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ORIGINAL ARTICLE

# Comparative study of the peroneus tertius muscle in pigs based on the origin, course, insertion and innervation

Y. Natsuyama et al., **Comparative study of the peroneus tertius muscle in pigs**

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**Abstract**

**Background:** That the peroneus tertius muscle (PT) is a separate entity has been debated. PT has been reported to be part of the extensor digitorum longus muscle, part of the extensor digitorum brevis, or a separate muscle. While pigs have a PT as well as primates, there are no reports of its association with the extensor digitorum longus muscle or extensor digitorum brevis.

**Materials and methods:** In this study, we used gross dissection and Sihler's staining to determine the origin, course, insertion, and innervation of the pig PT.

**Results:** The PT and extensor digitorum longus muscles jointly originated from the femur and ran between the tibialis cranialis and peroneus longus muscles. The PT was inserted at the retinaculum of the metatarsal extensors, tarsal bone, and second metatarsal bone. The branches of the common fibular nerve to the extensor digitorum longus muscle were distributed to the PT.

**Conclusions:** The innervations suggest that the PT and extensor digitorum longus muscles of the pig were derived from the same muscle mass during development but were named separately due to differences in their morphology. Furthermore, morphological features suggest that pig PT and human PT are probably different muscles.

**Keywords: common fibularis nerve, hindlimb, fibularis tertius, Shiler's stain, comparative anatomy**

## INTRODUCTION

In humans, the peroneus tertius muscle (PT) originates from the distal fibula, merges into the common tendon with the extensor digitorum longus muscle (EDL), and inserts at the fifth and fourth metatarsal bones [4, 12, 16]. Two issues regarding PT have been controversial since its first description: whether it is exclusively human and whether PT is a separate entity

[22]. PT has long been considered a unique human structure [5, 9, 17]. However, many studies have reported the presence of PT in primates (except humans) [8, 10, 21], and the idea that its structure is unique to humans has been denied. Regarding the latter issue, it has been reported that the PT is part of the EDL [12, 22], extensor digitorum brevis muscle [6], or a separate muscle [22]. The presence of PT has also been observed in pigs and horses (degenerated), as well as in primates, but there are no reports of its association with the EDL or extensor digitorum brevis muscle. A new perspective on the use of pigs for comparison with humans may provide new insights.

Therefore, in the present study, we used macroscopic anatomy and histological methods to conduct a detailed analysis of the origin, course, insertion, and innervation of the pig PT and conducted a comparative anatomical analysis with the human PT structure.

## MATERIALS AND METHODS

Pigs have entirely lost their first digit [1]. Therefore, in this study, the second digit is referred to as the hallux.

### Preparation of specimens and gross anatomy

A total of 20 lower limbs of pigs were used in this study, of which four adult pigs were purchased from Nansyu Natural Pork (Kagoshima, Japan) and 16 fetal pig lower limbs were purchased from Bio Corporation (Minnesota, USA). For fetal pigs purchased from the United States, merchants have performed 10% formalin circulation fixation and injected red latex into the arterial system and blue latex into the venous system to identify the blood vessels [2, 14, 15]. Fetal are suitable for Sihler’s staining, while adult pigs are suitable for gross dissection for nerve identification.

For gross anatomy, the quadriceps femoris, sartorius, hamstrings, and adductor muscles were identified and removed after removing the skin and subcutaneous soft tissue. Then the muscles on the anterior surface of the crus included the PT, EDL, tibialis cranialis, extensor hallucis longus, peroneus longus, and extensor digitorum lateralis muscles, which were observed, taking care not to injure the common fibular nerve. Lastly, the patellae ligament was removed to identify the origin of the PT.

For nerve observation, the tibial and common fibular nerves were identified in the popliteal fossa and the common fibular nerve was dissected beyond the hock joint after muscles observation. The structure of the nerve bundle was observed immediately after the common fibular nerve penetrated the gastrocnemius muscle. The peroneus longus and EDL were divided and reflected laterally to reveal the nerves.

In adult pigs, cranial crus muscles were also examined in cross-sections to reveal the layered structure of the PT and EDL around the neuromuscular joint.

**Modified Sihler's Staining**

To visualize the distribution of intramuscular nerve supply in the lower limbs of fetal pigs, we performed a modified Sihler's staining procedure [13, 19] on seven fetal pig lower limbs.

