

# Time-dependent Expression of Caspase-3 and Degeneration of Lateral Rectus Muscle on Experimental Esotropia in Rabbits

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## Abstract

**Background:** To identify correlation between caspase-3 expression and degeneration of lateral rectus muscle on experimental esotropia in rabbits by using time dependent analysis. **Methods:** Twenty four rabbits were divided into 4 groups, first month sham (S1); second month sham (S2); first month esotropia (E1); and second month esotropia (E2) model groups. Immunohistochemical and haematoxyllin eosin staining were performed. **Results:** In first month observation no significant difference in *caspase-3* expression between S1 ( $1.56 \pm 1.03$ ) and E1 ( $3.93 \pm 1.66$ ) groups ( $p > 0.05$ ) and in the degeneration score of lateral rectus muscle between S1 ( $1.33 \pm 1.36$ ) and E1 ( $3.83 \pm 2.04$ ) groups ( $p > 0.05$ ). In second month observation no significant difference in *caspase-3* expression between S2 ( $1.80 \pm 1.08$ ) and E2 ( $6.50 \pm 3.26$ ) groups ( $p > 0.05$ ); but there was significant difference in the degeneration score of lateral rectus muscle between S2 ( $1.16 \pm 1.16$ ) and E2 ( $4.50 \pm 1.04$ ) groups ( $p < 0.05$ ). There was a significant correlation between *caspase-3* expression and the degeneration score of lateral rectus muscle ( $p=0.020$ ;  $r=0.471$ ). **Conclusion:** On the experimental esotropia with prolonged lateral muscle contraction in rabbits, the increasing trend of caspase-3 expression has a correlation with the increasing degeneration score of lateral rectus muscle.

**Keywords:** *caspase-3, degeneration of lateral rectus muscle, esotropia, rabbit*

## Introduction

Esotropia is a type of strabismus that occurs when one or both eyes turn inward due to imbalance of the convergent and divergent tone of medial and lateral rectus muscles. The convergent tone of the medial rectus muscle causes the increased contraction of the lateral rectus muscle as its compensation that results in decreased visual function and binocularity as well as decreased quality of life.<sup>1,2</sup>

Prolonged strabismus often changes extraocular muscle structure as well as causes muscle degeneration which potentially makes difficulties in its surgical management and often requires repeated surgery.<sup>3,4</sup>

Based on extrapolation from the mechanism of general muscle contraction it was suspected that the increased contraction of lateral rectus muscle in esotropia might increase cytosolic calcium level that had impact on cell survival and apoptosis through various pathways in intracellular organelles.<sup>5,6</sup>

Although the change of extraocular muscle structures is found in strabismic eye, the effect of prolonged esotropia on the degeneration of lateral rectus muscle through apoptosis pathway has not been studied. This study was conducted on rabbits as due to ethical and technical concern it was not possible to conduct this study in humans. The purpose of this study was to identify correlation between caspase-3 expression and degeneration of lateral rectus muscle on experimental esotropia in rabbit by using time dependent analysis.

## Materials and Methods

### Animal

Twenty-four New Zealand white rabbits weighing 3.4-4.0 kg were used in this experimental study. All animals were strictly cared in accordance with the Animal Ethics and Animal Welfare Regulations. This study was carried out during August 2019 – February 2020 at Faculty of Veterinary Medicine and approved by the Institutional Review Board of Faculty of

Veterinary Medicine, Universitas Airlangga no:2. KE.153.07.2019.

**Surgery for esotropia model and sham**

After calculating sample size, the rabbits were randomly divided into two groups, the experimental group (n=12) and control group (n=12). Surgery was performed on rabbits in the experimental group to create a model of esotropia by cutting and shortening the medial rectus muscle. The surgery was only performed on the right eye under general anesthesia. After surgery, esotropia model rabbits were randomly divided into two observation groups, the 1<sup>st</sup> month group (E1) (n=6) and the 2<sup>nd</sup> month postoperative observation group (E2) (n=6). In rabbits control group, sham procedure was performed by cutting without shortening the length of

medial rectus muscle. Similar to the experimental group, sham rabbits were divided into two observation groups as described above. At the time of observation, the collection of 4 mm lateral rectus muscle specimen was carried out under general anesthesia in both experimental and control groups of rabbits.

