Histological structure of nerve fiber and blood vessels in regenerated tail of Tokay gecko (*Gekko gecko* (Linnaeus, 1758)

Cite as: AIP Conference Proceedings **2260**, 030010 (2020); https://doi.org/10.1063/5.0015764
Published Online: 16 September 2020

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Histological Structure of Nerve Fiber and Blood Vessels in Regenerated Tail of Tokay Gecko (*Gekko gecko* (Linnaeus, 1758)

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Abstract. *Gekko gecko* (Tokay Gecko, Tokek) has the ability to perform autotomy and regenerate its tail. There is not a lot of research that has been conducted on tail regeneration of *G. gecko* in Indonesia. Wound healing and regeneration methods in geckos are relatively similar to mammals, so geckos can be a biomedical research model suitable for regeneration research. Therefore, further research on the regeneration of tails using geckos needs to be done. The purpose of this research is to study the structure and development of nerve fibers and blood vessels in regenerated tail of *G. Gecko*. A total of 33 geckos were autotomized and then kept in a fiber plastic terrarium. Measurements of body weight and length were taken, and then 2 cm of the tail were taken from the tip of the autotomy on 0, 4, 8, 12, 16, 20, 28, 48, 56, 70, and 84 days after autotomy with three repetitions. Histological slides were made using the paraffin method after going through decalcification process to soften the bone tissue and stained with Hematoxylin-Eosin and Bodian's Gold Chloride staining. The structure and development of nerve fibers and blood vessels were observed descriptively and comparatively. The results showed the nerve fibers firstly appear on the 8 dpa regenerated tail and reach the highest number on the 16 dpa regenerated tail. Their number is subsequently decreasing afterward. Meanwhile, the number of blood vessels per field of view in the regenerated tail are decreasing after their first appearance on the 12 dpa, which has the greatest amount of blood vessels, and reach the lowest number on the 84 dpa.

INTRODUCTION

The nervous system plays a role in integrating a body's communication network. In general, the nervous system is divided into the Central Nervous System (CNS) which consists of the brain and spinal cord, and the Peripheral Nervous System (PNS) consisting of cranial nerves, spinal nerves, peripheral nerves and ganglia. The circulatory system plays a role in pumping and delivering blood and substances carried by the blood to all of the body tissues. Organs that play a role in the circulatory system are the heart, arteries, capillaries, and veins [1]. The nervous system and blood vessels associated with each other in the body of animals since the embryonic development. This association serves to flow oxygen and nutrients to the nerves and other tissues, and nerves control the course of vascularization in the body [2].

Gekko gecko, or commonly known as tokay gecko, is a species of the Gekkonidae family whose entire group has a fragile tail [3]. *G. gecko* has a rough backside and many large nodules on the body, has red or orange spots on the dorsal part, and are capable of performing caudal autotomy [4,5]. Autotomy comes from the Greek *autos* = "self" and *tomos* = "cutting" which refers to the voluntary action of self-amputation that occurs in a controlled manner along the fracture plane [6]. Caudal autotomy is a common and effective mechanism of avoiding predators used by most groups of lizards based on biological adhesion between segments [7,8].

Regeneration is an ability possessed by organisms to repair, replicate, and grow tissues, structures, and organs that are lost or damaged without forming a scar and the growth is integrated by the original tissue (not the damaged one) [9–11]. An autotomized gecko tail will experience a tissue-specific program that is almost perfect for replicating lost

The 6th International Conference on Biological Science ICBS 2019
AIP Conf. Proc. 2260, 030010-1–030010-11; https://doi.org/10.1063/5.0015764
Published by AIP Publishing. 978-0-7354-2020-5/$30.00

or damaged tails, which is called tail regeneration [10]. The tail will undergo a tail regeneration period which is divided into several phases. The speed and response of regeneration after autotomy may be different among types of animals [12]. Regenerated tail of lizard has different forms in terms of morphology and histology [11]. The nervous system in the original tail of the lizard consists of the spinal cord and dorsal root parasagittal with the spinal cord association. During autotomy, the spinal nerves are pulled out and then detached [13].

In general, functional recovery after nerve injury involves a series of complex steps, each of which can delay or damage the regenerative process if it is disturbed. The regeneration sequence is divided into several anatomical zones, namely the body of nerve cells, segments between the nerve cell body and the injury site, the injury site, the distal segment between the injury site, and the organs [14]. The difference between the nerves of the regenerated tail and the original tail lies in the spinal cord of the regenerated results that does not have white matter and gray matter. Besides, the dorsal root ganglia do not regenerate. Therefore, the spinal nerves grow from the proximal intact tail ganglia to stimulate new limbs [13]. The failure of the lizard's tail regeneration can be caused by the massive reduction of the nerve fibers on the tail [15].

Blood vessels that grow in regenerating tails do not have sphincter [16]. Sphincter in the regenerating tail serves to cause slight bleeding or fluid loss that occurs when the tail is released [12]. Nerve tissue growth is also more common in the early phases of blastema formation. The nerve tissue components that are seen in the regenerated tail are ependymal tubes and axons originating from the original spinal cord of the tail and dorsal root ganglia [16].