Sihler's staining was performed as previously described by Liu et al. [13]. The modified method is described briefly as follows:

1. Maceration and depigmentation (four weeks): 3% aqueous potassium hydroxide(KOH) solution add 0.2 mL of 3% hydrogen peroxide to 100 mL 3% KOH solution for depigmentation;
2. Decalcification (eight days): Sihler's solution I: one volume of glacial acetic acid, one volume of glycerin, and 12 volumes of 1% aqueous chloral hydrate;
3. Staining (two weeks): Sihler's solution II: one volume stock Ehrlich's hematoxylin, one volume of glycerin, and six volumes of 1% aqueous chloral hydrate;

4. Destaining (three hours): Sihler’s solution I: one volume of glacial acetic acid, one volume of glycerin, and 12 volumes of 1% aqueous chloral hydrate;

5. Neutralization (three hours): 0.05 lithium carbonate solution;

6. Clearing (three days each): Aqueous glycerin 50% and 100% glycerin.

**Histological evaluation**

As it was difficult to identify the PT and EDL borders by gross anatomy in fetal pigs, these muscles were harvested as a tissue block and evaluated by histology. Before tissue embedding, it was re-fixed by immersing in 10% formalin for 24 h, then washed thoroughly for 4–5 h under running tap water, dehydrated, and routinely embedded in paraffin. Ten-micrometer-thick sections were cut, and the slides were stained with Masson’s trichrome. Sections were imaged and photographed using an optical microscope.

Masson’s trichrome staining was performed according to the methods described in our previous studies [14, 15]. Briefly:

1. deparaffinization and rehydration: xylene for 10 min, 3 times; 100% ethanol for 5 min, 3 times; 95% ethanol for 5 min; 90% ethanol for 5 min; 80% ethanol for 5 min; 70% ethanol for 5 min; tap water for 10 min;

2. hematoxylin staining: hematoxylin was applied for 8 min; rinsed with tap water; immersed for 15 s in 70% alcohol and 1% hydrochloric acid to destain;

3. Fuchsin staining: Fuchsin was applied to the MM for 5 min and LM for 3 min; immersed for 15 s in 70% alcohol and 1% hydrochloric acid to destain;

4. phosphomolybdic-phosphotungstic acid differentiating (11 min); and

5. Aniline blue staining and dehydration: Aniline blue was applied for 7 min; rinsed with tap water; immersed for 15 s in 1% acetic acid; immersed in 95% ethanol for 45 s; 100% ethanol for 5 min, 3 times; and xylene for 5 min, 3 times.

All findings were photographed and illustrated using Adobe Illustrator 2023 (Adobe Inc., USA).

## RESULTS

### Gross observation of the origin, course, and insertion of the PT

The PT originated from the extensor fossa of the femur (Fig. 1B, D) by a common tendon with the EDL. The origin tendon descended within the stifle joint capsule and was covered by the peroneus longus and tibialis cranialis muscles, as shown in figure 1A, C. The tendon of origin ended at the point where the peroneus longus and tibialis cranialis muscles met the surface of the PT. At this point, the tendon became the muscle belly (Fig. 1A, C). The PT belly ran superficial to the EDL and was attached to the tendon of insertion deep in the retinaculum of the crural extensors. The PT tendon of insertion ran medially to the EDL and laterally to the tibialis cranialis muscle. It was inserted at the retinaculum of the metatarsal extensors, tarsal bone, and second metatarsal bone (Fig. 2A, D).

The EDL originated from the femur with the PT and ran deep to the PT and superficial to the extensor hallucis longus muscle. In this study, the three insertion tendons of the EDL were considered fundamental and were labeled EDL1, EDL2, and EDL3 from the medial side (Fig. 2). The EDL1 was inserted slightly medial to the third digit and spread to the thin aponeurosis. The EDL2 bifurcated near the end of the metatarsal bone, inserted slightly lateral to the third digit, and slightly medial to the fourth digit (Fig. 2C, F). These tendons were thicker because they merged with the extensor digitorum brevis. The EDL3 split into two: one part ran deep to the EDL1 and EDL2, and superficially to the extensor digitorum brevis muscle, and inserted slightly lateral to the second digit. The other part inserted slightly medial to the fifth digit (Fig. 2).

