

ORIGINAL ARTICLE

Gastrocnemius Muscle Histopathology In Spastic Rats**Marina Indriasari¹, Ferial Hadipoetro², Bambang Pontjosoeryanto³**¹ Department of Physical and Rehabilitation Medicine, Sekarwangi Hospital, Sukabumi, Indonesia² Department of Physical and Rehabilitation Medicine, Faculty of Medicine Universitas Muhammadiyah, Jakarta, Indonesia³ Veterinary Faculty of Bogor Agricultural University, Bogor, Indonesia**ABSTRACT**

Introduction: Spasticity caused by the injury of the central nervous system. Muscle with spasticity causes muscle fibers to change in size and distribution.

Methods: To analyze the structure of the spasticity of the muscles, study use the excisional biopsy of the gastrocnemius muscle of spastic rats. Experimental study was conducted on 42 spastic rats in 6 groups with different doses of calcium. Staining of gastrocnemius muscle tissue fibers using hematoxylin-eosin. Calcium staining using alizerin red.

Results: Hematoxylin-eosin staining shown the muscle cell nucleus, cytoplasm or endoplasm appears roseate. Analysis of the spastic condition found that the distance between the muscle fibers dilated and the diameter smaller. Calcium deposits appear in spastic muscle tissue, by purplish-blue. The pearson correlation test showed a significant correlation between muscle fiber distance and spasticity after intervention ($p<0.05$). The spearman correlation test showed a significant correlation between spasticity after intervention ($p=0.008$) and each changes in spasticity ($p=0.015$).

Conclusions: HE staining used to determine the arrangement of muscle tissue in spastic gastrocnemius muscles and alizerin red determined calcium deposits in spasticity muscle tissue. There is a significant correlation between the distance between muscle fibers with spasticity and between changes in spasticity after the intervention.

Keywords: alizerin red staining, gastrocnemius muscle, hematoxylin-eosin staining, histopathology, spastic rats

ABSTRAK

Pendahuluan: Spastisitas terjadi akibat adanya cedera sistem saraf pusat. Otot yang mengalami spastisitas menyebabkan serabut otot mengalami perubahan ukuran dan distribusinya.

Metode: Penelitian ini dilakukan dengan biopsi eksisi otot gastroknemius tikus spastik untuk menganalisis struktur otot dengan spastisitas. Penelitian eksperimental ini dilakukan pada 42 tikus spastik dalam 6 kelompok dengan dosis kalsium yang berbeda. Pewarnaan serabut jaringan otot gastroknemius menggunakan hematoksin-eosin. Pewarnaan kalsium menggunakan alizerin merah.

Hasil: Pewarnaan Hematoksin-eosin didapatkan gambaran inti sel otot, sitoplasma atau endoplasma yang berwarna merah muda. Analisis terhadap keadaan spastik didapatkan jarak antar serabut otot lebih melebar dan diameter terlihat lebih kecil. Deposit kalsium tampak dalam jaringan otot yang spastik berwarna biru keunguan. Dengan uji korelasi pearson didapatkan adanya korelasi bermakna antara jarak antar serabut otot dengan spastisitas setelah intervensi ($p<0,05$). Uji korelasi spearman mendapatkan hasil adanya korelasi bermakna antara spastisitas setelah intervensi ($p=0,008$) dan antar perubahan spastisitas ($p=0,015$).

Kesimpulan: Pewarnaan HE dapat digunakan untuk mengetahui susunan jaringan otot dan bagian dari sitoplasma pada otot gastroknemius dengan spastisitas. Alizerin merah dapat digunakan untuk mengetahui adanya deposit kalsium dalam jaringan otot dengan spastisitas. Adanya hubungan yang bermakna antara jarak antar serabut otot dengan spastisitas dan antar perubahan spastisitas setelah intervensi.

Kata Kunci: histopatologi, otot gastroknemius, pewarnaan alizerin merah, pewarnaan hematoksin-eosin, tikus spastik.

