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Changes of Muscle-related Genes and Proteins After Spaceflight in *Caenorhabditis elegans**

WANG Chi[1,3], SANG Chen[1)**], Akira HIGASHIBATA[2], Noriaki ISHIOKA[2], RONG Long[1], YANG Chun[1], SUN Yan[1], YI Zong-Chun[1], ZHUANG Feng-Yuan[1]

([1]*Bioengineering Department, Beijing University of Aeronautics & Astronautics, Beijing* 100191, China;
[2]*Japan Aerospace Exploration Agency, 2-1-1, Sengen, Tsukuba, Ibaraki* 305-8505, Japan;
[3]*CGMC, CNRS-UMR 5534, University Claude Bernard Lyon-1*, 69622 *Villeurbanne Cedex*, France)

Abstract The molecular mechanism underlying muscular atrophy and gravisensing during spaceflight is still unknown. The major effects of spaceflight on body-wall muscles of *Caenorhabditis elegans* (*C. elegans*) in the structures and functions were examined, and five important muscle-related genes and three proteins were studied after nearly 15-day spaceflight. The changes for the wall-muscles were observed *in situ*. Decreased muscle fiber size was observed with myosin immunofluorescence and duller dense-body staining in flight samples, which suggested that muscular atrophy had happened during spaceflight. However, F-actin staining showed no differences between the spaceflight group and ground control group. Otherwise, after returning to the earth the *C. elegans* displayed reduced rate of movement with a lower ratio (height/width) in crawl trace wave, which indicated a functional defect. These results demonstrated that *C. elegans* muscular development was changed in response to microgravity, and changes also occurred at the level of gene transcription and protein translation. Expression of dys-1 increased significantly in body-wall muscles, while hlh-1, myo-3, unc-54 and egl-19 RNA levels decreased after spaceflight. Dystrophin (encoded by dys-1) is one of important components in dystrophin-glycoprotein complex (DGC). Increased dys-1 expression after flight implied that the muscular cell would accept more gravity signals by DGC in microgravity in order to keep mechanical balance within the cells. It is concluded that DGC was involved into the mechanical transduction in body-wall muscles of *C. elegans* when gravity varied, which potentially played a vital role in gravisensing. The changes of hlh-1, myo-3, unc-54 and egl-19 suggested that they had the effects of promoting microgravity-induced muscular atrophy in structure and function aspects. Result of Western blotting showed that the level of myosin A in spaceflight group decreased, further confirmed that atrophy happened during flight.

Key words *Caenorhabditis elegans*, dystrophin, myosin A, spaceflight

Muscular atrophy is one of the most serious problems during human spaceflight. At early time of space exploring, people already noted that the weight of astronauts decreased markedly [1]. Partly it is resulted from the loss of fluid, and another reason is the loss of muscle mass. Microgravity-induced muscular atrophy happened rapidly, with up to 37% reduction in rat muscle mass within only one week [2]. In humans, it seems to vary among individuals and the degree of atrophy appears to be greater than that induced by bed-rest. 10%～20% of muscle mass was lost in short missions. While on long-term flight, it rises up to 50% without countermeasures [3, 4]. This might be due to disuse of postural muscles，because they don't need to resist gravity force in spaceflight, thereby which breaks the balance of muscle metabolism leading to an accelerated breakdown of contractile proteins and decreases protein synthesis[5~8]. It is quite rational, but the detailed changes about gravisensing and mechanical transduction in cellular and molecular levels are still unclear.

It is important to have an ideal model for studying muscular atrophy in space. The studies that are easily accomplished in a laboratory will become extremely difficult in space. *Caenorhabditis elegans* (*C. elegans*), a small nematode, provide a lot of advantages for

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**Corresponding author.
Tel: 86-10-82338062, E-mail: sangchen@buaa.edu.cn
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space life research. It was the unique organism that survived in space shuttle Columbia, which was destroyed while reentering the Earth's atmosphere. In the past more than 40 years of space life research, the scientists exerted utmost efforts to promote the technology *in vivo* model, but there are many difficulties to carry out a satisfied experiment because of limited technical conditions. Whereas, applying this model animal, *C. elegans*, permits researchers observing the molecular changes in the whole animals with relative simple measures. First of all, the culture system is quite simple. *C. elegans* Maintenance Medium (CeMM), a culture liquid, allows long-duration experiment. 10 000 worms in 10 ml medium can be kept for maximum of about 30 days[9]. Secondly, the worms can be observed easily under microscope due to its transparent body. Thirdly, the lifespan of *C. elegans* is not very long (meanly 13 days at 25℃), so the experimental time is saved. Moreover, it is easier to implement gene operation in *C. elegans*, and the genes/proteins of worms in body-wall muscles have been studied extensively and resemble those of human beings in both structures and functions[10].

