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Cite as: AIP Conference Proceedings **2260**, 030009 (2020); https://doi.org/10.1063/5.0015759
Published Online: 16 September 2020

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The Development of Integument and Muscle in Regenerated Tail of Tokay Gecko (*Gekko gecko* Linnaeus, 1758)

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Abstract. *Gekko gecko* has the ability to perform autotomy and regenerate its tail, including the integument and muscle. Further study regarding both developments of muscle and integumentary tissue in the regenerated tail of *G. gecko* is needed. This study aims to determine the muscle and integumentary tissue in several stages of tail regeneration in *G. gekko*. The regenerated tail was collected by inducing autotomy according to specified period: 4, 8, 13, 16, 20, 28, and 56 days post-autotomy for the integument; and 12, 14, 16, 18, 20, 28, 42, 56, 70, and 84 days post-autotomy (dpa). The samples were fixed using neutral-buffered formalin and decalcified. The samples were processed using paraffin method and stained with Periodic Acid Schiff-Alcian Blue (PAS-AB) for the muscle tissue, Mallory Acid Fuchsin (MAF) for the integument tissue, and Hematoxylin-Eosin (H&E) for both tissues. The samples were then observed using microscope and analyzed descriptively. The results showed that muscle tissue started to develop on the 14th dpa, marked by the presence of myoblast which will form myotube on the 20th day. The muscles underwent a maturing phase where myotubes started to group and formed myofibrils on the 42th until 84th dpa. The regenerated tail has a smaller diameter and elongated shape of muscle cells. The epidermal tissues started to proliferate in the 4th dpa. Blastema started to form on 8th dpa. Epidermis became flattened on the 13th dpa, and keratinization was beginning to start on the 16th dpa.

INTRODUCTION

Indonesia is a tropical country and has an extensive tropical rainforest. There are various types of flora and fauna that are widespread in the tropical rainforest. This variation caused this fauna to have various types of adaptation to support survivability and competition in nature. One interesting type of self-defense is autotomy, an ability possessed by the Gekkota clade. Autotomy is a unique ability where an individual can cut off a part of his own body as a self-defense mechanism. Autotomy can occur in various locations depending on the organism [1]. Organisms that are able to break off their tails as a self-defense mechanism, such as the family Gekkonidae, Eublepharidae, and Scincidae (clade Gekkota), are called caudal autotomy [2–7]

Caudal autotomy occurs when muscles contract around a fracture plane that lies between the vertebrae or intravertebral [8–10]. The network around the fracture plane will be cut off so the tail starts to break as well. At that time, the sphincter muscle in the tail artery begins to contract to avoid massive bleeding [11]. An autotomized tail will then regenerate and form a new tail that almost resembles the original tail. The tail has various functions in supporting their life to survive, namely for balance, agility, social status, and as a place to store fat. Therefore, tail regeneration needs to occur [5,11–14].

Regeneration occurs when there is injury or loss of most organs or tissue. The regenerative capability is limited to only a few groups of animal such as urodeles, anuran, lizards, and fish like zebrafish [7,15]. There are several types of regeneration. Based on its frequency, regeneration is divided into two, namely physiological regeneration and

The 6th International Conference on Biological Science ICBS 2019
AIP Conf. Proc. 2260, 030009-1–030009-11; https://doi.org/10.1063/5.0015759
Published by AIP Publishing. 978-0-7354-2020-5/$30.00

reparative regeneration. Based on cell multiplication, there are two types of regeneration, namely epimorphosis and morphallaxis. Regeneration in the autotomized lizard's tail is classified as epimorphosis and reparative regeneration which involves cell multiplication within regeneration process to repair the damaged tissue [1,16].

There are several members of lacertilians who have the ability to do caudal autotomy that has been commonly studied regarding the process of cell regeneration in the tail after autotomy, such as *Eublepharis macularius*, *Podarcis sicula*, and *Anolis carolinensis* [4,15,17–20]. Generally, the three species have four stages of regeneration: wound healing (0-10 days after autotomy), blastema cone formation (10-15 days after autotomy), tail growth (15-25 days after autotomy), and tail maturing (25-60 days after autotomy) [1,15,18]. Gecko (*Gekko gecko*) is a reptile that belongs to the class Gekkonidae, so it has the ability to do caudal autotomy [21]. However, there has not been much further research on tail regeneration in *Gekko gecko*, so this study aims to determine the development of muscle and integument at each stage of tail regeneration in *Gekko gecko*.

METHODS

Acclimatization and Sample Collection

The study was conducted at the Laboratory of Structure and Development of Animals, Faculty of Biology, Gadjah Mada University, Yogyakarta from March to October 2018. Geckos obtained from both animal market and wild catches were maintained and acclimatized in a wooden cage for approximately one month. Each cage was inhabited by 1-2 adult geckos. Geckos were fed with crickets and given drinks for every three days. Autotomy was done by twisting the tail until it was released in the fracture plane. Autotomized tails were treated with iodine antiseptics. The tails were then allowed to regenerate until a certain time and then autotomized again to get samples of the regenerated tail. Samples of the regenerated tail of gecko were taken after 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 days post-autotomy (dpa), and also 4, 6, 8, 10, and 12 weeks post-autotomy(wpa).

Tissue Processing

Samples were fixed using NBF (neutral-buffered formalin). The samples were then decalcified using 3% HNO_3 solution in 70% alcohol and subsequently washed using 70% alcohol which was replaced 6 times every 3 hours. Afterward, the samples were carried out in stages of dehydration using graded alcohol (80%, 90%, 95%, 100%). Dehydrated samples were dried with paper towel and put in toluene. After 5-6 hours of toluene soaking, the samples were put into toluene-paraffin solution. The samples were then carried out in the paraffin infiltration process, which was replaced 3 times every 1 hour in the oven. Finally, the samples were transferred into a mold and embedded in freshly melted paraffin. Finally, the paraffin embedded samples were left overnight to become paraffin blocks.

Observation

The paraffin blocks were trimmed and sectioned in 6 microns of thickness with rotary microtome. The paraffin sections were placed on glass slides with distilled water contained glycerin albumin. The glass slides were placed on a hot plate and then left overnight on the hot plate. The slide was stained using Hematoxylin-Eosin (H&E) staining, Mallory Acid Fuchsin (MAF), and Periodic Acid Schiff-Alcian Blue (PAS-AB) using Bancroft and Cook Protocols [22] with some modifications. After tissue staining has been completed, observations were made by descriptive analysis using a microscope.