The tibialis cranialis muscle originated from the tibia, ran medial to the PT, and inserted at the second metatarsal bone (Fig. 1, 2A, and D). The extensor hallucis longus muscle originated from the fibula and the crural interosseous membrane. It ran deep to the EDL, pierced the PT tendon just beyond the RC, emerged superficially, and inserted slightly medial to the second digit (Fig. 2A, D). The peroneus longus muscle originated from the lateral condyle of the tibia and fibula, ran laterally to the PT, passed over the sole of the foot, and inserted at the tarsal bones and the second metatarsal bone (Fig. 1, 2B and E). The extensor digitorum lateralis muscle originated from the lateral aspect of the fibula, ran deep to the peroneus longus muscle around the external capsule, and divided the tendon into two parts. One ran deep to the EDL3 and inserted slightly lateral to the fourth digit, spreading the thin aponeurosis. The other inserted slightly lateral to the fifth digit (Fig. 2B, C, E, and F).

Differences in tendon and muscle thicknesses were observed between fetal and adult pigs, but no differences in origin, course, or insertion were found.

**The distribution of the common fibular nerve**

The common fibular nerve consisted of six nerve bundles at the gastrocnemius piercing, numbered 1–6 in dorsal order (Fig. 3, 4). Nerve bundle no. 1 was distributed throughout the extensor digitorum lateralis muscle. Nerve bundle no. 2 descended between the extensor digitorum lateralis and extensor hallucis longus muscles, ran superficial to the retinaculum of crural extensors, and was distributed in the skin of the dorsal foot. Nerve bundle no. 3 was distributed in the peroneus longus muscle. Nerve bundle no. 4 entered between EDL1 and EDL2 and between EDL2 and EDL3. It was distributed throughout the EDL and PT. The branch to the PT did not run between the EDLs but ran internally with a branch to the EDL, so the EDL had to be excised to observe the nerve of the PT (Fig. 3, 4). Nerve bundle no. 5 was distributed in the tibialis cranialis muscle. Nerve bundle no. 6 was distributed throughout the extensor hallucis longus muscle, descended between the tibialis cranialis muscle and extensor hallucis longus muscle, ran deep to the retinaculum of crural extensors (Fig. 3, 4), and was then distributed in the extensor digitorum brevis muscle. No differences in the distribution of nerve bundles were observed between fetal and adult pigs.

**Gross observation of Sihler's staining**

The fetal pig nerve was stained purple by Sihler's staining. The muscle fibers of the EDL and PT were also slightly visible (Fig. 4). The nerves that enter between EDL1 and EDL2, and between EDL2 and EDL3, are distributed in the PT while branching to the EDL. In Figure 4B, the lower arrowhead indicates the nerve entering between EDL1 and EDL2, while the upper arrowhead indicates the nerve entering between EDL2 and EDL3.

**DISCUSSION**

In this study, we used gross dissection and Sihler's staining methods to conduct a detailed analysis of the origin, course, insertion, and innervation of the PT in fetal and adult pigs. The PT originated from the extensor fossa of the femur via a common tendon with the EDL, descended within the stifle joint capsule, and was covered by the peroneus longus and tibialis cranialis muscles. The PT was inserted at the retinaculum of the metatarsal extensors, tarsal bone, and second metatarsal bone, and the muscular branches of the common fibular nerve to the EDL were distributed to the PT (Fig. 5).

Regarding the distribution of the nerves, the muscular branches of the EDL were distributed in the PT. It can be interpreted that the morphological characteristics described above were stable, as they were the same in both fetal and adult pigs. Because each muscle is supplied by a nerve in the early stages of development, two different muscles supplied by a single nerve are considered to be derived from a single muscle mass [20]. Based on the above interpretation, it is suggested that the PT and EDL of the pig were derived from the same muscle mass but were named separately as the PT and EDL due to differences in their morphology. If the pig PT and human PT are identical muscles, the nerve distribution in this study would support the hypothesis that the human PT is part of the EDL. However, the morphology and nerve distribution of the PT suggest that the hypothesis that it is part of the extensor digitorum brevis muscle or that it is a separate muscle is incorrect.

