

Original Article

Ultrastructural pathological alterations in ocular muscles in a cat model with exotropia

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Abstract: To study the ultrastructural alterations of the ocular muscles in a cat model with exotropia. A total of 18 cats (4-6 weeks old) were included in the study and randomly divided into experimental group (n=12) and sham group (n=6). The experimental group underwent surgery to generate the exotropia model. 4 and 2 cats of each group were euthanatized respectively, at the end of 1st month, 2nd month, and 3rd month after strabismus surgery, and the right medial rectus muscles were harvested, followed by staining and examination of ultrastructural changes under electron microscope. The proportion of irregularly arranged myofibrils and abnormal Z lines, and the number of mitochondria per unit area were recorded. In experimental group myofibril irregular arrangement percentage was shown to be markedly higher than that of the sham group (t=2.882, 6.085, 8.641, P<0.05). There was significant difference of abnormal rate of Z line in the medial rectus muscles between the experimental and sham group at the 2nd and 3rd month after surgery (t=8.230, 34.272, P<0.01). No significant difference of abnormal rate of Z line was observed between experimental and sham group at 1st month after surgery, but the number of mitochondria per unit area was found to be significantly higher in experimental group than that of sham group at 1st month after surgery (t=4.90; P<0.01). At 2nd month and 3rd month after surgery, the number of mitochondria tends to be lower in experimental group than that of sham group; however, statistically significant difference can be observed only at 3rd month (t=-5.49; P<0.01). There was also significant positive correlation between the duration of strabismus and proportion of irregularly arranged myofibrils and proportion of abnormal Z lines (r=0.997 and r=0.987, respectively; P<0.05); and negative correlation between duration of strabismus and number of mitochondria (r=-0.953; P<0.05). High proportion of irregular arrangement of myofibrils and Z line in the medial rectus muscle were present in the ocular muscles of cat model with exotropia, the abnormal number of mitochondria per unit area was also noticed. In addition, the ultrastructural alterations tend to be enhanced with the extension of strabismus.

Keywords: Exotropia cat, ocular muscles, strabismus, mitochondria, ultrastructure

Introduction

Concomitant exotropia in children can hamper the development of binocular single vision or even damage the single vision already established. However, the etiology and pathogenesis of the condition has yet not been fully understood. Pathological examination of weak extraocular muscles has revealed that sparse, damaged, irregularly arranged myofibrils; mitochondrial vacuolation and shrinkage were present, and other pathological changes [1]. These findings suggest that tissue anaplasia would be related to the pathogenesis of strabismus [2, 3]. Therefore, Better understanding of the

pathogenesis of concomitant exotropia will be of help for researchers working on developing new therapies. The aim of this study was to investigate the pathological and ultrastructural changes in the internal rectus muscle by establishing a cat model of concomitant exotropia, and to determine whether these pathological changes were related to the duration of strabismus.

Materials and methods

Model preparation

A total of 18 healthy normal cats, regardless of sex, weighing 400-500 g, aged 4-6 weeks were

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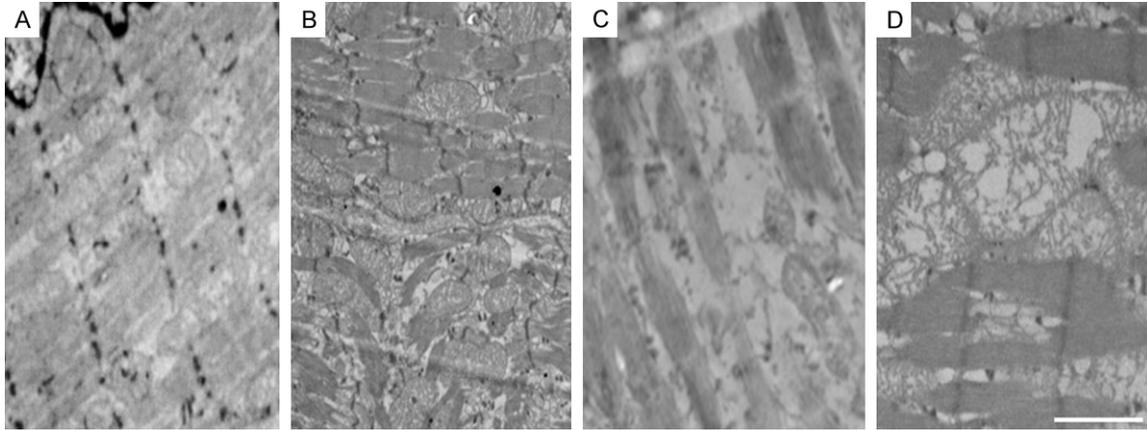