METHODS

This study has acquired ethical approval from Laboratorium Penelitian dan Pengujian Terpadu (LPPT) Universitas Gadjah Mada with certificate number: 00017/04/LPPT/V/2016. This research was conducted from June 2016 to August 2017. Specimens of the adult tokay gecko were collected from the animal market and the neighborhood around the Special Region of Yogyakarta. The specimen's maintenance and observations were carried out in the Laboratory of Animal Structure and Development at the Faculty of Biology, Universitas Gadjah Mada, Indonesia. The specimens were acclimatized for 2 weeks in the integrated wooden framed and iron wired cage consisting of 20 individual cages with the measuring of 30 cm x 30 cm x 30 cm each. The cage was placed in a room that is exposed to sunlight and has a good air circulation. The specimen's tail autotomy was induced by clamping the thumb and index finger on the tail, then rotating the tail until voluntarily separated in the fracture plane. The original tails were left to regenerate in several sequence of time, which are 4, 8, 12, 16 and 20 days post-autotomy (dpa) and also 4, 6, 8, 10, and 12 weeks post-autotomy (wpa).

Morphological Observation

The body mass and length of the geckos were measured. Tails that have regenerated in a certain time sequence was observed. Tail's pictures were taken using a Canon DSLR camera. The image taken is the dorsal and posterior part of the tail. Morphological observation was conducted objectively by comparing each sample of the tail that has been regenerated at different ages.

Tissue Processing

The regenerated tail samples were fixed with neutral buffer formalin for 12 hours. The samples were decalcified by immersing them in alcoholic 3% HNO_3 solution and then washed with 70% alcohol several times in every 3 hours. Histological slides of regenerated tail samples were subsequently proceed to the paraffin method [17–20] procedure. In brief, the samples were dehydrated using upgraded concentration of alcohol (80%, 90%, 95%, 100%) and then cleared in toluene immersion. After 5-6 of toluene soaking, the samples were treated with series of paraffin infiltration in the oven. The paraffin infiltrated samples were subsequently embedded in freshly melted paraffin on embedding mold and were left overnight. The paraffin blocks containing samples were trimmed and sectioned diagonally with rotary microtome in 6 microns of thickness. The paraffin sections were placed on the water bath containing glycerin albumin and were then transferred into glass slides. The slides were then stained using Bodian's Gold Chloride and Hematoxylin Eosin. Afterward, the histological slides of regenerated tail's were observed using a microscope.

Data Analysis

The histological slides of the regenerated tails were observed qualitatively and quantitatively, especially the nerve fibers (axons) and blood vessels. The development and growth of nerve fibers and blood vessel of each stages were observed. The results of the study were histological descriptions of each development stage. Quantitative data, including the number of nerve fibers and blood vessel that appeared in each time sequence, were then analyzed using ANOVA (Analysis of Variable) charts and tables.

RESULT AND DISCUSSION

Nerve Fibers in Regenerated Tail of Tokay Gecko.

From the observation, it is shown that the axons appear like small channels that extend longitudinally from proximal to distal (Figure 1). The ependymal tube is composed of layers of ependymal cells which are columnar and coated by cartilage. The tail spinal cord can regenerate producing a simple ependymal tube surrounded by several hundred descending axons and several special fluid-connecting neurons [21].

A blood clot occurs at the tip of 4 dpa regenerated tail. The remaining severed tail is still attached to the clot. The blood clot consists of tissue fluid, erythrocytes, dead cells, and tissue debris. The process of wound healing is more focused at this stage and was initiated by the formation of the wound epithelium. The nerve fibers have not been extended yet. The wound epithelium partially covers the wound on the gecko's tail. At this stage, the blastema begin to appear in an irregular polygonal shape. In Gekkota, blastema is formed as an apical aggregation of proliferating cells [22].

There is an extension of axons from the original tail to the regenerated tail at the 8 dpa (Figure 1 and Figure 2). The number of nerve fibers found is still small of which directions cannot be determined due to very small growth of the regenerated tail. The nerve fibers have not innervated the growing blastema. The 8 dpa regenerated tail shows a blood clot and the epithelium of the wound had covered the entire wound at the tip of the tail. The epithelium formed several layers and thickness. When all parts of the wound are covered by the epithelium, the blastema will be preserved by blood vessels and nerve tissue (ependyma tubes and axons) [16]. On the 12 dpa regenerated tail, the blastema spread at the tip of the tail and enlarged. From this stage, the nerve fibers innervate the blastema and the number of nerve fibers increase as the enlargement of blastema. In addition, the ependymal tube begins to grow distally. The middle part of the tail canal grows from the spinal cord and nerve fibers from the dorsal root ganglia found in the original tail innervated the blastema in gecko's regenerated tail [16].

The tip of the tail forms a dome with the ependymal tubes growing on the 16 regenerated tail and increasingly penetrate the blastema toward the distal. The number of nerve fibers are also increasing which helps in the treatment of blastema and formation of epithelium and dermis tissue. The diameter of the regenerated tail was greater than of which length, therefore, the tail has not elongated, but the tissues in the tail regenerate began to differentiate. The amount of nerve fibers in this stage is the highest among all stages.

The 20 dpa regenerated tail begins to elongate towards the caudal indicated by the diameter of the tail regenerate which is smaller than of which the length. The most widespread nerve fibers are found in the cranial part of regenerated tail and become less in the caudal part. The number of nerve fibers is less than the previous stage (Figure 5). The nerve fibers continue to extend towards the end part of regenerated tail and innervate the blastema.

The 28 dpa regenerated tail is getting longer and wider. The cranial, median and caudal parts of regenerated tail are overgrown with relatively equal amounts of nerve fibers. Nevertheless, the number of the observed nerve fibers in one field of view is decreasing. The ependymal cell layer has been coated by a cartilage tube. The cartilage tube also surrounds the nerve fibers. The nerve fibers begin to innervate the newly formed muscle tissue and adipose tissue in 42 dpa regenerated as all tissues experience differentiation (Figure 3). The number of nerve fibers on 42 dpa regenerated tail is decreasing (Figure 5). Nerve fibers continued to differentiate and innervated the newly formed tissues.