How was the balance of muscular metabolism finally broken? Which pathways or receptors are responsible for sensing gravity and induce the muscular atrophy? Up to now, the mechanism of sensing and transferring gravity information in cellular levels remains unknown. Recently some researches have focused on dystrophin-glycoprotein complex (DGC), a kind of transmembrane protein compound[11~13]. Dystrophin encoded by dys-1 is one of important proteins in DGC. Absence of dystrophin leads to loss of the entire DGC and is associated with muscular degeneration[14]. In *C. elegans*, dys-1 is the unique homologue of the MyoD family. Structural studies of dystrophin demonstrate that it binds F-actin with its N-terminal, and the C-terminal binds a group of other proteins thereby building a huge DGC complex. α-dystroglycan, one of the extracellular components of DGC, links this complex to the extracellular matrix (ECM). It is shown that DGC forms a connection between the ECM and the cytoskeleton[11~13]. Many studies have proved that DGC is important for maintenance of mechanical integrity in muscular cells. It acts as a biological converter changing mechanical stimuli into biochemical responses[15, 16]. In human beings, Duchenne and Becker muscular dystrophy (DMD and BMD), two progressive neuromuscular degenerative diseases are caused by mutations of dystrophin gene. However, in *C. elegans*, mutation of dys-1 does not lead to significant changes in muscular structure. When in addition to dys-1 the genes egl-19 (encoding a Ca^{2+} ion channel) or hlh-1 (encoding a transcription factor regulating the expression of muscle genes) are mutated at the same time, the muscular structure will change dramatically[17~19]. It implied that synergistic interactions existed between these genes in *C. elegans*. Transcription of unc-54 and myo-3 in the body-wall muscles are regulated by CeMyoD (Hlh-1), and egl-19 could induce the muscular atrophy through affecting the Ca^{2+} metabolism in muscles. So in order to reveal the functions of these genes, the expression of dys-1, hlh-1, unc-54, myo-3 and egl-19 in spaceflight are detected in this study.

1 Methods

1.1 Materials and instruments

CeMM[20] and space culture bags were provided by Japan Aerospace Exploration Agency (JAXA), opticell™ cell culture plates (Biocrystal, USA), TRIZOL (Invitrogen, Burlington, USA), Superscript™ Ⅲ Reverse Transcriptase (invitrogen, Burlington, USA), SYBR premix Ex Taq real time PCR kit (Takara, Japan), primers of dys-1, hlh-1, unc-54, myo-3 and egl-19 of *C. elegans* (Shanghai Sangon, China), ABI PRISM 7000 Real-time PCR system (Biosystem, Foster City, USA), Laser confocal scanning microscope (Leica & Zeiss, Gemany), Collegenase (Sigma, Chicago, USA), Rhodamine-Phalloidin (Invitrogen, California, USA), MH24 anti-vinculin and 5.6 anti-myosin A monoclonal antibodies (Developmental Study Hybridoma Bank, Iowa City, USA) and FluoProbes 546 Anti-Mouse IgG and FluoProbes 488 Anti Mouse IgG (interchim, Montlucon, France) were used in this study.

1.2 Experimental procedure

The *C. elegans* strain Bristol N2 was cultured in opticell™ cell culture plates with CeMM for 19 days at 20℃. Approximately 5 000 mixed-stages of worms and 5 ml fresh CeMM were transferred into culture bags before launching. Fifteen culture bags were put into three plastic tubes evenly. On September 9, 2006, the worms were sent into space by China biosatellite Shijian-8. This mission lasted 14 days and 19.5 h. During space-flown, the temperature remained at (21 ± 4)℃. After landing, the worms were frozen in liquid nitrogen. Samples in ground control were

obtained later in the laboratory under identical conditions.

1.3 Basic information of the experimental *C. elegans*

First of all, the worms both in control and flight groups were observed under microscope after dilution, and then counted the number of dead worms and live ones for calculating the survival rates [live number/ (live number + dead number) ×100 %]. On the other hand, the animals were allowed to crawl on 1.8 % agar surface dyed with ink. Ratios (height/width) of crawl trace waves were obtained from 10～30 traces per group. At last, video records of *C. elegans* were used to determine the movement rates. 15 worms were randomly selected from each group for counting.