Immunohistochemistry staining for caspase-3 expression and interpretation

Immunohistochemistry (IHC) examination for caspase-3 expression was performed by following the method that has been previously done using *anti-caspase3 antibody* ab44976 reagent Abcam USA. Immunoreactive scoring was assessed according to Remmele index scoring method, as follows <sup>7</sup> (Table 1)

**Table 1 Immuno Reactive Score**

A (percentage of positive cells)	B (intensity of staining)	IRS (multiplication of A and B)
0= No positive cells	0 = no color reaction	0-1 = negative
1=<10% Positive cells	1 = mild reaction	2-3 = mild
2=10-50% positive cells	2 = moderate reaction	4-8 = moderate
3=51-80% positive cells	3 = intence reaction	9-12 = strongly positive
4=>80& positive cells	Final IRS Score (A x B) : 0-12	

Hematoxyllin eosin staining and interpretation

Hematoxyllin eosin (HE) staining was performed by following the method that has been previously done. The degree of lateral rectus muscle damage on this examination was assessed according to the modified Yao method, with scoring as follows <sup>1</sup> (Table 2):

**Table 2. Score of the degeneration of lateral rectus muscle**

Score	Interpretation
0	Normal, if the thickness of the muscle cells (coir) is relatively uniform with the same direction of coir, muscle filaments is dense with little extracellular matrix
1	< 1/3 of the muscle tissue observed varies in thickness and the spacing between muscle filament is wider than normal
2	The same as score 1 with affected muscle tissue between 1/3 - 2/3 of the area
3	The same as score 2 with affected muscle tissue > 2/3 of the area
4	< 1/3 of the muscle tissue was atrophic, fibrotic and degenerative
5	The same as score 4 with affected muscle tissue between 1/3 - 2/3 of the area
6	The same as score 5 with affected muscle tissue > 2/3 of the area

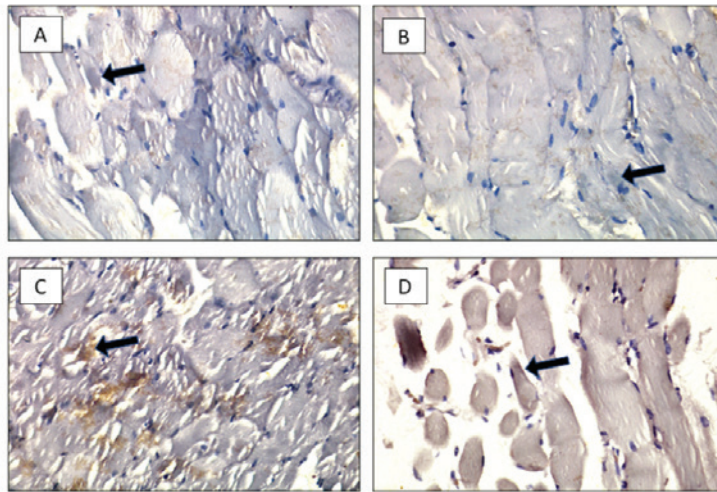
## Statistical Analysis

Datas were presented with mean  $\pm$  standard deviation. Normality test was performed using Shaphiro-Wilk test. Homogeneity test was performed using Lavene test. Furthermore, data difference was tested using ANOVA and the linear regression test was carried out. This study used a significance level of 0.05

## Results and Discussion

### *Expression of caspase-3 as an executor of apoptosis*

The expression of caspase-3 in myocyte was semiquantitatively calculated and shown in brown chromogen color in immunoreactive cells, as can be seen as follows (Fig.1)

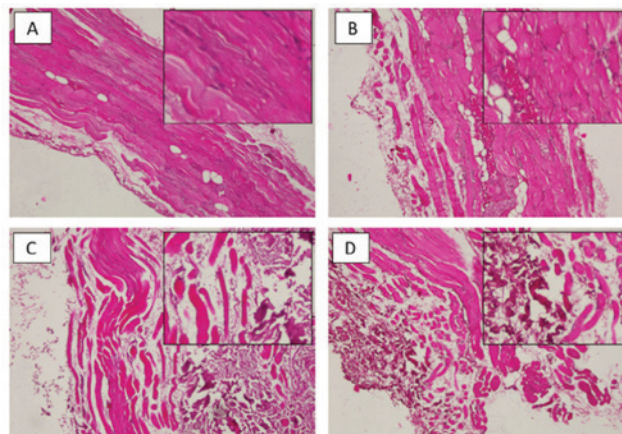


**Figure 1.** Caspase-3 expressions of sham group in 1<sup>st</sup> month observation/S1 (A), in 2<sup>nd</sup> month observation/S2 (B), esotropia group in 1<sup>st</sup> month observation/E1 (C), and in 2<sup>nd</sup> month observation/E2 (D). IHC Magnification 400x; microscope Nikon

The expression of caspase-3 in S1, S2, E1, and E2 groups were  $1.56 \pm 1.03$ ;  $1,80 \pm 1,08$ ;  $3,93 \pm 1,66$ ; and  $6,50 \pm 3,26$  respectively.

