures were performed in accordance with the Analytical Manual for Standard Tables of Food Composition in Japan and The JOCS Standard Methods for the Analysis of Fats, Oils and Related Materials (Japan Oil Chemists' Society). From here on, we use the abbreviation "MFGM" to signify a diet containing MFGM as an active ingredient, unless otherwise specified.

2.2. Animals

Male Balb/c mice (6 month-old) were purchased from Charles River Japan (Yokohama, Japan) and maintained under controlled conditions of temperature (23 ± 2 °C), humidity (55 ± 10%), and lighting (0700 to 1900 h). The mice were fed laboratory chow (CE-2; CLEA Japan, Tokyo, Japan) and had free access to drinking water to acclimate to the housing conditions for 1 week. All animal experiments were conducted in the Experimental Animal Facility of Kao Corporation's R&D Department. The study was approved by Kao's Animal Care Committee. All experiments strictly followed the guidelines of the committee.

2.3. Experimental design

After the acclimatization, the rotarod test was carried out for measuring the basal motor coordination function (details are described in Section 2.4 *Rotarod test*). Subsequently, the mice were divided into five groups with similar body weights and rotarod performance (n = 10/group). For the habitual voluntary running exercise groups, mice in two of the five groups were individually housed in plastic cages equipped with a running wheel (SW-15 mg; MELQUEST, Toyoama, Japan). Mice from the remaining groups were individually housed in normal cages, and these mice represented the non-running controls. Each cage had a nest box (Shepherd Specialty Papers, Watertown, TN) for environmental enrichment. In these exercise groups, the voluntary exercise volume (number of rotations) of each mouse was monitored for 1 week, and, subsequently, mice were regrouped within these groups according to their voluntary exercise volume without affecting all other factors. At the beginning of the intervention, five groups were allocated as follows: Young, Sedentary-control diet (Cont-Sed), Sedentary-MFGM diet (MFGM-Sed), Voluntary running-control diet (Cont-VR), and Voluntary running-MFGM diet (MFGM-VR). Mice were allowed *ad libitum* access to water and one of the following powder diets until sacrifice at the age of 14 months: the control diet containing 10% fat (w/w), 20% casein, 55.5% potato starch, 8.1% cellulose, 4% minerals, 2.2% vitamins, and 0.2% methionine (for the Cont-Sed and Cont-VR groups) or the MFGM diet consisting of the control diet supplemented with 1% MFGM (for the MFGM-Sed and MFGM-VR groups). Dietary intake was measured throughout the experimental period by subtracting the weight of the remaining food from the initial weight of the food given on the previous feeding day. Voluntary exercise volume of each mouse in the VR groups was recorded until sacrifice. Mice from the Young group were allowed *ad libitum* access to water and the control diet until sacrifice at 6-month of age.

2.4. Rotarod test

The motor coordination of mice was evaluated at 6-month and 13-month of age by using a rotarod (MK-610A, Muromachi Kikai, Tokyo, Japan) according to the method described by Graber et al. (2013). The test device was set to an acceleration program that increases the rotation speed from 4 rpm to 40 rpm within 5 min. Mice were acclimated to the test protocol by performing three trials per day on three consecutive days. On day 4, the performance of each mouse was tested by measuring the time remained on the rotarod. The average of three trials was calculated. The test was performed at rest condition in all mice by locking the running wheels on the day before the measurement to avoid the effect of fatigue in the VR mice.

2.5. Grip strength test

The grip strength of the forelimbs was evaluated at 6-month and 14-month of age by using a grip strength meter (47106 Grip Strength Meter for Mouse, Ugo Basile, Varese, Italy) according to the method described by Menalled et al. (2010) and Voikar et al. (2013). Briefly, a mouse was placed over a base plate, in front of a grasping bar. After grasping the bar with the forelimbs, the tail was pulled backward at a constant power and speed until the mouse loses its grip on the bar. The peak-pull force was recorded. Five measurements were performed for each mouse and the average of the three middle scores was calculated as the grip strength. The test was performed at rest condition for all mice by locking the running wheels in the VR mice on the day before the measurement as in the case of the rotarod test.