1.4 Observation of F-actin, myosin A and dense-body in body-wall muscles

For this purpose, Rhodamine-Phalloidin staining and myosin A/dense-body immunofluorescence were performed according to references[21, 22], and the slices were observed by laser confocal fluorescence microscope.

The samples were divided into three parts. One of them was used for F-actin staining. Phalloidin can bind to filamentous actin, which allows the visualization of thin filament in muscular cells. Briefly, the whole animals were fixed in 4% formaldehyde for 3 h at 22℃ with occasional inversion, permeabilized with 100 % acetone for 5 min at –20℃, incubated with Rhodamine-Phalloidin (final Conc. 2 U/ml) for 1 h at 22℃ in dark box, and finally mounted on a slide for observation.

Another two parts of worms were prepared as similar to that above but permeation with collagenase for 1 days and 2 nights at room temperature, and immunostaining was performed as described[22]. The target proteins were combined with MH24 anti-vinculin or 5.6 anti-myosin A monoclonal antibodies, and then marked by FluoProbes 546 Anti-Mouse IgG or FluoProbes 488 Anti Mouse IgG. Lastly, the samples were mounted on slices and were observed under microscope.

1.5 Real-time PCR

Expression of five muscle-related genes was detected by real-time PCR. Sequences of dys-1 (AJ012469), hlh-1 (NM_001026722), unc-54 (NM_061195), myo-3 (NM_073664) and egl-19 (NM_171379) were obtained from GenBank and the primers were designed by the software named Primer Express (Version 2.0, Applied Biosystems, USA) and evaluated by Oligo 6.65. The sequences are shown in Table 1.

Table 1 Sequences of primers for muscle-related genes

Primers	Sequence (5′→3′)	Length	Position(5′→3′)	Product size /bp	t_m/℃
dys-1F	GTGAATCCGCCGCATTTG	18	9725～9742	95	56
dys-1R	AGCGCCTTCCGATATTGTTG	20	9800～9819		60
hlh-1F	ACGGATTCGGACGACGATAG	20	787～806	108	58
hlh-1R	CGCGACAATCTGTCCAAAGAG	21	874～894		64
unc-54F	GATGCCGAATCCCAAGTCAA	20	6678～6697	103	60
unc-54R	CGGCTTCGTGTTGGTAGTTCT	21	6760～6780		64
myo-3F	AAAGGTCAAGCCAATGCTCAA	21	5703～5723	99	60
myo-3R	TCTTCAACCAAATCGGCAAC	20	5782～5801		58
egl-19F	TGCACTGGACGATTCAACATC	21	3846～3866	93	62
egl-19R	CTTGCCAGGCTTCTCCAGTT	20	3930～3949		62
gpd2-F	CCGTCAACGATCCATTCATCT	21	97～117	105	62
gpd2-R	GGTAGTCTCCCTCGTGAGCAA	21	181～201		64

F: Forward primer, R: Reverse primer.

Total RNA was isolated from whole animals using standard TRIZOL method according to manufacturer's protocol. The quantity and purity of total RNA were analyzed at 260/280 nm. Then equal amounts of total RNA were reversely transcribed into cDNA by Superscript™ Ⅲ Reverse Transcriptase. Finally two-step real-time PCR reactions were carried out using 8-strip PCR tubes in 20 μl reaction volume with SYBR premix and optimized concentrations of specific primers. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as reference gene. GAPDH is the protein product of gpd-2 and can keep stable expression in different mechanical conditions, so it can be used as an internal standard for this real-time PCR.

1.6 Western blotting of myosin protein

In order to compare the level of myosin A in the

flight group and the control group, Western blotting was performed. Total proteins were isolated from worms by TRIZOL method according to manufacturer's protocol. The quantity and purity of total proteins were analyzed at 260/280/230 nm. The equal amounts of total proteins were boiled for 5 min after mixing with blue buffer, and then centrifuged for 30 s at 13 000 *g*. 50 μl of mixture was loaded into each lane of 6% polyacrylamide gel. After electrophoresis, proteins were transferred onto BA83 nitrocellulose membrane (Schleicher & Schuell) in transfer buffer (Tris 25 mmol/L, glycine 250 mmol/L, SDS 0.1%) for 1.5 h at 70 mA. Then myosin A was combined with 5.6 anti-myosin A monoclonal IgG at a dilution 1∶20 000. Afterward, peroxidase-coupled goat anti-mouse IgG (Biorad) was used at a dilution of 1∶3 000. Eventually, blots were revealed using ECL kit (Amersham) as recommended by the supplier.