RESULTS AND DISCUSSION

Muscle Development

G. gecko has an incomplete reparative epimorphic regeneration characterized by the presence of wound epithelium [1,23]. Re-epithelialization starts from the first day to the fourth day post-autotomy (dpa). After re-epithelialization, a new blastema can be observed on 8 dpa and continues to proliferate without differentiation into other tissues on 12 dpa (Fig. 1).The formation of blastema, a group of undifferentiated cells, is a common follow up process of epimorphic

regeneration after wound healing stage. The origin of blastema cells is still being debated but is likely to originate from the dedifferentiation of wound tissue or the presence of stem cells [4,9,15,24]. Blastema cells in regenerated tail of *G. gecko* can only be observed when re-epithelialization has been completed.

Blastema cells can be formed due to the process of dedifferentiation of cells around the autotomy wound tissue. Apart from dedifferentiation, new cells in the muscle can originate from inactive cells that are reserved, called satellite cells. These satellite cells function as promoters of new muscle cells and will actively proliferate only if there is damage to muscle cells [25]. The blastema cells will change or differentiate back into the original tissue because the blastema cell has a memory of the original tissue [15,23,26].

In the 14 dpa regenerated tail, myoblasts have been found in the tissues which begin to aggregate on the 16 dpa regenerated tail forming myotubes. Myoblast is a mesenchymal cell that promotes myogenesis which later will aggregate to form myotubes. The presence of myoblasts in the tissue indicates the start of myogenesis stage [4,25]. The tail regeneration of *G. gecko* has the same sequence of myoblast formation compared with that of *Eublepharis macularius* [4].

The myoblast will then form a multinucleate tube-shaped myotube that resembles fine threads. In the formation of myotubes, myoblasts go through 3 stages in the level of cell membranes: adhesion between myoblast cells, rearrangement of actin and myosin, and destabilization of lipid bilayers [27]. A series of myotube formation from changing blastema cells to myoblasts to the formation of myofibrils are processes that have been programmed by genes belonging to myogenic regulatory factor (MRF) [28]. The myoblasts have differentiated into myotubes in the 18 dpa regenerated tail (Fig. 2). In addition, the size of the myotube was longer and thicker than the newly formed myotube in the 14 dpa regenerated tail. The existence of a narrowed curve in the muscle can be observed in this tail regenerate, indicating the formation of myosepta. The myotube elongation can still be observed even in the 20 dpa regenerated tail.

The presence of myofibrils in the regeneration of muscle tissue indicates that the regeneration of the muscle is in the final stages. Regenerated tail at 28 dpa or 4 weeks post-autotomy (wpa) has more developed muscle tissue compared with 20 dpa regenerated tail (Fig. 2). Myotube in this regenerated tail already has a structure similar to myofibrils. Muscle tissue in this regenerated tail was still not perfect because there are still a number of blastema cells and myofibrils still appear and still in the process of conversion to myofibrils. This muscle formation undergoes a relatively equal time range if compared to *E. macularius* [4]. In the regenerated tail at 6 wpa, myofibrils have developed well and can be clearly observed (Fig. 3) and has a larger diameter than that of the 4 wpa regenerated tail. In addition, striation can also be observed in myofibrils at this stage. In this stage of regenerated tail, blastema cells were not found and other tissues have developed well, or it can be said that it is entering the maturing phase.

The myofibrils of the 8 wpa regenerated tail have bigger diameter and longer than that of 6 wpa regenerated tail. In addition, there were longitudinally elongated muscle development and laterally transverse muscle extension. Blastema cells have not been found and other tissues have developed well such as adipose tissue, cartilage, and dermis. The 10 wpa regenerated tail has longer and denser myofibril as seen in PAS-AB staining. In the final stages of 12 wpa regenerated tail, myofibrils appear to have a perfect structure (Fig. 4). Myofibrils look similar with muscles in the original tail. The muscles of 12 wpa regenerated tail have the same diameter compared with 10 wpa regenerated tail but they are slightly different in length. Other tissues appear to be in a mature and perfect condition, indicating that the regeneration stage is complete and only the growth process occurs.

The muscle cells of the regenerated tail have a centrally located nucleus (Fig. 5) [29,30]. Meanwhile, the muscle cells nucleus of original tail, like that of the other skeletal muscles, is located on the edge of the muscle. It is still debatable among the scientists about the reason of regenerated muscle cells have centrally located nucleus, but it is possibly related to sarcomere formation [31]. The results obtained in this study are relatively similar to studies conducted on *E. macularius*, which shows that the nucleus of regenerated muscle cells is at the edges and has smaller muscle diameter (Fig. 6) [32].

Integument Development

After autotomy, the tip of the tail resembles an open wound with the dermis, muscles, adipose tissue, spinal cord, and marrow bone exposed traumatically [23]. In the fourth-day regenerated tail of *G. gecko*, the regeneration begins with the formation of a temporary scar or clot of exudate tissue and blood that functions as a physical barrier between the tissue and the external environment and restores homeostasis to the injured tissue (Fig. 7) [7]. Under the blood clot that covers the tissue, epidermal cells at the edge of the wound begins to proliferate. There are growth factors named fibroblast growth factors (FGFs) in the basal layer of the normal epidermis, where cell proliferation occurs,

and in the injured epidermis of the tail, [33]. The previously formed exudate clot disappears and the wound surface of the tail appears relatively flat in the 8 dpa regenerated tail. The original integument is degraded and being covered by a scar tissue around the wound site to partially cover the autotomy surface and the site of exudate clot formation. The destruction of the integument serves to reduce the diameter of the wound [23]. Wound epithelium proliferate and begin to spread throughout the surface of the autotomy surface until it clotted [32]. At this stage, the apical epithelial cap is formed and the blastema located inside the wound epithelium is well-formed (Fig. 7).This apical cap is the pioneer of blastema [34]. Blastema will continue to appear until the 20 dpa (Fig. 8), and it is relatively the same as the regeneration time span that occurs in *E. macularius* [4].