Furthermore, based on the idea that strong movement of the stifle joint is more important in pigs than the control of micro-movements of the hock joint, the PT and EDL can be interpreted as the same muscle in terms of their function. The two muscles originate from the same point, and insert into the hock joint in a balanced manner, medially and laterally. These results support the proposal of König and Liebich [11] that they act as reciprocal devices for hindlimbs. On the other hand, morphological results suggest that the muscle called the PT may be a different muscle in humans and pigs. The PT originated from the femur, ran between the tibialis cranialis and peroneus longus muscles, and was inserted at the retinaculum of the metatarsal extensors, tarsal bone, and second metatarsal bone.

The PT originates from the femur in pigs and from the fibula in humans [16]. It runs between the tibialis cranialis muscle and peroneus longus muscles in pigs and between the EDL and peroneus longus muscles in humans [16]. It is inserted at the second metatarsal bone in pigs and at the fifth metatarsal bone in humans [16]. In primates, including humans, the PT is thought to play a role in making walking on land more efficient by pronating the foot to bring the plantar surface closer to the horizontal plane [3, 7, 12, 22]. The origin, course, and insertion of the pig PT indicate that it has the function of supination of the foot. This function is different from that of the human PT.

Muscles are typically named based on a number of criteria, including the origin, insertion, action, location, fiber direction, number of divisions, size, and shape [18]. The PT is a muscle named because it is the third peroneus muscle following the peroneus longus and peroneus brevis, which originate from the fibula [3]. As previously stated, the pig PT did not originate from the fibula, and its morphological characteristics, including its action, differ from those of the human PT. Therefore, the name is inappropriate for pigs. Unfortunately, we were unable to compare the innervation of the human PT because there was no information available on it. Therefore, we cannot conclude that pig and human PT are different from all perspectives.

This study was limited by the fact that it did not include an analysis of nerve fibers in muscle fascicles. Since there is no detailed information available on the distribution of nerves in the human PT, a comparison with the pig PT is not possible. Therefore, comparison of the innervation of muscle fascicles between the pig PT and human PT is insufficient.

## CONCLUSIONS

Considering the distribution and function of the nerve bundles, it can be interpreted that the pig PT and EDL are the same muscle. In terms of morphology, pig PT and human PT are considered distinct muscles. Further comparative anatomical research is needed to determine how pig PT should be named.

## Article information and declarations

**Data availability statement:** The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

**Ethics statement:** All experiments and procedures involving animals were conducted in accordance with the animal experimentation guidelines of the affiliated university or institution.

**Author contributions:** SY acquired the funding. YN and SY designed and conceived the study. YN and TY performed experiments. YN, TY, RL, SU, KR, KT, RK, and MI analyzed the data. KS and YK provided adult pig specimens. YN and SY wrote the article. All authors have contributed to the final version of this manuscript. All authors have read and approved the final manuscript.

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**Conflict of interest:** The authors declare no conflicts of interest in association with the present study.