1.7 Statistical analysis

All data were given as ($\bar{x} \pm s$). One-way ANOVA analysis was used by SPSS software to assess statistical significance between flight and control group. $P<0.05$ was considered to be significant.

2 Results

2.1 Changes of reproduction and movement

There was no significant difference in survival rate between flight group and ground control group. After nearly 15-day spaceflight the movement rate of animals decreased compared to ground control group ($P>0.05$ *vs* ground control, Table 2). Mean ratio of crawl trace wave in spaceflight worms also changed, but there was no significant difference between two groups ($P>0.05$ *vs* ground control, Table 2, Figure 1).

Table 2 Summary of survival rate, movement rate and ratio of crawl trace wave in worms after spaceflight

Groups	Survival rate /%	Movement rate/min	Ratio (height/width) of crawl trace wave
Spaceflight	78.78 ± 13.27	76.40 ± 11.89	0.41 ± 0.05
Ground control	83.53 ± 9.99	82.40 ± 18.21	0.44 ± 0.07

$\bar{x} \pm s$. *$P<0.05$ spaceflight group *vs* ground control.

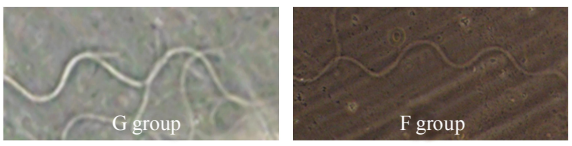

Fig. 1 The crawl wave of *C. elegans* in spaceflight group and ground control group

G: Ground control; F: Spaceflight group.

2.2 Morphological observation

Although it is reported that there are some significant changes in muscular structure in rats and humans after spaceflight[2, 23], space-induced muscular atrophy in morphological aspect in *C. elegans* has not been shown. Therefore in this experiment, F-actin, myosin A and dense-body in body-wall muscles of worms were stained and were observed under a confocal microscope. The myosin A immunofluorenscence showed that muscle fiber size of flight worms decreased, and intensity of fluorescence in dense-body staining after spaceflight became weaker, which suggested that the quantity of dense-body decreased compared to ground control group. However, there was no significant difference in F-actin staining between two groups (Figure 2).

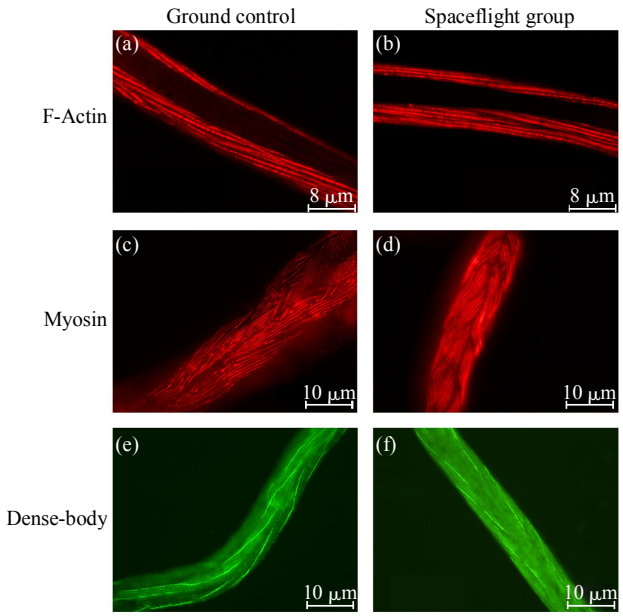

Fig. 2 The effects of spaceflight on muscular structure of *C. elegans*

Pictures of nematodes body-wall muscles in fluorescence microscopy were showed after staining with phalloidin-rhodamine [(a) and (b)], anti-myosin A antibodies [(c) and (d)] or anti-dense-body antibodies [(e) and (f)]. (a), (c) and (e) revealed the body-wall muscles of *C. elegans* in ground control group and (b), (d) and (f) present the muscular structures of animals in spaceflight group.

2.3 Expression of five muscle-related genes

In order to compare the expression of these genes, relative quantitative PCR was performed. The results demonstrated that the linear value of dys-1 in flight group increased 64.19% from (0.176 3 ± 0.005 0) to (0.234 7 ± 0.001 2) as compared to ground control, whereas the linear values of hlh-1, unc-54, myo-3 and egl-19 decreased 71.31%, 70.39%, 81.85% and 75.13% respectively (Figure 3).