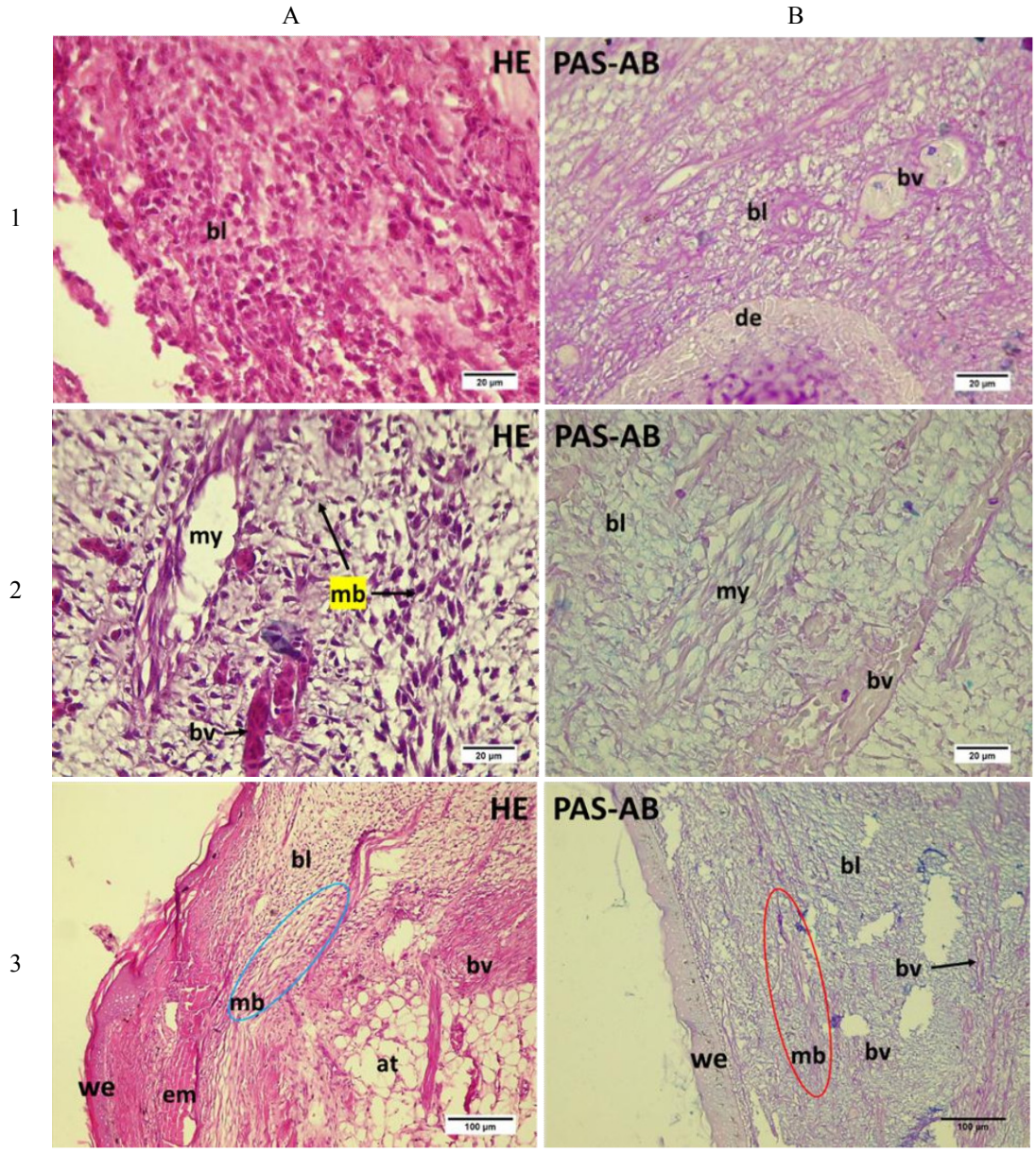

FIGURE 1. The muscle tissue structure of regenerated tail of *G. gecko* at 12 dpa (row 1), 14 dpa (row 2), and 16 dpa (row 3) stained with Hematoxylin-Eosin (A) and Periodic Acid Schiff-Alcian Blue (B) . *at: adipose tissue; bl: blastema cell; bv: blood vessels; de: dermis; em: epaxial muscle; mb: myoblast; my: myotube; we: wound epithelium.*