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centrally, but the treatment of spasticity is given peripherally, either to the nerves or muscles.¹

Changes in muscles and nerves are interconnected. Spasticity can cause changes in the microscopic features of muscles. Skeletal muscles that are shortened due to central nervous system disorders cause the changes in size and distribution of muscle fibers. Increased use of skeletal muscle causes muscle fiber hypertrophy.²

Changes in intracellular Ca^{2+} concentration play an important role in the excitation-contraction-relaxation cycle of skeletal muscle. Abnormal changes in intracellular Ca^{2+} concentration

INTRODUCTION

Spasticity that occurs in skeletal muscles is a secondary condition due to brain injury. Individuals with spastic muscles will experience a decrease in the range of motion and muscle strength and joint stiffness, which may cause decreased functional activity. Spasticity occurs

cause abnormal muscle contractions, including spasticity. The increase in intracellular Ca^{2+} concentration results in excessive muscle contraction causing spastic. Sarcoplasmic reticulum Ca^{2+} - ATPase (SERCA) pumps active Ca^{2+} ions back into the lumen of the sarcoplasmic reticulum (SR) to induce muscle relaxation. Reducing the activity of this pump can cause a prolonged increase in Ca^{2+} which can cause spastic. Calcium stored in the muscle is thought to be excess, which is thought to affect the hyperexibility of muscle fibers due to the continuous inflow of Ca^{2+} into the sarcomere.³

The literature on the causes and treatment of spasticity mostly focuses on the nervous system because primary injuries that cause spasticity occur in the central nervous system. Several studies have assessed skeletal muscle activities using electromyography (EMG). This study used a surgical procedure (i.e., excisional biopsy) to determine the structure of skeletal muscles, especially gastrocnemius muscles of spastic rats, to analyze the condition of spastic gastrocnemius muscles.

METHODS

Design, location and time

This experimental study was conducted on 42 spastic rats that were divided into six groups, and they had been killed. The study was conducted at the laboratory of the Faculty of Veterinary Medicine, IPB University. The histopathological examination was performed in June 2019 and February 2020.

MATERIALS AND TOOLS

Materials

A total of 42 experimental animals, namely male Sprague dawley rats aged 10-12 weeks weighing 200-250g. The material for spastic induction was erythrosine B (ErB) solution at a dose of 80mg/KgBW. Experimental animal feed made at PT Indofeed Bogor. The material used to make spastic rats die under anesthesia was 2mg/kgBW of ketamine xylocaine. Gastrocnemius muscle tissues were taken from the left side of the rats' limbs. The muscle tissues were stored in a 10% neutral buffered formalin (NBF) solution. Gastronomic muscle fibers were stained using hematoxylin and eosin. The solutions required were absolute ethanol, xylol, paraffin, glycerin 99.5%, albumin, hematoxylin, lithium carbonate, and eosin. Alizarin red was used in the staining of calcium stored in gastrocnemius muscle tissue.

Tools

A 3mL syringe was used to insert the intravenous ketamine xylocaine solution. Tweezers and scalpels were used for excision of gastrocnemius muscle. Gastrocnemius muscle tissue was fixated in paraffin and glass preparations. The vacuum and blocking machines, freezer, microtome machine, microtome knives, and water bath were needed to make tissue preparations. Microscope slides, cover glasses, special shelves for staining, and ovens were also needed. The gastrocnemius muscle tissue that had been stained was observed under a transmission electron microscope with a magnification of 100 μM .

RESEARCH PHASES

Feed preparation

Feed was prepared in 2 types, namely standard feed and intervention feed. Standard feed contains 200mg calcium in 20g feed (P3). The intervention feed contained 50mg (P1) and 100mg (P2) calcium in 20g of feed. Calcium solution preparations were made in 3 preparations, namely calcium 100mg, 200mg and 300mg dissolved in distilled water up to 3mL.

Maintenance of experimental mice and ErB Induction

Rats aged 10-12 weeks were adapted for 14 days first. Rats were weighed before and after adaptation. During adaptation, they were given a standard diet containing 200mg of calcium in 20g of feed and drinking ad libitum. On day 15, mice were induced with ErB at a dose of 80mg/KgBW.⁴

Assessment of spasticity

Induction was carried out by inserting 1 mL of ErB dissolved in aquadest through the lateral tail vein. Immediately after induction there were physical changes in the rats, namely changes in the skin and mucosa and body fluids to a pink color. Spasticity was seen significantly with an Asworth scale 4 value and the limb resistance in passive flexion was 100%. Spasticity was assessed before and after the intervention started the next day.