Genes	Spaceflight	Ground	Spaceflight/Ground
dys-1	0.234 7 ± 0.001 2	0.017 6 ± 0.005 0	1.641 9 ± 0.136 0
hlh-1	0.286 9 ± 0.009 5	0.039 5 ± 0.001 8	0.286 9 ± 0.009 5
unc-54	0.296 1 ± 0.015 8	0.701 0 ± 0.009 3	0.296 1 ± 0.015 8
myo-3	0.181 5 ± 0.030 8	0.021 0 ± 0.003 9	0.181 5 ± 0.030 8
egl-19	0.017 9 ± 0.000 9	0.082 3 ± 0.039 0	0.248 7 ± 0.097 8

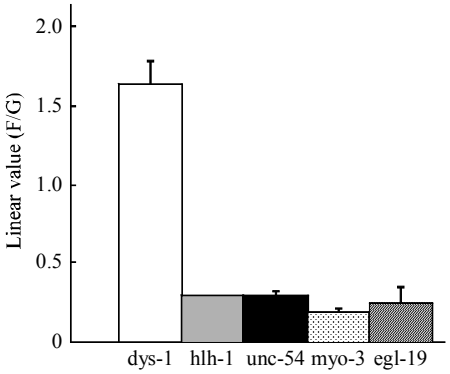

Fig. 3 Real-time quantitative PCR analysis of muscle-related genes in spaceflight worms

The expression of dys-1, hlh-1, unc-54, myo-3 and egl-19 are showed. The mRNA level of each gene was standardized by GAPDH (gpd-2) mRNA, which was used as reference gene. In spaceflight worms, the expression of dys-1 increased significantly, however the others decreased at the same time. F, spaceflight group; G, ground control. The right table is the data of real-time PCR.

2.4 The level of myosin A measured by Western blotting

In order to check the level of myosin A in body-wall muscles in *C. elegans*, we used Western blotting. Observed from the film, we can find the bands in 250 ku, which stands for the level of myosin A. After analyzing with software of image J, we found the quantity of myosin A in flight group (gray value is 152.146) decreased significantly compared to ground control (gray value is 189.906), suggesting a marketable loss of myosin A in body-wall muscles of *C. elegans* after spaceflight.

3 Discussion

In this study, we observed overall culture growth and movement character of *C. elegans* responding to spaceflight. The lifespan of *C. elegans* is meanly 13 days at 25℃. Duration of 14 days and 19.5 h spaceflight means that all the worms we got finally had spent all their lives in microgravity conditions. Hence, our data revealed the effects of long-term spaceflight on *C. elegans*. The survival rate of worms declined slightly after nearly 15-day flown, but there is no significant difference between flight group and ground control. It proved that spaceflight had no significant influence on survival and reproduction of worms. Otherwise, the animals in space-flown group moved slower and the ratio (height/width) of crawl trace wave on agar plate decreased. It showed that ability of locomotion in *C. elegans* had become weaker compared to that in ground control, which was corresponded to previous studies [5]. Spaceflight had changed the muscular development in *C. elegans*.

Using immunofluorenscence staining, myosin A and dense-body, the main structures of body-wall muscles in *C. elegans*, were observed directly. The worm's photo of Myosin A staining in spaceflight group showed that the size of muscular cells decreased obviously, while the length of cells were similar, which provided a direct evidence for muscular atrophy. Otherwise, the staining of dense-body demonstrated no significant changes between flight and ground control group, but the density of fluorescence became duller in flight worms. It suggested the quantity of dense body declined, which revealed that the anchors of thin filament became less. However, the F-actin staining displayed no significant differences in two groups. According to our data, even the thin filament in spaceflight group had no significant differences in two groups, but their anchors (dense-body) to the cutile in space group decreased as compared with that ground control group. That is an interesting finding and is deserved to be studied further. In other previous studies, the scientists have found the genes coding F-actin had no changes after 10 days spaceflight[5]. So we inferred that the effects of microgravity on different filaments (myosin is belonging to thick filament, while F-actin is the main structure of thin filament) varied.

The expression of muscle-related genes is the key point in microgravity-induced muscular atrophy. Our results demonstrated that the expression of dys-1, one of important component of DGC, increased significantly, whereas the genes of hlh-1, unc-54, myo-3 and egl-19 declined at the same time, which would contribute to muscular atrophy induced by spaceflight in different pathways.