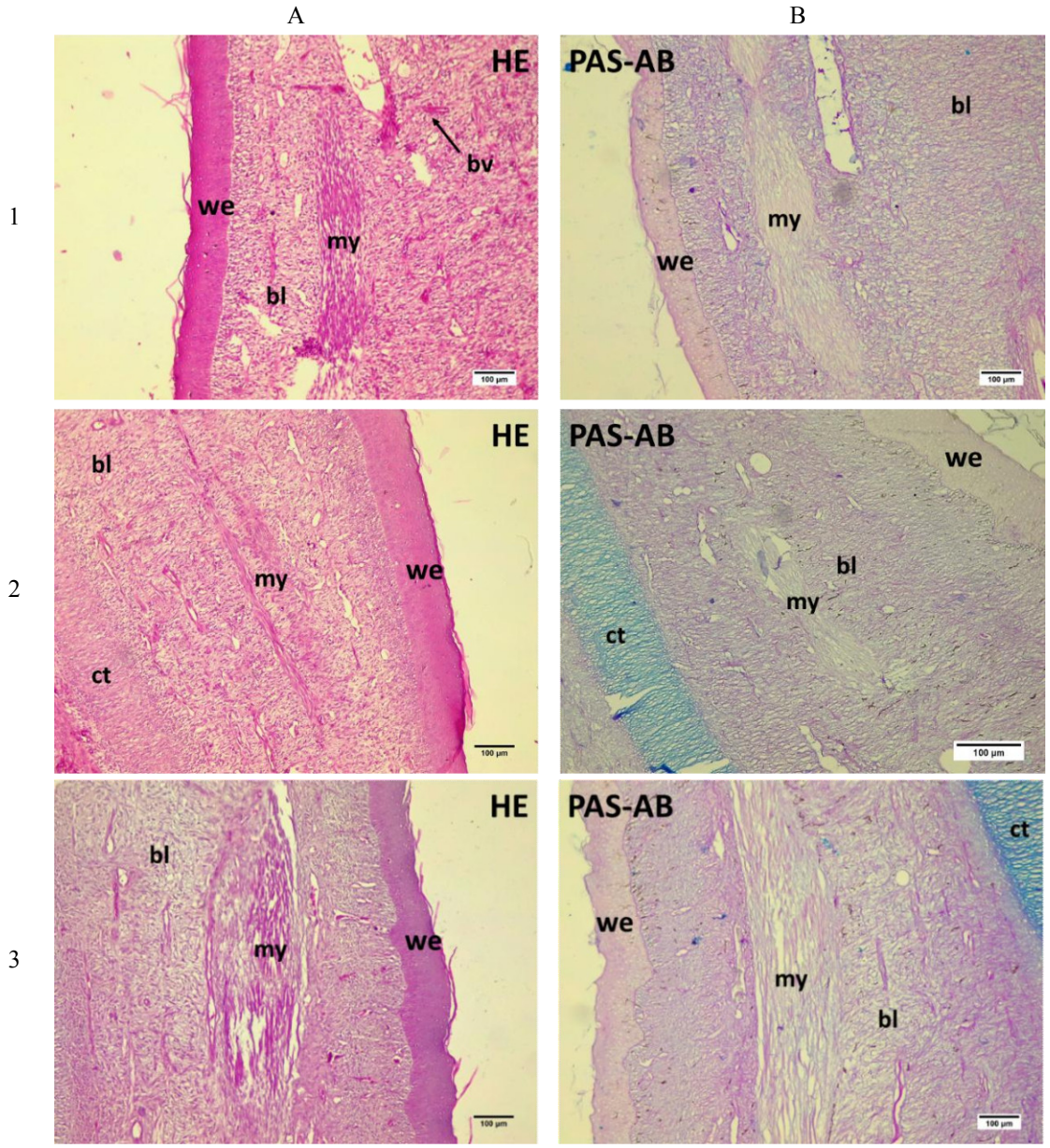

FIGURE 2. The muscle tissue structure of regenerated tail of *G. gecko* at 18 dpa (row 1), 20 dpa (rows 2), and 4 wpa (rows 3) stained with Hematoxylin-Eosin (A) and Periodic Acid Schiff-Alcian Blue (B). *bl: blastema cell; bv: blood vessels; ct: cartilage tube; my: myotube; we: wound epithelium.*

The keratinization and formation of scales of the epidermis can be observed in 20 dpa regenerated tail (Fig. 8). The outer layer of the wound epithelium become keratin and epidermal scales are formed. The formation of scales begins when the basal layer of the wound epithelium invades the blastema. There is a possibility of B-catenin expression involvement in the differentiation of epidermal scales. The dynamic tenascin-C expression also shows involvement in the regeneration [35]. The superficial layer of epidermal cells were increasingly flattened at this stage, so they have different structure compared with the basal layer of epidermal cells near the blastema. The formation of dermis can also be observed in this stage. In reptiles, dermis consists of fibrous connective tissue [36].

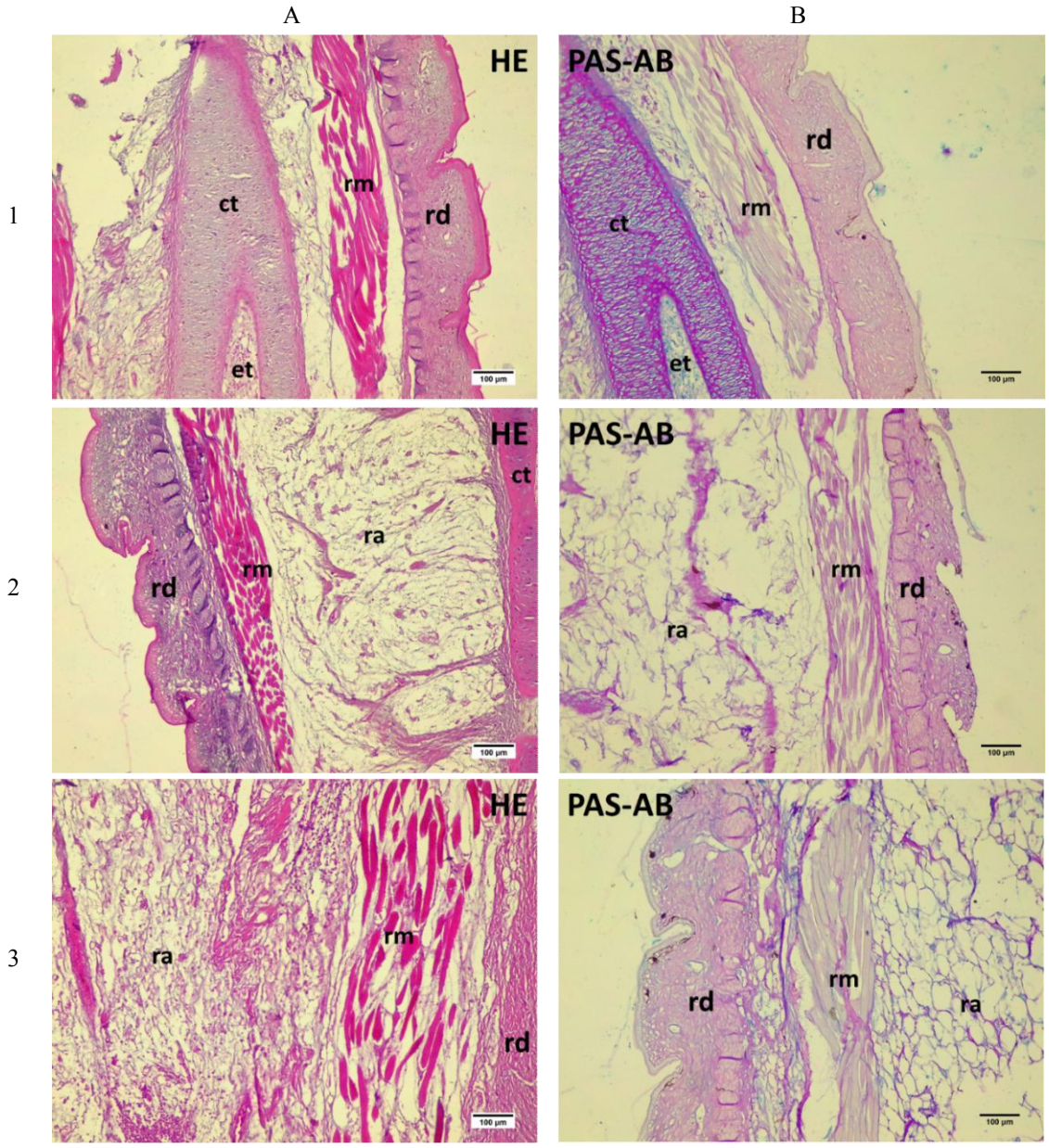

FIGURE 3. The muscle tissue structure of regenerated tail of *G. gecko* at 6 wpa (row 1), 8 wpa (row 2), and 10 wpa (row 3) stained with Hematoxylin-Eosin (A) and Periodic Acid Schiff-Alcian Blue (B). *ct: cartilage tube; et: ependymal tube; ra: regenerated adipose tissue; rd: regenerated dermis; rm: regenerated muscle.*

The formation of the dermis was clearly visible under the layers of the epidermis in the 4 wpa regenerated tail (Fig. 9). The regenerated epidermis has the same thickness as the epidermis of the original tail and it cover the newly differentiated dermis. The epidermal cells layer can also be clearly divided at this stage, which are the *stratum corneum* containing α- and β-keratin, *stratum granulosum*, and *stratum basale* [36].