### Degeneration of lateral rectus muscle

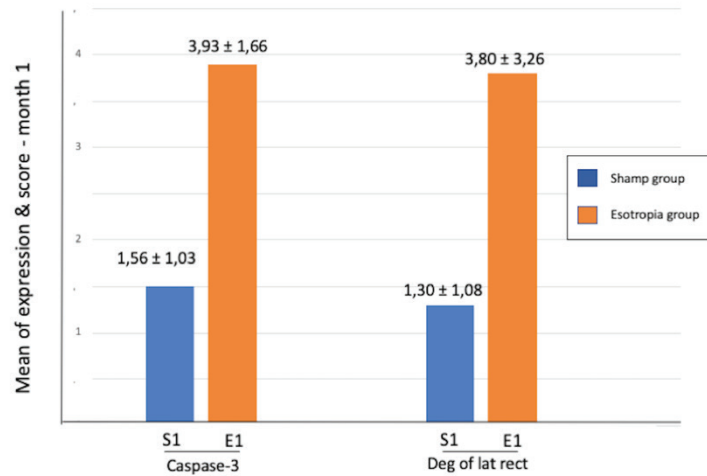
The degeneration of lateral rectus muscle was assessed semiquantitatively and can be seen as follows (Fig.2)



**Figure 2.** The degeneration of lateral rectus muscle of sham group in 1<sup>st</sup> month observation/S1 (A), in 2<sup>nd</sup> month observation/S2 (B), esotropia group in 1<sup>st</sup> month observation/E1 (C), and in 2<sup>nd</sup> month observation/E2 (D). HE Magnification 400x

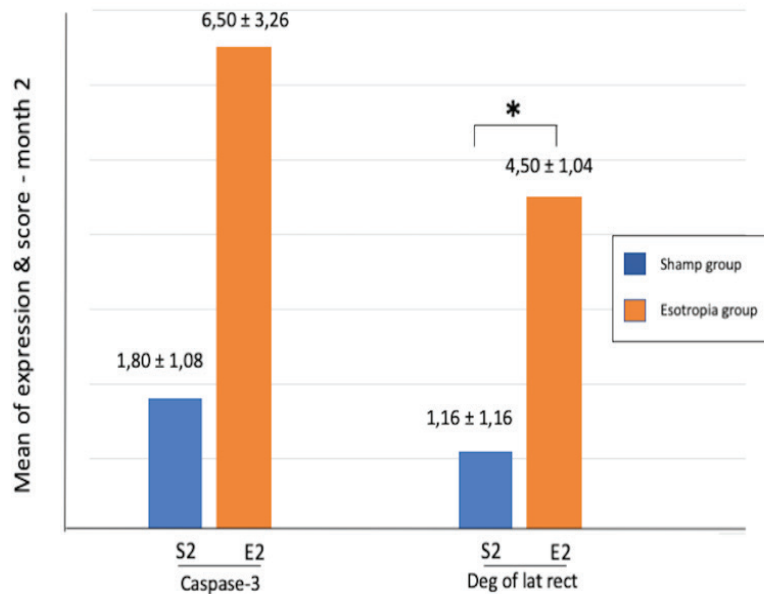
Degeneration score of lateral rectus muscle in S1, S2, E1, and E2 groups were as follows:  $1.33 \pm 1.36$ ;  $1.16 \pm 1.16$ ;  $3.83 \pm 2.04$ ; and  $4.50 \pm 1.04$ , respectively.

In first month observation there was no significant difference in caspase-3 expression between S1 and E1 groups ( $p > 0.05$ ) and in the degeneration score of lateral rectus muscle between S1 and E1 groups ( $p > 0.05$ ) (Figure 3)



**Figure 3.** The bar diagram of 1<sup>st</sup> month observation on the expression of caspase-3 and degeneration score of lateral rectus muscle (Deg of lat rec=degeneration of lateral rectus muscle; S1=1<sup>st</sup> month observation on sham group; E1=1<sup>st</sup> month observation on esotropia model group)

In 2<sup>nd</sup> month observation there was no significant difference in *caspase-3* expression between S2 and E2 groups ( $p > 0.05$ ); but there was significant difference in degeneration score of lateral rectus muscle between S2 and E2 groups ( $p < 0.05$ ) (Figure 4).



**Figure 4.** The bar diagram of 2<sup>nd</sup> month observation on the expression of caspase-3 and degeneration score of lateral rectus muscle (Deg of lat rec=degeneration of lateral rectus muscle; S2=2<sup>nd</sup> month observation on sham group; E2=2<sup>nd</sup> month observation on esotropia model group).