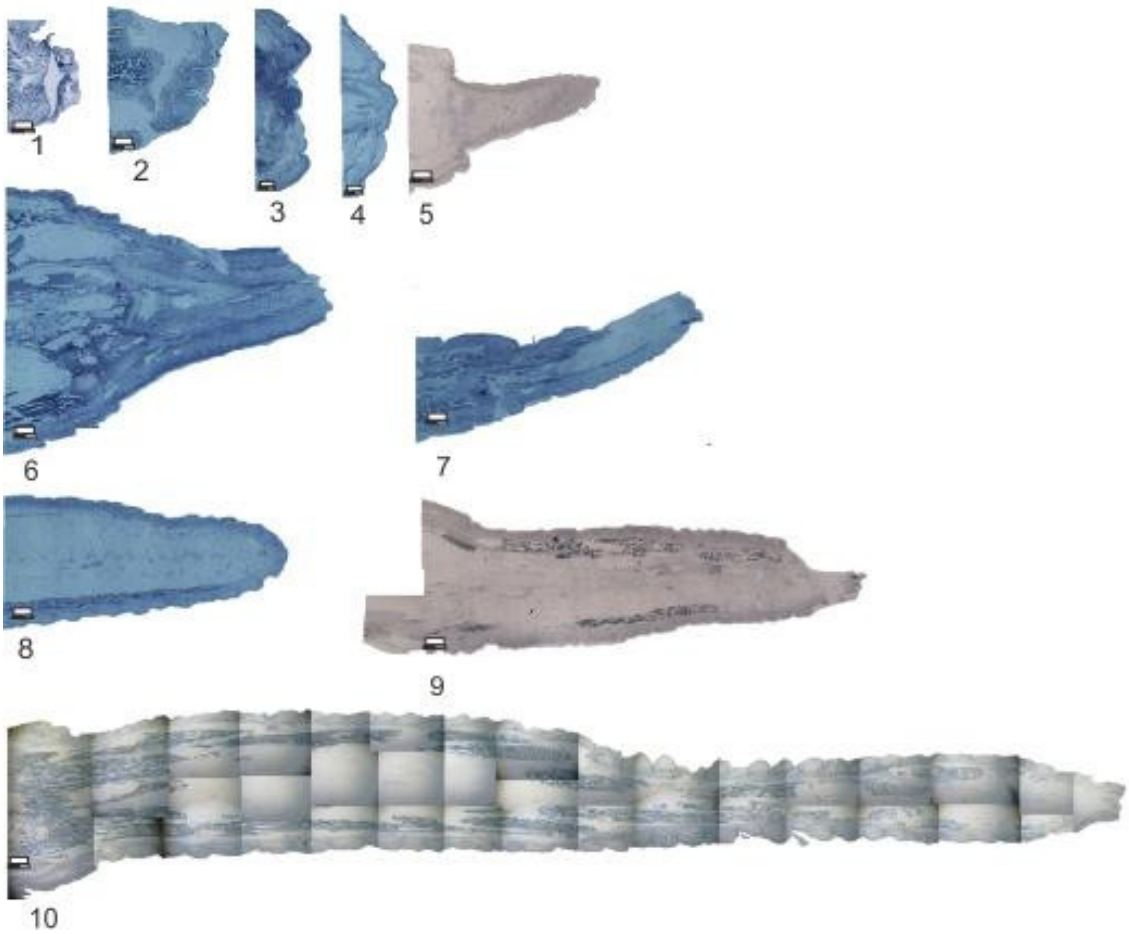

FIGURE 1. Histological overview of regenerated tail of tokay gecko (*Gekko gecko*) at 4 dpa (1), 8 dpa (2), 12 dpa (3), 16 dpa (4), 20 dpa (5), 28 dpa (6), 42 dpa (7), 56 dpa (8), 70 dpa (9), and 84 dpa (10) with Bodian's Gold Chloride (scale bar: 200 µm).

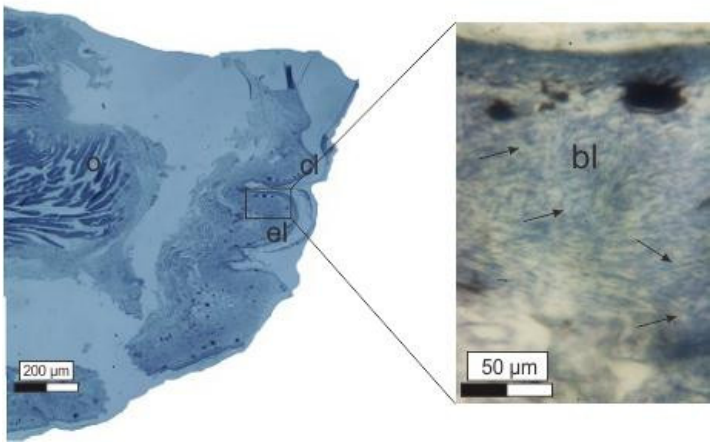

FIGURE 2. Histological structure of of regenerated tail of tokay gecko (*Gekko gecko*) at 8 dpa in low magnification (left) and high magnification (right). Elongation of nerve fibers is indicated by arrows. Bodian's Gold Chloride for nerve fibers staining. *bl: blastema, cl: clot, el: epithelium wound.*