The epidermal scales with uniform scales that have emerged from the 20th day were getting thicker and clearer in the 8 wpa regenerated tail with MAF staining (Fig. 9). The epidermis of the regenerated tail has the same thickness with that of the original tail, while the dermis of the regenerated tail is thicker than that of the original tail. Moreover, the transition area of the regenerated tail and the original tail is well-defined due to the presence of continued epidermal downgrowth.

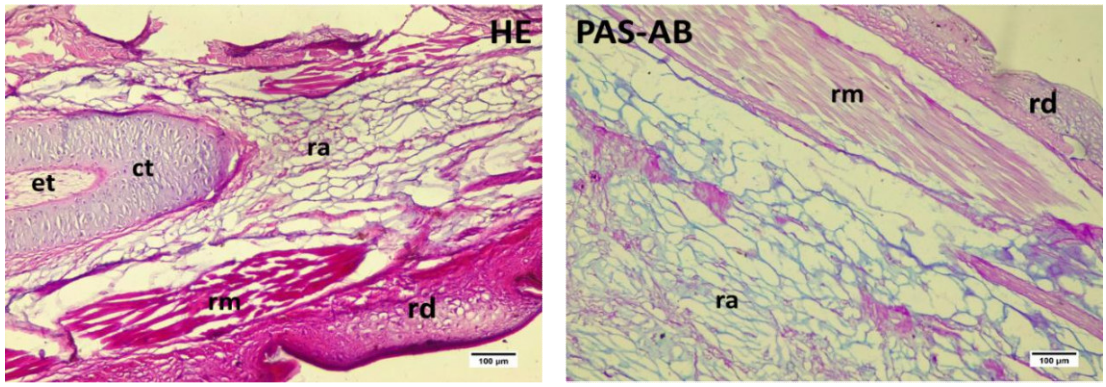

FIGURE 4. The muscle tissue structure of regenerated tail of *G. gecko* at 12 wpa stained with Hematoxylin-Eosin (A) and Periodic Acid Schiff-Alcian Blue (B). *ct: cartilage tube; et: ependymal tube; ra: regenerated adipose tissue; rd: regenerated dermis; rm: regenerated muscle.*

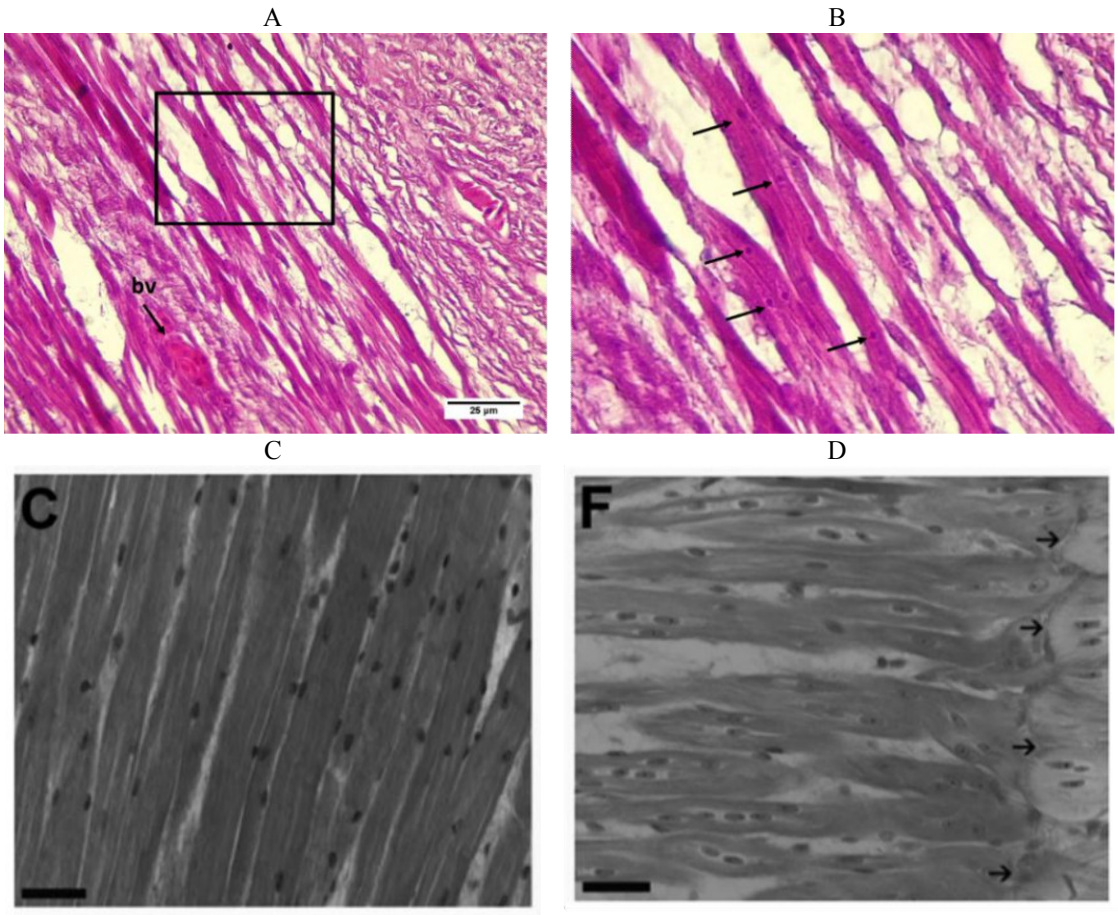

FIGURE 5. Muscle cells structure in regenerated tail of G. gecko 4 wpa (A and B) compared with the original muscle (C) and regenerated muscle (D) in *Eublepharis macularius* in the study of Gilbert et al. (2013). Hematoxylin-Eosin Staining (A and B); Mason's trichrome coloring, bar scale of 20 μm (C and D).