Intervention

A total of 42 rats were divided into 6 groups, including G1 with P1, G2 with P2, G3 with P3, G4 with P3 and 100mg calcium supplementation, G5 with P3 and 200mg calcium supplementation and G6 with P3 and supplementation. calcium 300mg. Calcium

supplementation is given through a sonde. The intervention was carried out for 15 days.

Gastrocnemius muscle sampling

Spastic rats received intracardiac injection using 2mg/kg BW of ketamine xylocaine.⁵ The skin of the left foot was incised along with the attachment of gastrosoleus complex. Gastrocnemius muscle was bluntly separated from soleus muscle. The gastrocnemius muscle tissue was stored in a container containing 5mL of 10% formalin solution.

Making tissue preparations

The tissue was sliced and arranged in tissue cassettes, dehydrated, and dried with a vacuum machine. The tissue was then blocked with paraffin liquid, and the block was cut in a 3-5 μ m thickness with a microtome machine. The tissue slices in the paraffin block were attached to microscopic slides.⁶

Hematoxylin-Eosin Staining

The staining of tissue preparations using hematoxylin and eosin was performed manually. The staining provided a clear balance of blue and red colors in the tissue, and thereby the components of cells in the muscle tissue could be identified.

Alizerin red staining

The staining using 0.25% Alizerin red was performed to identify the presence of calcium in muscle tissue. The muscle tissue was dehydrated and blocked with paraffin using 50% ethanol for 30 minutes at pH 7.0.⁷ The calcium staining in the gastrocnemius muscle by Alizerin red showed an orange-red color. The slide was cleansed with acetone-xylene and covered with permount.⁸

Observation of the preparations using a microscope

The transmission electron microscope (TEM) was used to observe the detailed internal structure of cells in the gastrocnemius muscles of spastic rats. The tissue preparations were observed under the microscope using the smallest magnification (10x10) first to strong magnification (10x40). The observation results were labeled according to the rats' groups and analyzed.

Histopathological analysis

The image of the muscle nucleus in each muscle cell was obtained using hematoxylin-eosin (HE) staining. The cytoplasm or endoplasm of muscle cells appeared pink. The analysis of the spastic state of the tissue indicated that the distance between muscle fibers or sarcomeres was wider, and the diameter of the muscle fibers or sarcomeres in the cross-section looked smaller.

Normally, skeletal muscle (including gastrocnemius muscle) is commonly called muscle fiber. Muscle fibers have a cylindrical

shape and many nuclei at the edges, bordering with sarcolemma. The muscle fibers in animals can reach 12cm with a diameter ranging from 10 μ to 150 μ . Calcium deposits in spastic muscle tissue were obtained using Alizarin red staining. The calcium deposits appeared in purplish-blue.

RESULTS AND DISCUSSION

Histopathological features of muscle tissue using HE staining

Muscle tissue is a tissue that mechanically experiences contraction and relaxation in muscle fibers. Muscle tissue consists of a long array of cells called muscle fiber.⁹ Hestianah et al. have said that special cells of muscle tissue have a special shape associated with contraction activity. The shape of these cells extends to form fibers. Muscle fibers are arranged in bundles, and their axes are parallel to the direction of contraction. In muscle fibers, there are many fibrous proteins in sarcoplasm, which are easy to absorb dyes for cytoplasm.¹⁰

Table 1. Mean muscle fiber diameter and distance between gastrocnemius muscle fiber

Group (G)	The diameter of muscle fibers	Distance between muscle fibers
G1(Ca 50)	4.89 \pm 0.97	0.47 \pm 0.38
G2(Ca 100)	6.19 \pm 2.53	0.71 \pm 0.32
G3(Ca 200)	4.62 \pm 0.98	1.14 \pm 0.49
G4(Ca 300)	5.43 \pm 1.05	1.17 \pm 0.77
G5(Ca 400)	4.76 \pm 1.83	1.05 \pm 0.29
G6(Ca 500)	4.06 \pm 1.94	1.02 \pm 0.53