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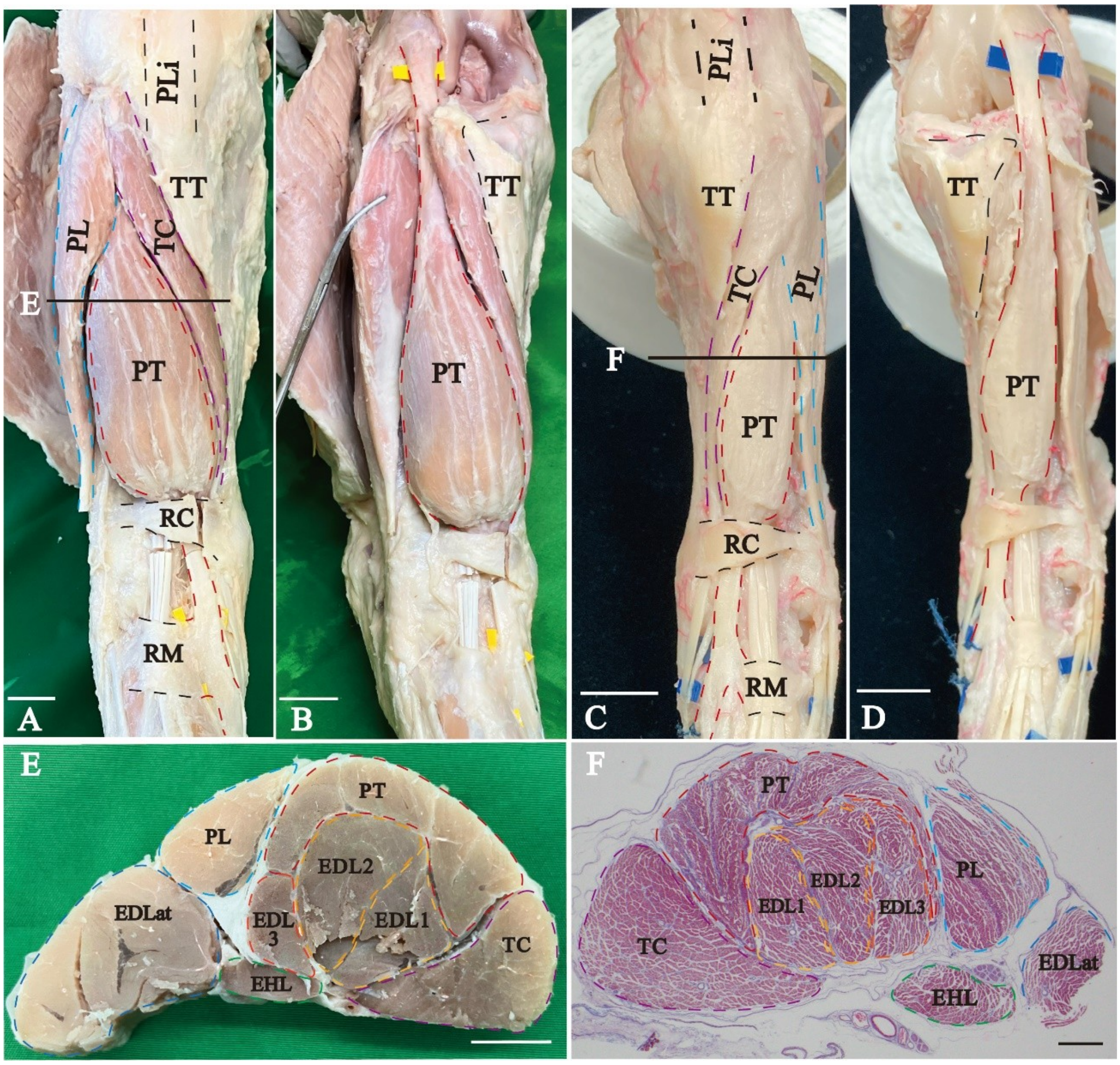


**Figure 1.** Photographs showing the course and origin. The anterior aspect of the right crus in an adult pig is shown in images A and B. In images C and D, the anterior aspect of the left crus in a fetal pig is shown. Image E is a cross-section of the black line in image A. Image F is a histological photograph of the cross-section of the black line in image A, and was stained using the Masson trichrome method. The dotted lines indicate the borders of the structure. EDLat — extensor digitorum lateralis muscle; EDL1–3 — extensor digitorum longus muscle; EHL — extensor hallucis longus muscle; RC — retinaculum of crural extensors; RM — retinaculum of metatarsal extensors; PL — peroneus longus muscle; PLi — patellar ligament; PT — peroneus tertius muscle; TC — tibialis cranialis muscle; TT — tibial tuberosity. Scale bar: (A, B) = 1.5 cm, (C, D) = 0.5 cm, (E) = 1.0 cm, (F) = 1.0 mm