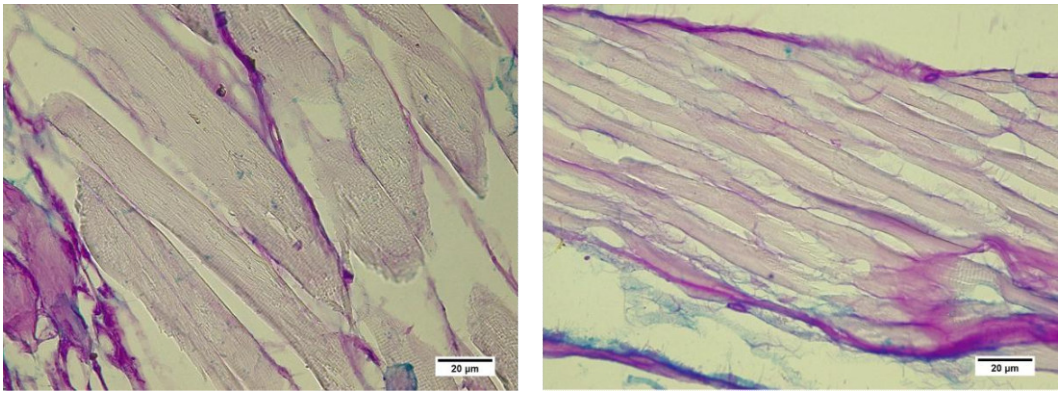

FIGURE 6. Comparison of muscle tissue structure in the original tail (left) and regenerated tail (right) of *G. gecko* with Periodic Acid Schiff-Alcian Blue staining.

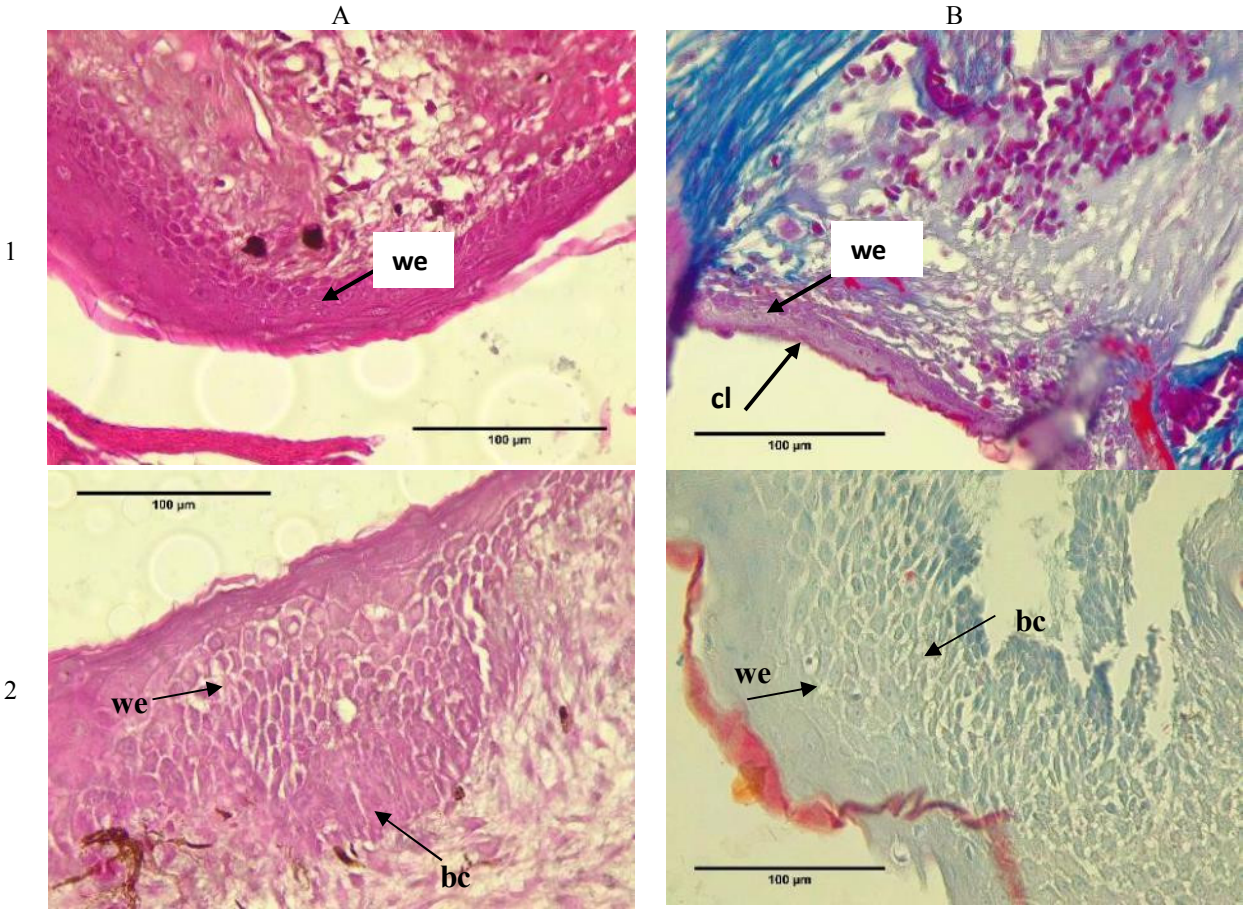

FIGURE 7. Integument structure of regenerated tail of G. gecko at 4 dpa (row 1) and 8 dpa (row 2) stained with Hematoxylin-Eosin (A) and Mallory Acid Fuchsin (B). cl: *clot*, we: *wound epithelium*, bc: *blastema cells*.