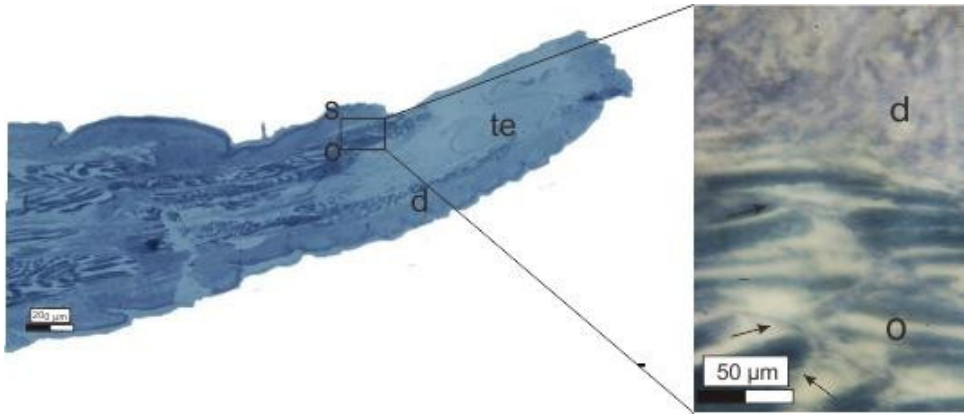

FIGURE 3. Histological structure of of regenerated tail of tokay gecko (*Gekko gecko*) at 42 dpa in low magnification (left) and high magnification (right). Elongation of nerve fibers is indicated by arrows. Bodian's Gold Chloride for nerve fibers staining. *d: dermis, o: muscle, s: scales, te: ependymal tube.*

The 56 dpa regenerated tail is elongating of which cranial width is similar with that of the original tail. The skeletal muscles begin to grow distally and the axons also innervate the distal part of them. The developed muscle at the base of the tail affect the presence of the innervated nerve fibers. The nerve fibers at the proximal part are more specialized than the nerve fibers at the distal part. The number of nerve fibers or axons on 56 dpa regenerated tail are relatively equal in each part (cranial, median and caudal). The appearance of 86 dpa regenerated tail begin to morphologically resemble the original tail, especially in the pattern and the color of the tail. It is caused by many pigment cells (melanocytes) found in the epithelium. Melanocytes are dark cells which have melanin-containing vesicles called melanosomes [23]. The nerve fibers in this stage of regenerated tail are spread throughout every part of the tail. The nerve fibers continued to innervate the tissues in the regenerate that form up to the distal part of regenerated tail (Figure 4). The innervation of nerve fibers to the tissue target maintain cell proliferation and tissues function.

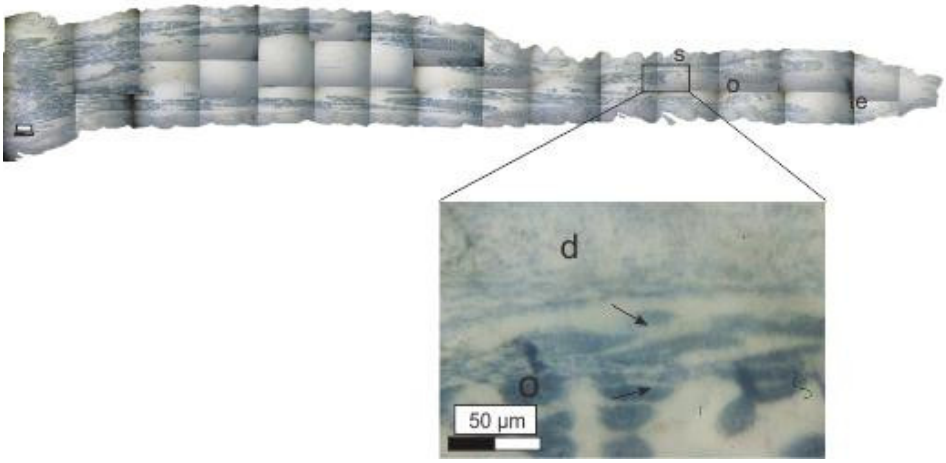

FIGURE 4. Histological structure of of regenerated tail of tokay gecko (*Gekko gecko*) at 84 dpa in low magnification (left) and high magnification (right). Elongation of nerve fibers is indicated by arrows. Bodian's Gold Chloride for nerve fibers staining. *d: dermis, o: muscle, s: scales, te: ependymal tube.*

The nerve fibers in regenerated tail of *G. gecko* have the different number per field of view on every stage of development (Figure 5). Nerve fibers firstly appear on the 8 dpa regenerated tail. Most nerve fibers were found on 16 dpa regenerated tail with a total of 22 nerve fibers per field of view, but then the number of nerve fibers decreased. The lowest number of nerve fibers is 6 nerve fibers in each field of view in 56 dpa regenerated tail. The number of

nerve fibers that most appeared on 8-16 dpa regenerated tails is caused by the nerve fibers that trigger the growth and multiplication of cells at the tail end, especially for blastema growth [16]. From the 20th day onwards, the number of nerve fibers was decreased due to thickening and lengthening of nerve fibers to accelerate the transmission of signals from the central nerve to the tail end.