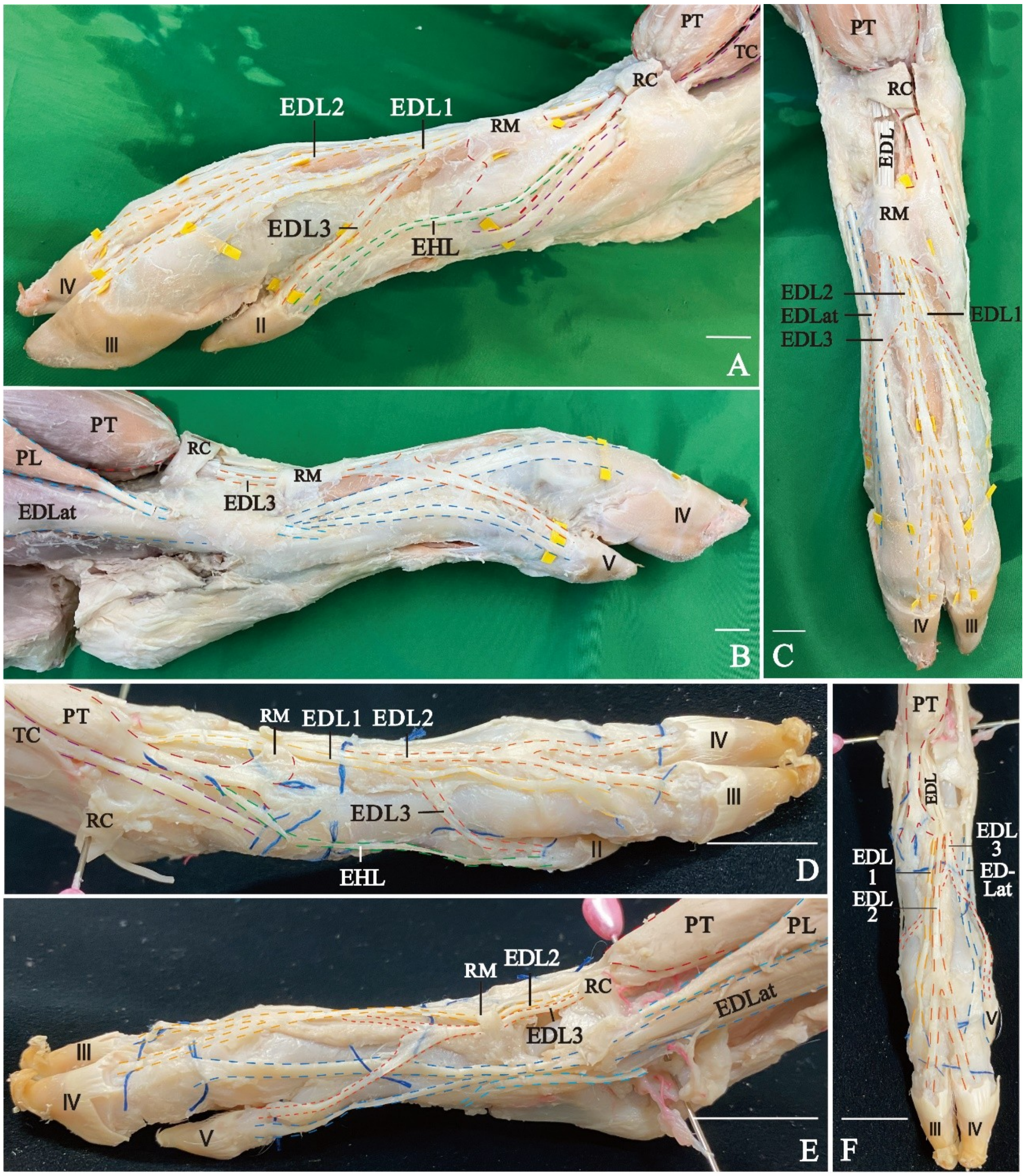


**Figure 2.** Photographs showing the insertion. Medial (A), lateral (B), and anterior aspect (C) of the right crus in adult pig; medial (D), lateral (E), and anterior aspect (F) of the left crus in fetal pig. In (E), the PL was cut and inverted at the level of the RC. The dotted lines indicate the borders of the structure. II–V — Digiti II–V; EDLat — extensor digitorum lateralis muscle; EDL1-3 — extensor digitorum longus muscle; EHL — extensor hallucis longus

muscle; RC — retinaculum of crural extensors; RM — retinaculum of metatarsal extensors; PL — peroneus longus muscle; PT — peroneus tertius muscle; TC — tibialis cranialis muscle. Scale bar: (A, B, and C) = 1.5 cm, (D, E, and F) = 1.0 cm