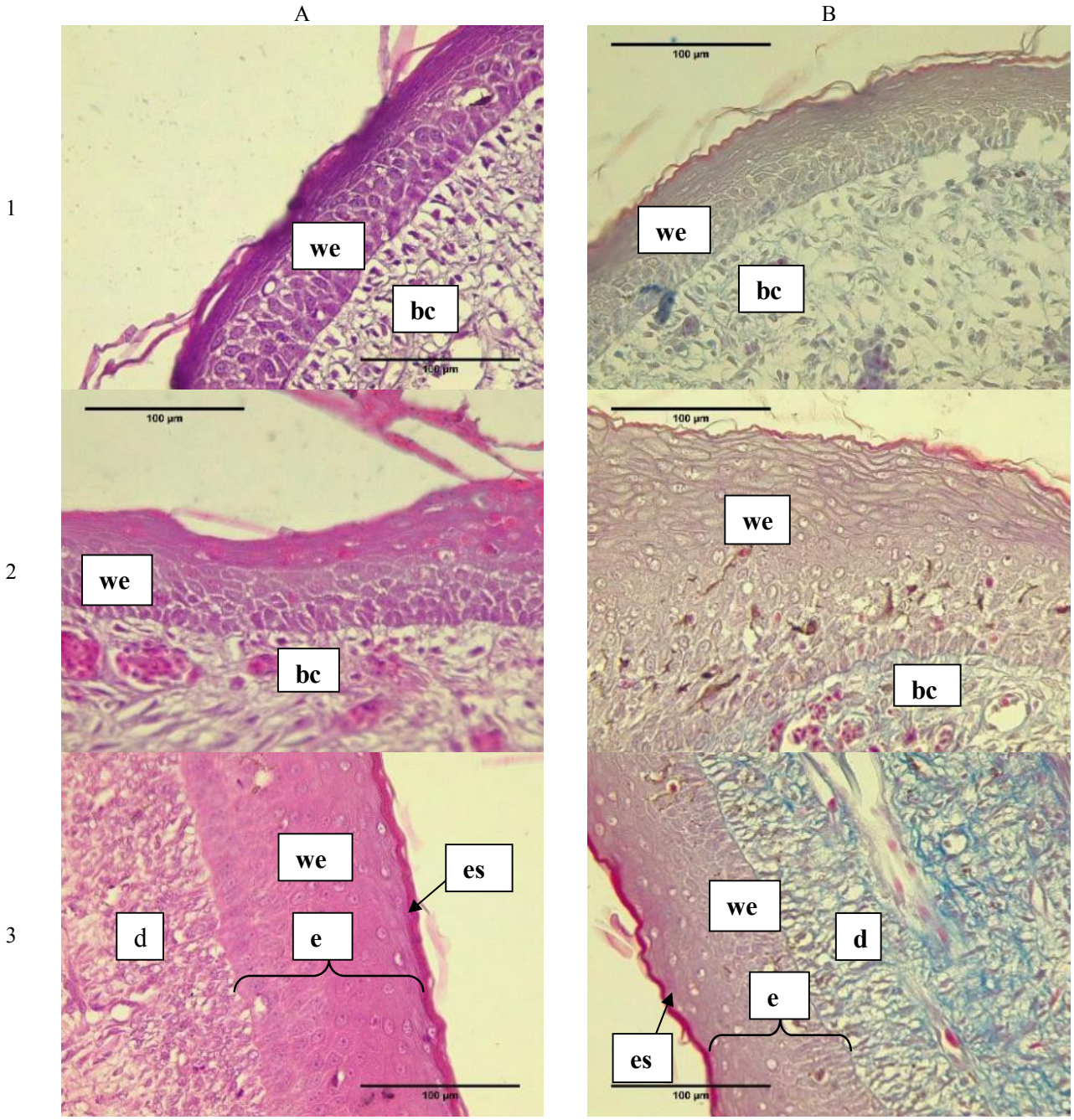

FIGURE 8. Integument structure of regenerated tail of *G. gecko* at 13 dpa (row 1), 16 dpa (row 2), and 20 dpa (row 3) stained with Hematoxylin-Eosin (A) and Mallory Acid Fuchsin (B). we: *wound epithelium*, bc: *blastema cells*, d: *dermis*, ep: *epidermis,* dan es: *epidermal scales*

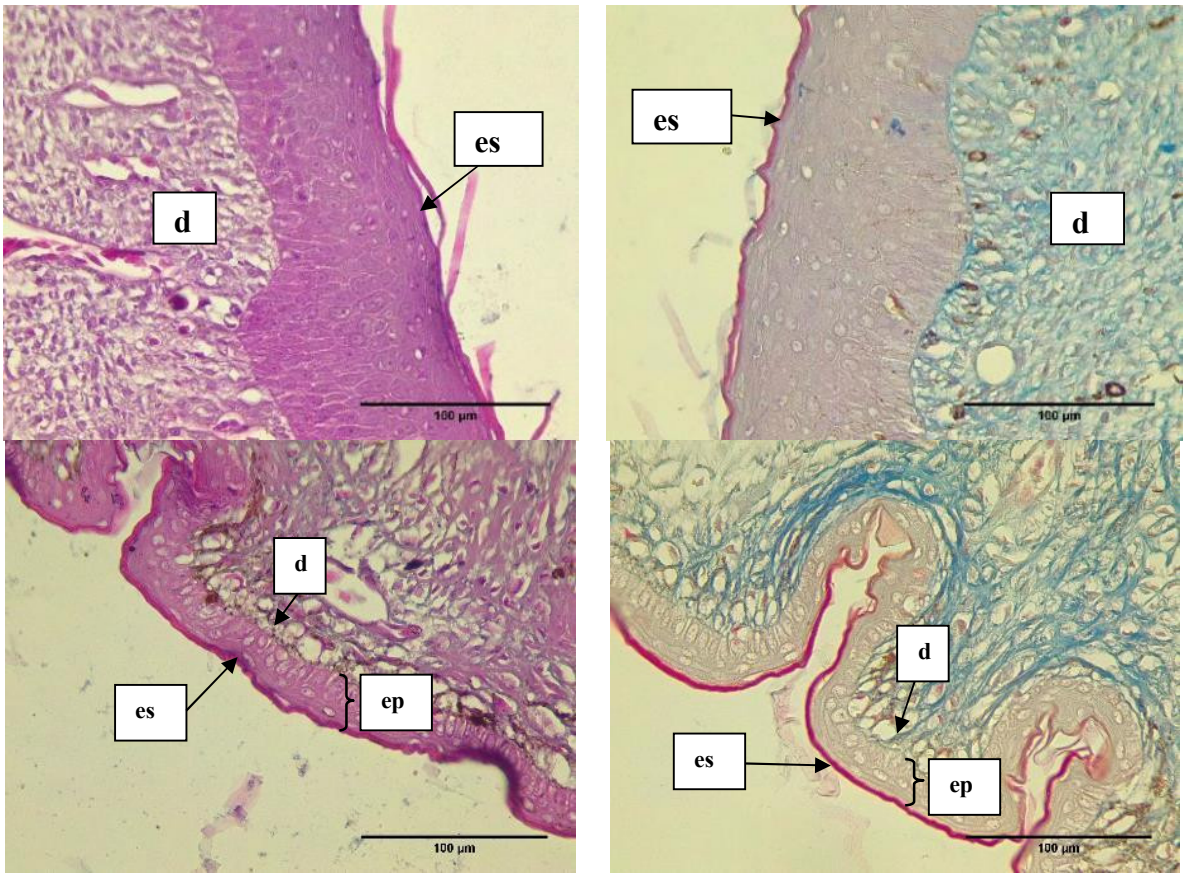

FIGURE 9. Integument structure of regenerated tail of *G. gecko* at 4 wpa (row 1) and 8 wpa (row 2) stained with Hematoxylin-Eosin (A) and Mallory Acid Fuchsin (B). d: *dermis*, es: *epidermal scales,* ep: *epidermis*