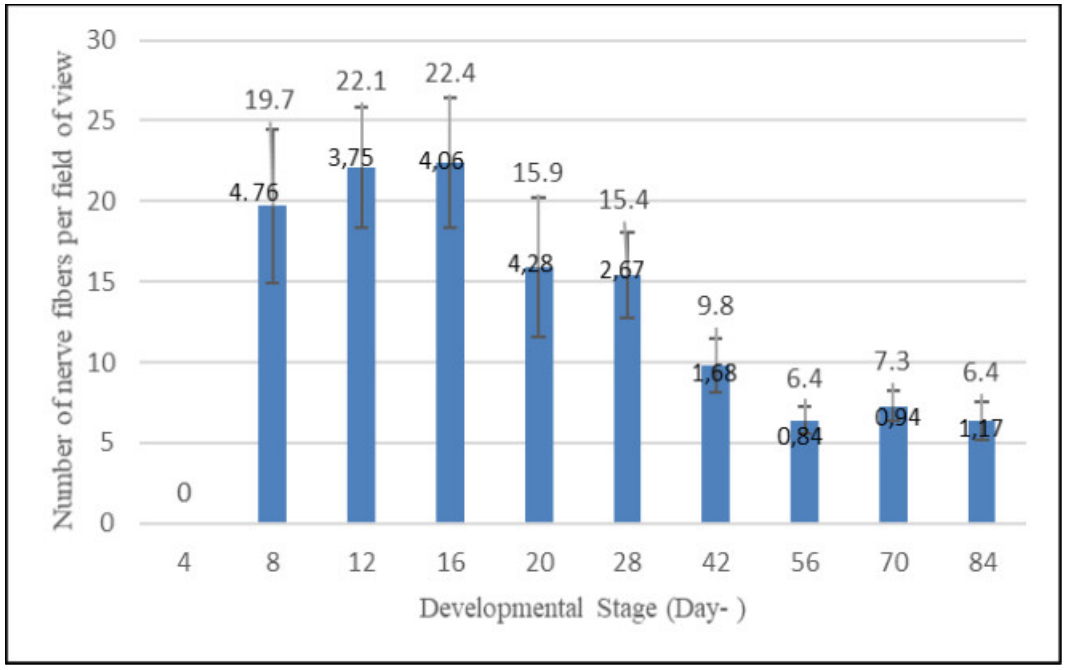

FIGURE 5. The number of nerve fibers per field of view in the regenerated tail of tokay gecko (*G. gecko)*

The peripheral nervous system of the lizard's regenerated tail includes the elongation of nerve fibers and ependymal tubes. Elongation of nerve fibers from the original tail ganglia will innervate the tissue in the regenerated tail and the ependymal tube serves to replace the spinal cord in the original tail. The growth of nerve fibers is in line with the growth of the ependymal tube and almost simultaneously with the development of the blastema at the distal part of regenerated tail. In this study, visible growth of nerve fibers in regenerated tail began on 8 dpa. The nerve fibers are still small and begin to innervate the blastema resulting on the larger and well-organized growth of blastema compared to the previous stage. The regenerated tail development is triggered by the regeneration of peripheral nervous system (including the ganglia dorsale), which includes the migration of cells originating from the spinal cord, specifically from ependymal cells [24,25]. Elongation of nerve fibers is originated from the original tail spinal cord, which is located close to the basal membrane in each ependymal cell.

The growth of nerve fibers in regenerated tail multiply until the 16 dpa and start to decrease on the 20 dpa. The 16 dpa regenerated tail shows differentiated blastema into epithelial tissue with 4 layers, namely the corneum layer, the granulosum layer, the spinosum layer, and the germinativum layer. The outermost layer, the corneum layer, contains a large amount of resistant β-keratin [13]. The epidermal component of lizard skin is a complex capable of producing epidermal generation cycles that synthesize α- or β-keratin [26]. In addition, the dermis began to appear at this stage. The number of nerve fibers on the 16 dpa regenerated tail is greater than any other stage, and it may affects the differentiation of blastema into specific tissue. Nerve fibers are associated with epithelium tissue, muscle tissue, and connective tissue. Axon genesis is in line with the growth of the blastema and the increasing length of the tail regenerate and is also in line with the increasing length of the nerve fibers. That way, each tissue in the regenerated tail can receive sensory and motor innervation from the original tail [16].

The number of nerve fibers is decreasing in the 20 dpa regenerated tail. However, the nerve fibers underwent elongation and begin to thicken and lengthen. Nerve fibers of the regenerated tail will pass through the mesenchymal cells between cartilage tubes, myoblast, and epithelial cells. The 42 regenerated tail start to form scales while of which number of nerve fibers tend to decrease due to nerve fibers differentiation and innervation towards the regenerated tail tissues. Some nerve fibers reach the dermis and immediately followed by skin keratinization.

Over time, nerve fibers experience elongation, thickening, and differentiation. The thickened nerve fibers affect the speed of signal transmission. The growth of nerve fibers is very important in the signal transmission from the original tail to the regenerated tail. In the original tail, peripheral nerves appear from each vertebrae segment along the tail and innervate the surrounding tissue. In contrast to the regenerated tail, the peripheral nerves of the original tail will elongate and invade the target tissue despite its distance [13,27]. Nerve fibers are able to interfere muscle differentiation by producing neurotrophic factors [28]. These neurotrophic factors can be produced by various structures in the peripheral nerves and can affect the peripheral nerves themselves, one of which is BNDF (Brain Derived Neurotrophic Factor). BNDF is produced by Schwann cells and degenerating muscle fibers (inhibition of a nerve) and plays a role in the formation of myelin membranes and elongation of the nerve fibers during regeneration in Rodentia [29].

Blood Vessels in Regenerated Tail of Tokay Gecko.

Types of blood vessels in tokay geckos are the same as blood vessels in general which consist of arteries, veins, and capillaries. Arteries in the regenerated tail of tokay gecko have no sphincter muscles [13] and the muscle walls are thinner than that of original tail. The veins in the tail regenerate are also different from the veins in the original tail. The veins in the regenerated tail do not have valves in their lumen and the muscle walls are thinner. The regenerative capillaries are relatively similar in structure. The original tail stump undergoes wound healing without scars, characterized by a leukocyte response but little inflammation and no fiber. Leukocytes, especially granulocytes secreting antimicrobial peptides, combined with exudate clot formation provide a temporary barrier to the entry of pathogens [13].