† The Shapiro-Wilk test (normality test)

Table 1 shows that the diameter of muscle fibers tends to decrease with the higher dose of calcium intake received. The distance between muscle fibers shows a tendency to increase in diameter with a higher dose of calcium intake. The Shapiro-Wilk test (normality test) indicated that the data variance regarding muscle fiber diameter and the distance between muscle fibers had a normal

distribution ($p>0.05$). The homogeneity test showed that there were no differences in variance between the groups of data compared ($p>0.05$). In other words, the data variance was the same. After performing ANOVA, no significant differences were found in the diameter of muscle fibers and the distance between muscle fibers between the groups ($p>0.05$).

Table 2. The results of the correlation test of the diameter of muscle fibers and distance between muscle fibers with spasticity

	Spasticity after intervention \int	Changes in spasticity \approx
The diameter of muscle fibers \approx	0.56**	0.69**
Distance between muscle fibers \int	0.01*	0.05*

*Significant; \int Pearson's test; **No significant; \approx ANOVA

Table 2 shows a correlation between the distance between muscle fibers and spasticity after the intervention ($p<0.05$). The correlation test used was Pearson's test because the data distribution of all groups was normal. Changes in muscle shape occur during contractions. The muscle will shorten and enlarge. The smallest unit of myofibril is called sarcomere, which will shorten during contraction and lengthen during relaxation. A change occurs when the muscle contracts, in which about 50% of the muscle fibers constrict. Muscle contraction is initiated by the release of calcium ions from the sarcoplasmic reticulum. The calcium ions then stimulate the activity of adenosine triphosphate (ATP), and the hydrolysis of ATP molecules into ADP and energy release occur. This energy is used for contraction. Calcium ions as catalyst will be recaptured by

sarcoplasmic reticulum. There are thousands of myofibrils in one skeletal muscle fiber, and each myofibril has hundreds of submicroscopic myofilaments. The nucleus in one skeletal muscle fiber is present in large numbers. The rat's nucleus is long, located at the edge, under the sarcolemma. Each muscle fiber is surrounded by endomysium. Some muscle fibers are enclosed in dense connective tissue with many collagen fibers called fascicles, and the sheath is called perimysium. Outside perimysium is filled with loose connective tissue, which gives leeway for the fascicles to move. Some fascicles combine to form muscles enclosed by dense connective tissue called epimysium, and the fascia is found around the epimysium. Sarcoplasm contains mitochondria (sarcosomes), ribosomes, Golgi apparatus, myofibrils, and endoplasmic reticulum.

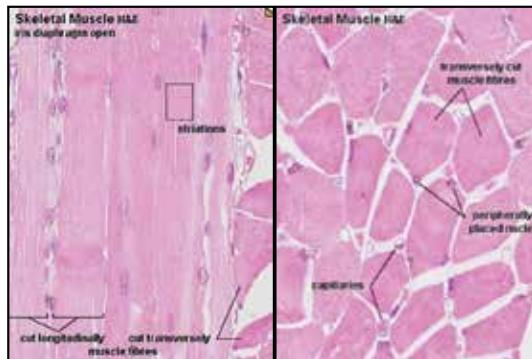


Figure 1. Histological feature of skeletal muscle in a normal rat



Figure 2. Histopathological feature of the gastrocnemius muscle in a spastic rat receiving 50 mg of calcium (S1.2)

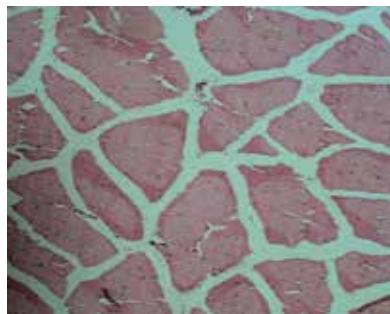


Figure 3. Histopathological feature of the gastrocnemius muscle in a spastic rat receiving 100 mg of calcium (S2.6)