Figure 1. A: Ultrastructure of the normal medial rectus (after dual staining with uranyl acetate and aluminum citrate $\times 15,000$). The myofibrils were arranged regularly; the Z line was clear; the mitochondria were located parallel and close to the myofibrils and in a circular or oval shape; the cristae were clearly visible. B: Ultrastructure of the medial rectus in a cat with strabismus (after dual staining with uranyl acetate and aluminum citrate $\times 8000$). The myofibrils were arranged irregularly and part of it was crooked; the Z line has become tortuous or vanished; the sarcomere with a fuzzy structure was present; C: Ultrastructure of the medial rectus in a cat with strabismus (after dual staining with uranyl acetate and aluminum citrate $\times 15,000$). The dissolved focus of muscle; the myofibrils were swollen; the Z line was blurred or even vanished; the damaged structure of sarcomere was present. D: Ultrastructure of mitochondria in a cat with strabismus (after dual staining with uranyl acetate and aluminum citrate $\times 15,000$). The mitochondria were swollen, uneven thickness of the outer membrane, decreased number of cristae, and degenerated vacuolar can be present.

selected for this study. Prior to inclusion, both eyes were confirmed to be normal and one will be ruled out if any systemic disease was examined. These cats were randomly divided into two groups: an experimental group ($n=12$) and a sham group ($n=6$). All cats were reared under the same environmental conditions of diet and housing during the experiment.

The cats in experimental group underwent surgery under general anesthesia for shortening and advancement of the right lateral rectus to generate the exotropia model. They were divided into 1st month, 2nd month, 3rd month group after strabismus according to the timing of the surgery, with each group having 4 cats. In the sham group, the cats were subject to surgery but with the right lateral rectus being intact. The sham group was also randomly divided into 3 groups to correspondingly match with 3 groups set in experimental group, and with each group having 2 cats. Collected were both binocular medial rectus muscles in sham group and right medial rectus in experimental group under deep anesthesia after 1st month, 2nd month and 3rd month after surgery. The study get approved by the Medical Ethics Committee of the Affiliated Hospital of Qingdao University, and all the relevant performance of animal

operation involved were strictly in accordance with the Animal Ethics and Animal Welfare regulations of the Affiliated Hospital of Qingdao University.

Specimen preparation

The harvested medial rectus muscle was cut into $2\times 1\times 1$ mm pieces, fixed in 3% glutaraldehyde solution, and stored at 4°C . For processing, the pieces were rinsed three times with phosphate buffer, followed by fixation for 2 hours in 1% osmium tetroxide solution, and subject to rinse for three times with phosphate buffer again. After dehydration with gradient propanol, the samples were embedded with epoxy resin 618. Ultrathin section was carried out after the positioning of the semi-thin section. After dual staining with uranyl acetate and aluminum citrate, the toluidine blue could be seen under a JEM-1200EX transmission electron microscope.

Outcome measures

In each section, three non-overlapping visual fields were examined [4, 5] for assessing: 1) The extent of regular arrangement of myofibrils and myomeres, the complete situation and the

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Table 1. The proportion of irregularly arranged myofibrils in the medial rectus muscle in the two groups

Groups	1 st month	2 nd month	3 rd month	F	P
Experimental	33.75±4.19	46.25±4.44	62.5±5.00	35.21	<0.01
Sham	22.5±3.51	22.5±3.43	27.5±3.14	1.33	>0.05
t	2.882	6.085	8.641		
P	<0.05	<0.01	<0.01		

Table 2. The rate of abnormal Z lines in the medial rectus muscle in the two groups

Groups	1 st month	2 nd month	3 rd month	F	P
Experimental	21.25±1.50	33.75±2.63	49.5±1.00	236.45	<0.01
Sham	20.75±0.95	21.75±1.25	21.5±1.29	0.779	>0.05
t	0.562	8.23	34.272		
P	>0.05	<0.01	<0.01		

location relation of myomere's band and dark band. The percentage of irregular arrangement of myofibril was analyzed statistically. 2) The abnormal rate of Z line: the abnormal alterations include blurred, tortuous, vanished of Z line. If there is any condition appearing, we can all take it as abnormal Z line. The abnormal Z line in every visual field divided by Z line measured in every visual field equals the abnormal rate of Z line. 3) To observe the morphological changes of mitochondria and count the number of mitochondria in per unit area (The size of this unit was set to be 31.14 μm^2 in the experiment).