Generally, the number of blood vessels per field of view in the regenerated tail are decreasing after their first appearance on the 12 dpa. The 12 dpa regenerated also has the greatest amount of blood vessels while the 84 dpa regenerated tail has the least blood vessels (Figure 6). The blood clots occur in 4 dpa regenerated tail (Figure 7). The blood vessels have not yet grown at the tip of the tail, but the wound has been covered by the wound epithelium so the injured tail does not bleed. Blood clots were found at the end of the tail [13]. The blood vessels still do not appear on 8 dpa regenerated tail. The wound epithelium covered all of the wound and the blastema grew larger.

Many blood vessels grow at the tip of the 12 dpa regenerated tail near the wound epithelium (Figure 7 and Figure 8). Vascularity in blastema is very common in this stage. This is related to the function of the circulatory system which supports the nutrition and oxygen of the newly formed tissue [1,30]. The blood vessels on the blastema vascularized in the early stages of regeneration after the wound was completely close to the proximal tail [16]. The blood vessels are relatively small but plentiful around the blastema. Figure 8 shows the appearance of blood vessels in the blastema of regenerated tail. Blood vessels appeared to spread towards the tip of the tail.

The number of blood vessels in 16 dpa regenerated tail is less than the previous stage (Figure 6 and Figure 9). Blood vessels spread at the tip of the tail and continued to vascularize the blastema with various diameter of blood vessels (Figure 9). The walls of the blood vessels around the blastema begin to thicken in this stage. The elongation of the20 dpa regenerated tail is accompanied by the elongation of blood vessels towards the tip of the tail (Figure 10). The blood vessels start to dilate, but the number of blood vessels observed is getting smaller in one field of view. The diameter of the blood vessels is getting bigger. At the distal end of the tail, blastema is still found and there are many blood vessels inside the blastema which vascularized the blastema and the surrounding tissue.

The regenerated tail get wider and longer on the 28 dpa. The blood vessels begin to vascularize the dermis. The number of blood vessels decrease in one field of view, but more new tissues are vascularized. The 42 dpa regenerated tail is also getting longer and of which scales are keratinized. Vascularization is abundant in the dermis and connective tissue. The blood vessels of 56 dpa regenerated tail On day 56 are visible with larger size while that of 70 dpa regenerated tail are longer at the distal parts. Vascularization start to reach the adipose tissue. The tissues on 84 dpa regenerated tail are mostly differentiated but the number of blood vessels per field of view is the least (Figure 11).

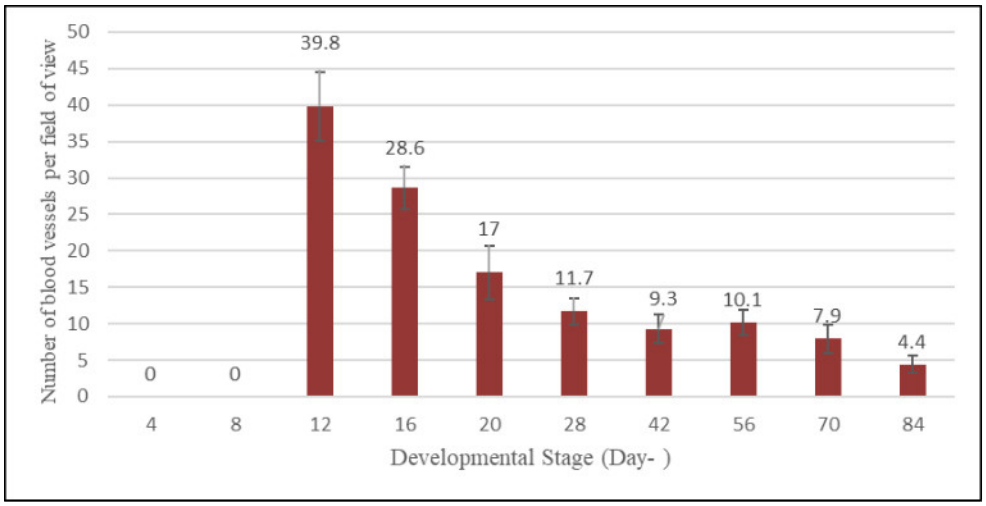

FIGURE 6. Number of blood vessels per field of view in the regenerated tail of tokay gecko (*G. gecko)*.