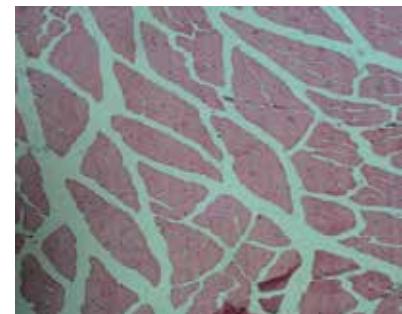


Figure 4. Histopathological feature of the gastrocnemius muscle in a spastic rat receiving 200 mg of calcium (S3.5)



Figure 5. Histopathological feature of the gastrocnemius muscle in a spastic rat receiving 300 mg of calcium (S4.4)

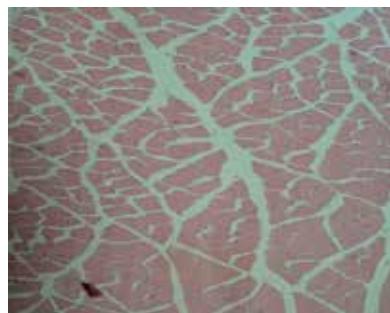


Figure 6. Histopathological feature of the gastrocnemius muscle in a spastic rat receiving 400 mg of calcium (S5.3)



Figure 7. Histopathological feature of the gastrocnemius muscle in a spastic rat receiving 500 mg of calcium (S6.3)

Histopathological features of muscle tissue using Alizarin red staining

The histological features of gastrocnemius muscle tissue in spastic rats after being stained with Alizarin red and observed using an electron microscope with a magnification of 10x40

showed calcium deposits in darker purplish-blue muscle tissue. This result was in line with a study by Harari, who obtained large dark fibers (hypercontracted fibers) or necrotic fibers in dogs with spastic quadriplegia after biopsy and Alizarin red staining were performed.¹¹

The values of calcium deposits in the gastrocnemius muscle tissue of experimental animals were calculated by multiplying the distribution of the amount of calcium with color intensity. The calculation results are presented in Table 3.

Table 3. Mean value of calcium deposits in gastrocnemius muscles

Group (G)	Mean±SD	P-
G2 (Ca 100)	1.57±0.79	0.001*
G3 (Ca 200)	2.42±0.98	0.001*
G4 (Ca 300)	1.72±1.89	0.001*
G6 (Ca 500)	6.14±3.34	0.001*

*Significant; - Kruskal-Wallis test

Table 3 shows that the largest calcium deposit in gastrocnemius muscle is found in G6 or the group of spastic rats receiving the highest calcium intake (600mg). G1 and G5 could not be statistically analyzed because the calcium deposit was not found when observed with an electron microscope with a 10x40 magnification. There were two groups with abnormal data distribution, and thereby the Kruskal-Wallis test was performed to analyze the differences. Significant differences ($p=0.001$) were found between the groups with different calcium intakes using the Alizerin red staining. There was a difference in the variance between the groups of data compared (the data variance was not the same) in the homogeneity test ($p=0.001$).

The Spearman's correlation test indicated significant differences between spasticity after the intervention ($p=0.008$) and changes in spasticity ($p=0.015$) with Alizerin red staining.

CONCLUSION

The HE staining was used to determine the composition of muscle tissue and parts of the cytoplasm. The observation using an electron microscope with a 10x10 magnification showed that the diameter of muscle fibers tended to decrease with the higher dose of calcium intake received, while the distance between muscle fibers showed a tendency to increase in diameter with a higher dose of calcium intake. A significant correlation was found between the distance of muscle fibers and spasticity after intervention ($p<0.05$).

The Alizerin red staining was used to detect calcium deposits in the muscle tissue. It can be concluded that calcium deposits in the gastrocnemius muscle of spastic rats were most significantly found in the group of spastic rats receiving the highest calcium intake. Significant differences were found between groups ($p=0.001$), and significant correlations were found between spasticity after the intervention ($p=0.008$) and changes in spasticity ($p=0.015$) with the Alizarin red staining.

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