Statistical analysis

Data were expressed as means \pm standard deviation (STDEV). Analysis of variance (ANOVA) was used for multiple comparisons of means among the groups. Pearson correlation analysis was used to assess the correlation between the proportion of irregularly arranged myofibrils, the proportion of abnormal Z lines, and the number of mitochondria per unit area. SPSS 16.0 software (SPSS, Chicago, Ill) was applied in statistical analysis, and the significance level was set at $P < 0.05$ for all analyses.

Results

Proportion of irregular arrangement of myofibrils

The medial rectus showed different degree of pathological changes. It was exhibited that the irregular arrangement of myofibril, the crooked

walking line, the nonparallel to the long axis of muscle cells were present (**Figure 1B**). The sarcomere with a fuzzy structure and the myofilament gets decreased, broken or even dissolved in several cases were also noticed (**Figure 1C**). In sham group, the normal extraocular muscle sarcomere was shown to be clear and complete, the muscle fibers arranged into order and myofibril ran regular. Sarcoplasmic reticulum can be seen clearly between them, without obvious myofibril bending, dissolving or necrosis (**Figure 1A**).

The percentage of irregular arrangement of myofibril in the medial rectus of experimental group was shown to be statistically significant higher than that of sham group at the 1st month, 2nd month, and 3rd month respectively after strabismus ($t=2.882, 6.985, 8.641, P < 0.05$). Within the experiment group, there was also significant difference of the proportion of irregular arrangement of myofibrils in the medial rectus between the 1st, 2nd and 3rd month ($F=35.21, P < 0.01$; **Table 1**), suggesting that irregular arrangement of myofibrils seems to be deteriorated with the duration of strabismus. Pearson correlation analysis exhibited that there was a positive correlation between the proportion of irregularly arranged myofibrils and duration of strabismus ($r=0.987; P < 0.05$). Expectedly, no marked difference can be observed of the proportion of irregular arrangement of myofibrils in sham group ($F=1.33; P > 0.05$; **Table 1**).

Proportion of abnormal Z lines

In the experimental group, the Z line was shown to be tortuous and blurred, even disappeared entirely in severe cases (**Figure 1C**). In contrast, in sham group, the Z line was presented to be clear and regular (**Figure 1A**). The abnormal rate of Z line of medial rectus in experimental group was found to be remarkably higher than that of sham group at the 2nd month and 3rd month after strabismus in comparison with sham group ($t=8.230, 34.272; P < 0.01$). No significant difference was observed between experimental group and sham group at the first

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Table 3. The number of mitochondria per unit area of the medial rectus muscle in the two groups

Groups	1 st month	2 nd month	3 rd month	F	P
Experimental	15±0.82	12±0.67	8.75±1.32	15.68	<0.01
Sham	13±0.82	13.25±0.96	13±0.82	0.11	>0.05
t	4.9	-1.99	-5.49		
P	<0.01	>0.05	<0.01		

month after strabismus (t=0.562; P>0.05; **Table 2**).

The abnormal rate of Z line of medial rectus was presented to be significantly higher as the strabismus time extends in experimental group. There was statistically significant difference among the 3 groups by one-way ANOVA analysis (F=236.45; P<0.05). Pearson correlation analysis showed that there was positive relationship between the abnormal rate of Z line and strabismus time (r=0.987; P<0.05). With the extension of time after strabismus surgery, there was expectedly no significant alteration of the abnormal rate of Z line in sham group (F=0.779; P>0.05; **Table 2**).

Number of mitochondria per unit area

In the experimental group, the mitochondria became irregular in shape or edematous, or displayed decrease in the number of cristae. With strabismus extending, the morphological changes increased, and mitochondrial outer membrane breaks, vacuole degenerated, and eventually became apparently visible (**Figure 1D**). Whereas the mitochondria in sham group did not show any obviously morphological changes (**Figure 1A**).

The number of mitochondria per unit area at the 1st month after strabismus was displayed to be significantly higher in the experimental group than that of the sham group (t=4.90; P<0.01). At the 2nd month, no marked difference was found between the experiment group and sham group (t=-1.99; P>0.05; **Table 3**). But there was statistically significant difference at the 3rd month (t=-5.49; P<0.01). The difference at 2 months was not statistically significant (**Table 3**). One way ANOVA analysis showed that the number of mitochondria was presented to be significantly decreased in the experiment group with the extension of strabismus (F=15.68; P<0.01). Pearson correlation analy-

sis showed that there was a significant negative correlation between mitochondrial numbers per unit area and strabismus time (r=-0.953; P<0.05). In the sham group, no significant alteration of the number of mitochondrial was found (F=0.11; P>0.05; **Table 3**).