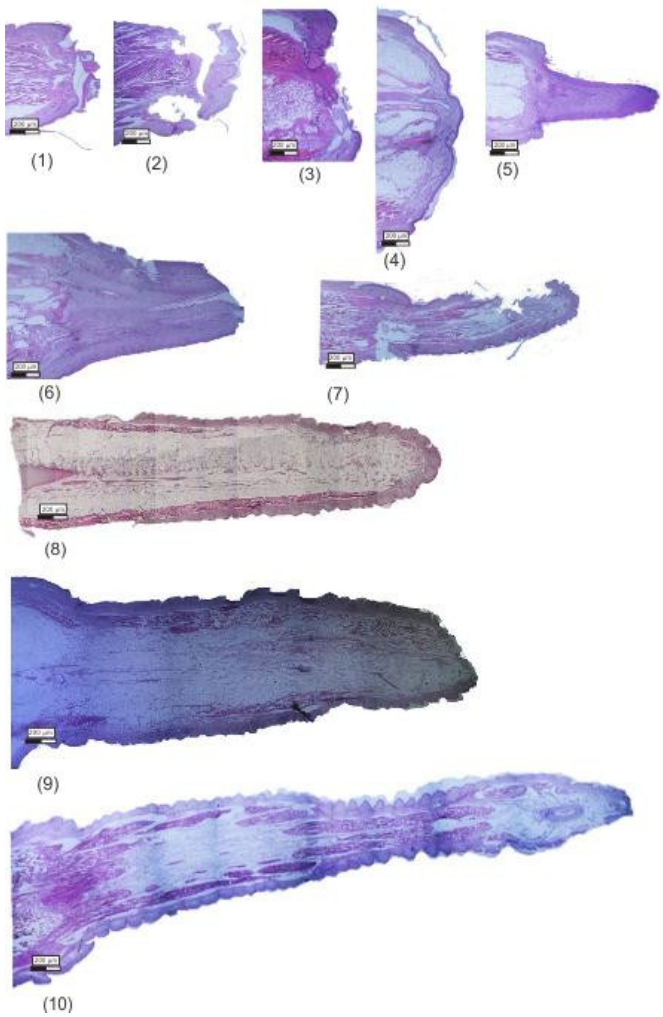

FIGURE 7. Histological overview of regenerated tail of tokay gecko (*Gekko gecko*) at 4 dpa (1), 8 dpa (2), 12 dpa (3), 16 dpa (4), 20 dpa (5), 28 dpa (6), 42 dpa (7), 56 dpa (8), 70 dpa (9), and 84 dpa (10) with Hematoxylin Eosin staining (scale bar: 200 µm).

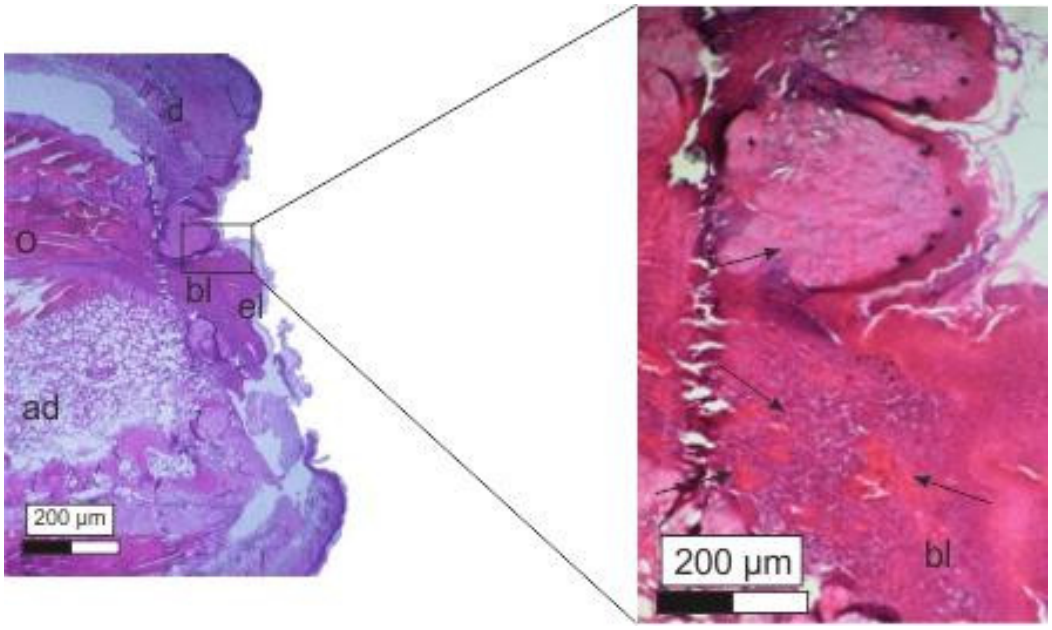

FIGURE 8. Histological structure of of regenerated tail of tokay gecko (*Gekko gecko*) at 12 dpa in low magnification (left) and high magnification (right). Blood vessels are indicated by arrows. Hematoxylin Eosin (HE) staining. *ad: adipose, bl: blastema, d: dermis, el: wound epithelium, o: muscle.*