Misaligned condition with inward turning of the eye in esotropia may cause increased lateral rectus muscle contraction wherein the activity can mimic an exercise that can cause disruption of calcium balance. The changes in calcium homeostasis will affect metabolism processes in mitochondria and endoplasmic reticulum.<sup>8,9</sup>

In this study, immunohistochemical examination of the lateral rectus muscles in the 1<sup>st</sup> month esotropia group showed an increase in caspase-3 expression compared with control group. This result indicated that the mitochondrial apoptotic pathway was activated. Mitochondria was important checkpoints of the apoptotic process, as they might release caspase cofactors.<sup>10</sup> Calcium ( $\text{Ca}^{2+}$ ), which is released from endoplasmic reticulum storage to cytosol due to the need of lateral rectus muscle contraction, also enters mitochondria. Caspase-3 as a marker of programmed cell death is activated upon the initiation of apoptosis. Caspase-3 activation and subsequent apoptosis occurs through the release of cytochrome c from mitochondria. The pore on mitochondrial outer membrane, through which cytochrome c is released, is controlled by Bcl-2 family proteins. Altering Bax (pro apoptosis) and BCL2 (anti apoptosis) ratio results in cytochrome c release to cytosol. As a consequence, organelle swelling and fragmentation are detected as parallel with the release of cytochrome c.<sup>8,11</sup> In the control group caspase 3 expression also increased even though it was lower than that in esotropia group. The sham procedure, in which shortening of lateral rectus muscles were not performed, also triggered apoptosis but not in similar way with its mechanism in esotropia group. In esotropia group, the increased lateral rectus muscles contraction produced higher amount of apoptosis compared with that in the control group.

In the 1<sup>st</sup> month observation, it was found that the increasing degeneration score of lateral rectus muscles in esotropia group was higher than that in the control group, although it was not statistically significant. This result was in line with the expression of caspase-3 in the same month of observation. The lateral rectus muscle, as the skeletal muscles anatomy in general, is a unique network consisting of myofibrils with many nuclei and interstitial tissue. Beside of its occurrence in myonuclei, apoptosis also occurs in interstitial tissue

with different apoptotic signals. As a consequence of increasing muscle contraction in shortening muscle in esotropia group, increasing exercise is followed by endoplasmic reticulum stress. It may cause perturbations and increasing of calcium release from endoplasmic reticulum which in turn produce apoptosis.<sup>12-14</sup> It is suspected that apoptosis occurs in lateral rectus muscle, which is indicated by increasing of caspase-3 expression followed by the degeneration of lateral rectus muscle. In the 2<sup>nd</sup> month observation, prolonged contraction of lateral rectus muscle may increase calcium release and further stimulate caspase dependent apoptotic pathways. The result in this study showed that increasing caspase-3 expression in esotropia group was higher than its expression in control group.

In the 2<sup>nd</sup> month of observation, prolonged contraction of the lateral rectus muscles caused significant increase of the score of lateral rectus muscle degeneration in esotropia group, which was significantly higher than its degeneration score in control group. The average score of 4 in modified semiquantitative Yao method scoring showed that atrophy, fibrosis and degeneration occurred in lateral rectus muscles. This result was in line with the finding in a research performed by Gong et al. (2017), who in different condition observed cat model exotropia and found that the longer the observation time (1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> months), the higher the irregular myofibril arrangement in the medial rectus muscle.<sup>15</sup> Similar to Gong's study, our research showed that in prolonged contraction of lateral rectus muscle (2<sup>nd</sup> month of observation) the higher the caspase-3 expression, the higher the degeneration score of lateral rectus muscle.

In this study it has also been proven that there was a significant correlation between *caspase-3* expression and the degeneration score of lateral rectus muscle ( $p=0.020$ ;  $r=0.471$ ). However, it is still necessary to prove several pathways that correlate prolonged contraction of lateral rectus muscle with apoptosis through endoplasmic reticulum, reactive oxygen species and inflammation in esotropic eye. Extrapolation from a number of studies suggest that calcium homeostasis dysregulation in lateral rectus muscle activates the apoptotic pathway and further it will induce several pathological processes, including muscle atrophy and degeneration.<sup>10,16</sup>

As a conclusion of the study, on the experimental esotropia with prolonged lateral rectus contraction in rabbits the increasing trend of caspase-3 expression has a correlation with increasing degeneration score of lateral rectus muscle. Further studies are needed.

**Conflict of Interest:** The authors declare no conflict of interest

**Financial Disclosure:** There is no financial disclosure.

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