Discussion

In our study, given the pathogenesis of strabismus has been largely unknown, we've established a cat model mimicking the development of strabismus under the help of surgery. Based on the cat model of strabismus we've established, ultrastructural alterations of the ocular muscles were investigated and examined mainly by using the electronic transmission microscope method. It was found that high proportion of irregular arrangement of myofibrils and Z line in the medial rectus muscle was present in the ocular muscles of cat model with strabismus, and the abnormal number of mitochondria per unit area was also noticed in comparison with sham group. Furthermore, the ultrastructural alterations tend to enhanced with the extension of strabismus, indicating that the time of surgery to interfere at the time of diagnosis of strabismus could be important to patients suffering strabismus.

In consideration of the cat that was most commonly selected to generate the animal model mimicking the human visual deprivation [6], we've determined to select the cat as model of interest to generate the strabismus model paralleling to human disease environment in our study at the outset. To mimick the development of strabismus of human and to successfully obtain the effect of model to the largest extent, we've particularly selected cat with 4-6 weeks, which was an important period for visual development of cat. Comparatively, there was no special requirement for sex or gender of cat.

Clinically, most patients with strabismus exhibit morphological and pathological changes in the extraocular muscles [2]. It has been well-accepted that weak eye muscles can lead to ophthalmic disorders, and in particular, strabismus [7]. Therefore, we've mainly focused on the ultrastructural alterations of ocular muscles of cat model of strabismus we've generat-

ed. on the other hand, keep balance and normal position of the eye not only depends on normal structure of ocular muscle and innervation, but also depends on muscle tone and muscular tension of normal muscle fibers, which leads to the suggestion that muscle fibers of extraocular muscles play an important role in eye movements [8]. Considering that the myofibril was the main contractile element of a muscle fiber; In the extraocular muscles, any damage to the myofibrils can lead to dysfunction of eye movement and strabismus. Consequently, in our study we've quantitatively evaluated the proportion of irregular arrangement of myofibrils. It was shown that the proportion of irregular arrangement of myofibrils tends to be deteriorated with the extension of strabismus, which totally makes sense and which was in completely agreement with what's been previously reported in clinic [9]. The sarcomere was the most basic functional unit of the muscle cell and was responsible for contraction and relaxation. In normal muscle cells, Z line from the same muscle cell can beat the same level [10]. The muscle fiber of the medial rectus in patients with concomitant strabismus was fine, the arrangement of myofibril was disorder, Z band was unclear, sarcoplasmic reticulum was expanded and part of muscle fiber plasmalemma was broken [4]. So it stands to reason that degeneration of medial rectus may be the main cause of concomitant strabismus [11, 12]. In the present study, we presented a number of ultrastructural pathological alternations in the medial rectus of the cat model, which was in line with the pathological findings of ocular muscle in previous reports [5, 13]. After the cat developed an exotropia, there have been degenerative pathological changes that can be observed on medial rectus, which tends to be increasingly serious with the extension of strabismus.

It has been reported that acquired strabismus due to high myopia may be a manifestation of mitochondrial myopathy [14], which leads to the implication that strabismus could be associated with mitochondrial abnormality. Therefore, it would be necessary to study the morphological changes as well as number of mitochondria under different situations of strabismus [15, 16]. In the present study, after 1 month of strabismus, the mitochondrial numbers were shown to be higher in the experimental group than that of sham group, indicating

that the function of medial rectus might have been affected. Mitsui et al. [17] suggested that multiple deletions of mitochondrial DNA of extraocular muscles may cause dysfunction of mitochondrial oxidative phosphorylation and result in abnormalities of the eye movement system. We found that as strabismus time increased, the morphological changes as well as number of mitochondria increased. We reasoned that, at the early time of strabismus, the mitochondria providing energy for the contraction and relaxation function of extraocular muscle could contribute to the mitochondria increase reflexively, in order to maintain the balance of the eye position. However, with the strabismus time being prolonged, pathological changes of mitochondria occurred and the number of mitochondria decreased gradually.

In conclusion, in this study, a cat model of concomitant exotropia has been established showing that after onset of strabismus, the number of mitochondria first increased at the outset and then decreased. Mitochondrial morphology changed progressively, and the myofibrils also showed degenerative changes. These changes could adversely affect the function of medial rectus. The longer the duration of strabismus was, the more serious the damage will be to myofibrils and the more obvious the vacuolar degeneration will be [18, 19].

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Disclosure of conflict of interest

None.

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