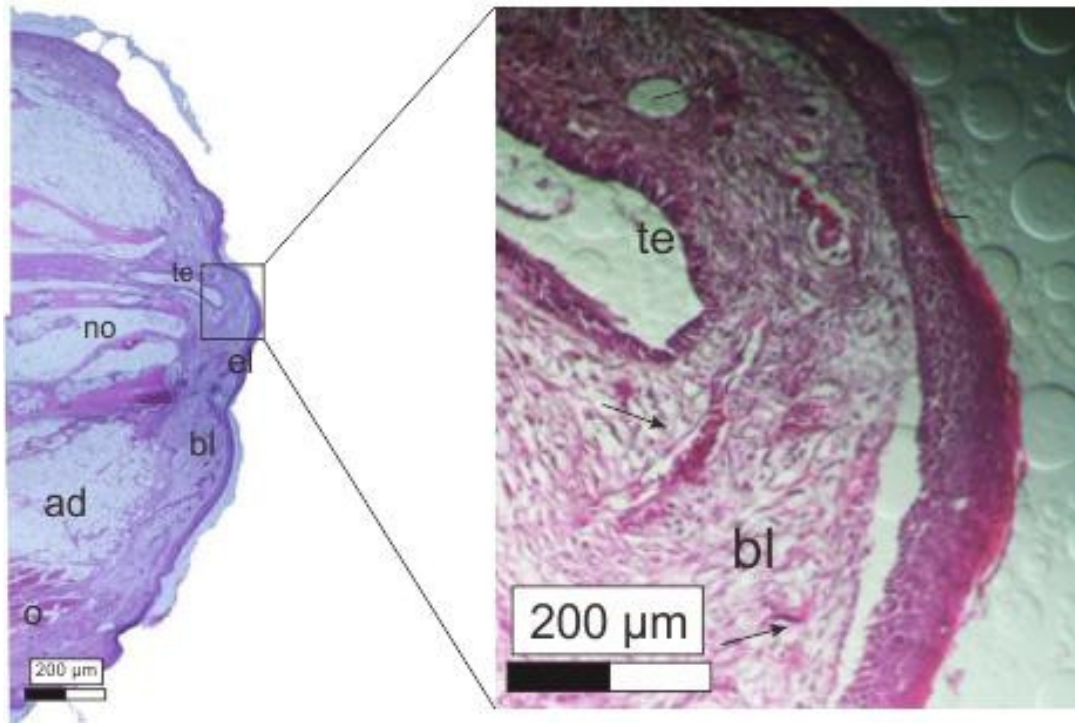

FIGURE 9. Histological structure of of regenerated tail of tokay gecko (*Gekko gecko*) at 16 dpa in low magnification (left) and high magnification (right). Blood vessels are indicated by arrows. Hematoxylin Eosin (HE) staining. *ad: adipose, bl: blastema, el: epithelium wound, no: notochord, o: muscle, te: ependymal tube.*

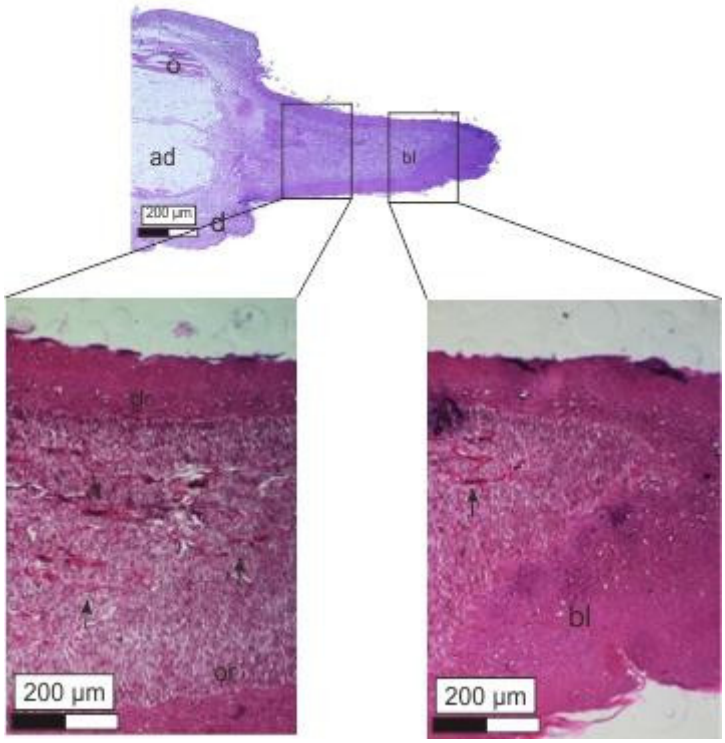

FIGURE 10. Histological structure of of regenerated tail of tokay gecko (*Gekko gecko*) at 20 dpa in low magnification (above) and high magnification (bellow). Blood vessels are indicated by arrows. Hematoxylin Eosin (HE) staining. *ad: adipose, bl: blastema, d: dermis, o: muscle.*

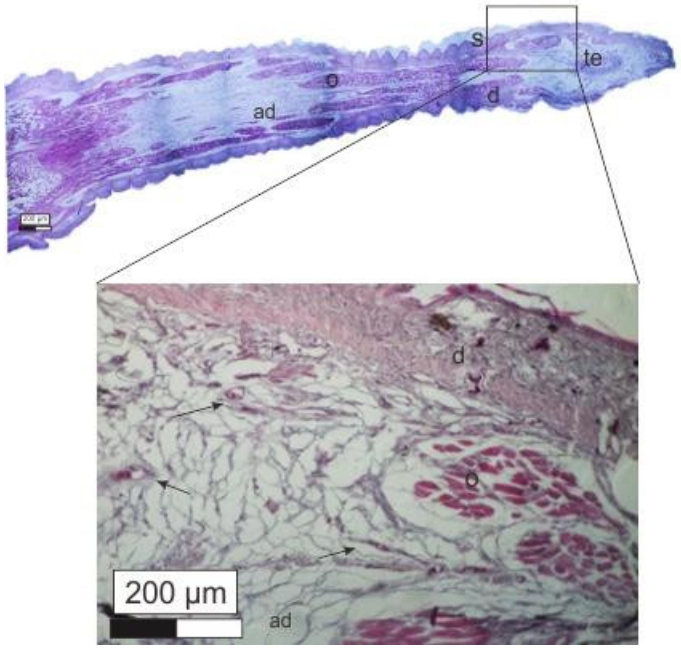

FIGURE 11. Histological structure of of regenerated tail of tokay gecko (*Gekko gecko*) at 84 dpa in low magnification (above) and high magnification (below). Blood vessels are indicated by arrows. Hematoxylin Eosin (HE) staining. *ad: adipose, d: dermis, o: muscle, s: scales, te: ependymal tube.*

CONCLUSION

The number of nerve fibers and blood vessels in the regenerated tail fluctuate among regeneration stages. Nerve fibers firstly appear on the 8 dpa regenerated tail and reach the highest number on the 16 dpa regenerated tail. Their number is subsequently decreasing afterward. Meanwhile, the number of blood vessels per field of view in the regenerated tail are decreasing after their first appearance on the 12 dpa, which has the greatest amount of blood vessels, and reach the lowest number on the 84 dpa.

ACKNOWLEDGEMENT

This research was partially funded by Hibah Penelitian Kolaborasi Dosen dan Mahasiswa Faculty of Biology Universitas Gadjsah Mada 2018.

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