It is well known that DGC links extracellular matrix proteins with intracellular cytoskeleton, and it can detects continuously mechanical stimuli from outside of cells and transfers them into biochemical signals, and then leads to physical adaptive reactions[16, 24]. Our data in this studies suggested that dys-1 gene expression was affected by microgravity at the level of transcription. DGC may increase in flight group since dystrophin (encoded by dys-1) is the major structure of DGC. Owing to the special functions of DGC on mechanical transmission, this result suggested that DGC will increase in microgravity in order to accept more gravity signals for keeping the mechanical balance within the muscular cells, so it play an important role in gravisensing.

Hlh-1, helix-loop-helix transcription factor, belongs to the members of myogenic regulatory factor family (MRFs), and Hlh-1 controls the expression of two myosin heavy chain MHC isoforms (MHC A and B encoded by myo-3 and unc-54, respectively)[25, 26]. Declined expression of hlh-1 will lead to muscular atrophy through affecting the transcription of myosin proteins as showed in Western blotting, which displayed the loss of myosin A in flight group worms. The gene of egl-19 encodes the α1 subunit of voltage-activated L-type Ca^{2+} channel in *C. elegans*. Silent mutants of egl-19 are lethal, whereas reduction of its function causes feeble contraction which suggests its important effects played by these ion channels in body wall muscle functions[27, 28]. Our results demonstrated that egl-19 was down-regulated in spaceflight group, which induced weaker locomotion from functional way.

In conclusion, our data showed that dys-1 was involved in responding to changes of gravity, and decreased hlh-1, unc-54, myo-3 and egl-19 expression promoted the muscular atrophy through structural and functional ways respectively. This might be one of molecular mechanisms in microgravity-induced muscle atrophy. The pathway of gravisensing is a complicated process even in this simple multicellular eukaryotic organism——*C. elegans*. More works have to be done, especially further studies on the relationship between different muscle-related genes and proteins.

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太空飞行后秀丽隐杆线虫肌相关基因和蛋白质变化 *

王 驰[1,3] 桑 晨[1)**] Akira HIGASHIBATA[2)] Noriaki ISHIOKA[2)]
荣 龙[1)] 杨 春[1)] 孙 艳[1)] 易宗春[1)] 庄逢源[1)]

([1)]北京航空航天大学生物工程系，北京 100083；
[2)]*Japan Aerospace Exploration Agency, 2-1-1, Sengen, Tsukuba, Ibaraki* 305-8505, Japan;
[3)]*CGMC, CNRS-UMR 5534, University Claude Bernard Lyon-1*, 69622 *Villeurbanne Cedex*, France)

摘要 太空飞行所致的肌萎缩和重力感知的分子机制至今尚不清楚．研究太空飞行对秀丽隐杆线虫(*C. elegans*)体壁肌细胞结构和功能的影响．经过近 15 天太空飞行后对其生存率和运动能力进行了观察，并检测了 5 个重要的肌相关基因的表达和 3 种蛋白质含量．太空研究是在动物的整体水平进行的，而不是就单个细胞的研究．经历太空飞行后线虫生存率没有明显变化，但运动频率变慢，爬行轨迹也发生了改变，提示线虫运动功能出现障碍，这些数据揭示：微重力下秀丽线虫肌肉发育发生了变化．肌球蛋白 A(myosin A)免疫荧光染色观察发现，太空飞行组肌纤维面积缩小，肌细胞致密体(dense-body)荧光亮度下降．这些形态学观察直接提示太空组线虫出现了肌萎缩．但是，肌动蛋白(F-actin)荧光染色显示两组并无明显差别．基因表达水平的分析结果显示，在太空飞行组动物中 dys-1 表达明显上调，同时 hlh-1, myo-3, unc-54 和 egl-19 基因表达下调．抗肌萎缩蛋白(dystrophin，由 dys-1 编码)是抗肌萎缩蛋白 - 糖蛋白复合物(DGC)的主要组成成分，而该复合物在微重力下增多，提示肌细胞是为了接受更多的力学刺激以维持细胞内外的力学平衡，所以该复合物在肌细胞的重力感知中起关键作用．基因 hlh-1, myo-3, unc-54 和 egl-19 表达下调，说明它们分别从结构和功能两个途径促进了微重力性肌萎缩的发生．最后，Western blot 结果提示，太空组线虫体壁肌内肌球蛋白 A 减少，进一步确证了太空飞行中线虫有肌萎缩发生．

关键词 秀丽隐杆线虫，抗肌萎缩蛋白，肌球蛋白 A，太空飞行
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