CONCLUSION

In conclusion, myogenesis in the regenerated tail of *G. gecko* begins at 14 dpa and reaches its final differentiation stage at the 4 wpa. The muscle of regenerated tail of *G. gecko* continues to grow and accomplishes a mature structure at 12 wpa. In the other hand, the integument development of regenerated tail of *G. gecko* begins with the formation of blood clots on the wound surface right after autotomy until 4 dpa. This process is followed by wound epithelium proliferation (from 4 dpa to 8 dpa), epithelial cap formation (from 8 dpa to 20 dpa),dermis formation (from 20 dpa to 4 wpa), and finally keratinization (from 20 dpa to 8 wpa).

ACKNOWLEDGEMENT

This research was partially funded by Hibah Penelitian Kolaborasi Dosen dan Mahasiswa Faculty of Biology Universitas Gadjah Mada 2019.

REFERENCES

1. A.W. Seifert, J.R. Monaghan, M.D. Smith, B. Pasch, A.C. Stier, F. Michonneau and M. Maden, Biol. Rev. Letters **87**, 330 (2012).
2. S.J. Downes and R. Shine, Ecology, Letters **82**, 1293 (2001).
3. D.G. Chapple, C.J. McCoull and R. Swain, J. Herpetol. Letters **36**, 480 (2002).

4. K.E. McLean and M.K. Vickaryous, BMC Dev. Biol. Letters **11**, 50 (2011).
5. P.A. Fleming and P.W. Bateman, African Zool. Letters **47**, 55 (2012).
6. L.J. Vitt and J.P. Caldwell, *Herpetology* (2014).
7. K. Jacyniak, R.P. McDonald and M.K. Vickaryous, J. Exp. Biol. Letters **220**, 2858 (2017).
8. J.P. Jamison, Herpetologica, Letters **20**, 145 (1964).
9. A.D. Bellairs and S. V. Bryant, in *Biol. Reptil.*, edited by C.G.A.F. Billet (John Wiley & Sons, Inc., New York, 1985), pp. 303–410.
10. A.E. Abdel-karim, Qatar Univ. Sci. J. Letters **13**, 288 (1993).
11. K.W. Sanggaard, C.C. Danielsen, L. Wogensen, M.S. Vinding, L.M. Rydtoft, M.B. Mortensen, H. Karring, N.C. Nielsen, T. Wang, I.B. Thøgersen and J.J. Enghild, PLoS One Letters **7**, (2012).
12. A.R. Clause and E.A. Capaldi, J. Exp. Zool. Letters **305A**, 965 (2006).
13. T.E. Higham, A.P. Russell and P. a Zani, Physiol. Biochem. Zool. Letters **86**, 603 (2013).
14. Z. Starostová, L. Gvoždík and L. Kratochvíl, Elsevier Letters **206**, 82 (2017).
15. L. Alibardi, Prog. Histochem. Cytochem. Letters **48**, 143 (2014).
16. K. Agata, Y. Saito and E. Nakajima, Dev. Growth Differ. Letters **49**, 73 (2007).
17. W.N.F. Woodland, Q. J. Microsc. Sci. Letters **65**, 63 (1920).
18. L. Alibardi, *Morphological and Cellular Aspects of Tail and Limb Regeneration in Lizards* (Springer-Verlag, Berlin, 2009).
19. M. Hutchinson and I. Williams, (2014).
20. Z. Nain, S.A. Islam, C. Hasan and S. Afroza, Cell Biol. **4**, 9 (2016).
21. P.W. Bateman and P.A. Fleming, J. Zool. Letters **277**, 1 (2009).
22. J.A. Stewart, Histopathology, Letters **26**, 95 (1995).
23. E.A.B. Gilbert, S.L. Delorme and M.K. Vickaryous, Regeneration, Letters **2**, 45 (2015).
24. S.B. Simpson, J. Morphol. Letters **114**, 425 (1964).
25. A.L. Mescher, *Junqueira's Basic Histology Text and Atlas*, 14th ed. (McGraw-Hill Education, New York, 2016).
26. M. Kragl, D. Knapp, E. Nacu, S. Khattak, M. Maden, H.H. Epperlein and E.M. Tanaka, Nature, Letters **460**, 60 (2009).
27. J.H. Kim, P. Jin, R. Duan and E.H. Chen, Curr. Opin. Genet. Dev. Letters **32**, 162 (2015).
28. C.F. Bentzinger, Y.X. Wang and M.A. Rudnicki, Cold Spring Harb. Perspect. Biol. Letters **4**, a008342 (2012).
29. B. Cadot, V. Gache and E.R. Gomes, Nucleus, Letters **6**, 373 (2015).
30. W. Roman and E.R. Gomes, Semin. Cell Dev. Biol. (2017).
31. A.L. Auld and E.S. Folker, Mol. Biol. Cell, Letters **27**, 2351 (2016).
32. E.A.B. Gilbert, S.L. Payne and M.K. Vickaryous, Physiol. Biochem. Zool. Letters **86**, 631 (2013).
33. L. Alibardi, Belgian J. Zool. Letters **142**, 23 (2012).
34. T.P. Lozito and R.S. Tuan, Connect. Tissue Res. Letters **58**, 145 (2017).
35. P. Wu, L. Alibardi and C.-M. Chuong, Regeneration, Letters **1**, 15 (2014).
36. K. V. Kardong, *Vertebrates: Comparative Anatomy, Function, Evolution*, 6th ed. (The McGraw-Hill Companies, Inc., New York, 2012).