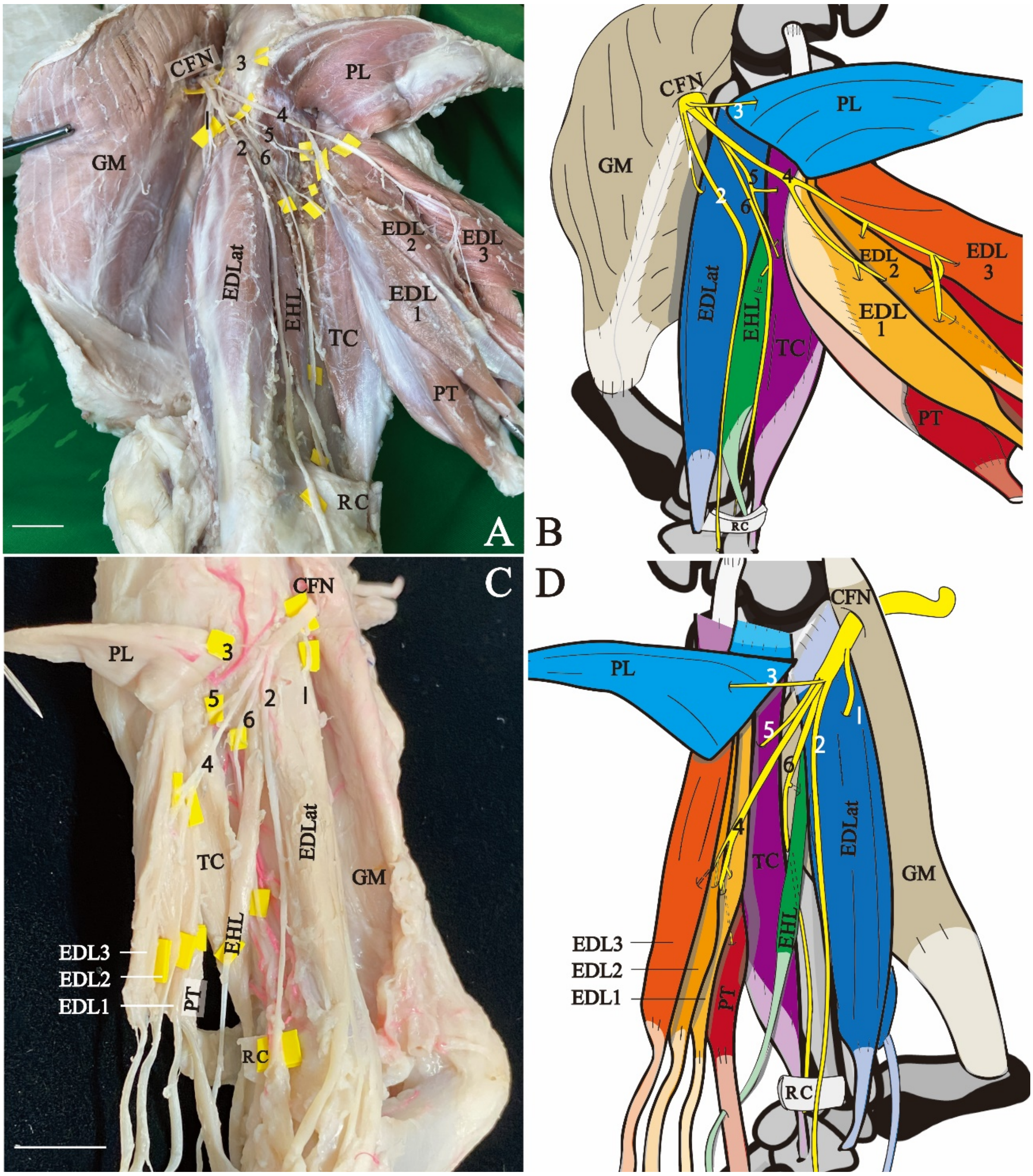


**Figure 3.** Photographs and illustration showing the distribution of the common fibular nerve. Lateral aspect of the right crus in adult pig (A, B), lateral aspect of the left crus in fetal pig (C, D). PL, EDL, and PT are cut and inverted at the level of the RC. 1–6 — the layer structure of

the common fibular nerve at the site at which the gastrocnemius is pierced, numbered in dorsal order on the nerve bundle; CFN — common fibular nerve; EDLat — extensor digitorum lateralis muscle; EDL1–3 — extensor digitorum longus muscle; EHL — extensor hallucis longus muscle; RC — retinaculum of crural extensors; GM — gastrocnemius muscle; PL — peroneus longus muscle; PT — peroneus tertius muscle; TC — tibialis cranialis muscle. Scale bar: (A) = 1.5 cm, (C) = 1.0 cm

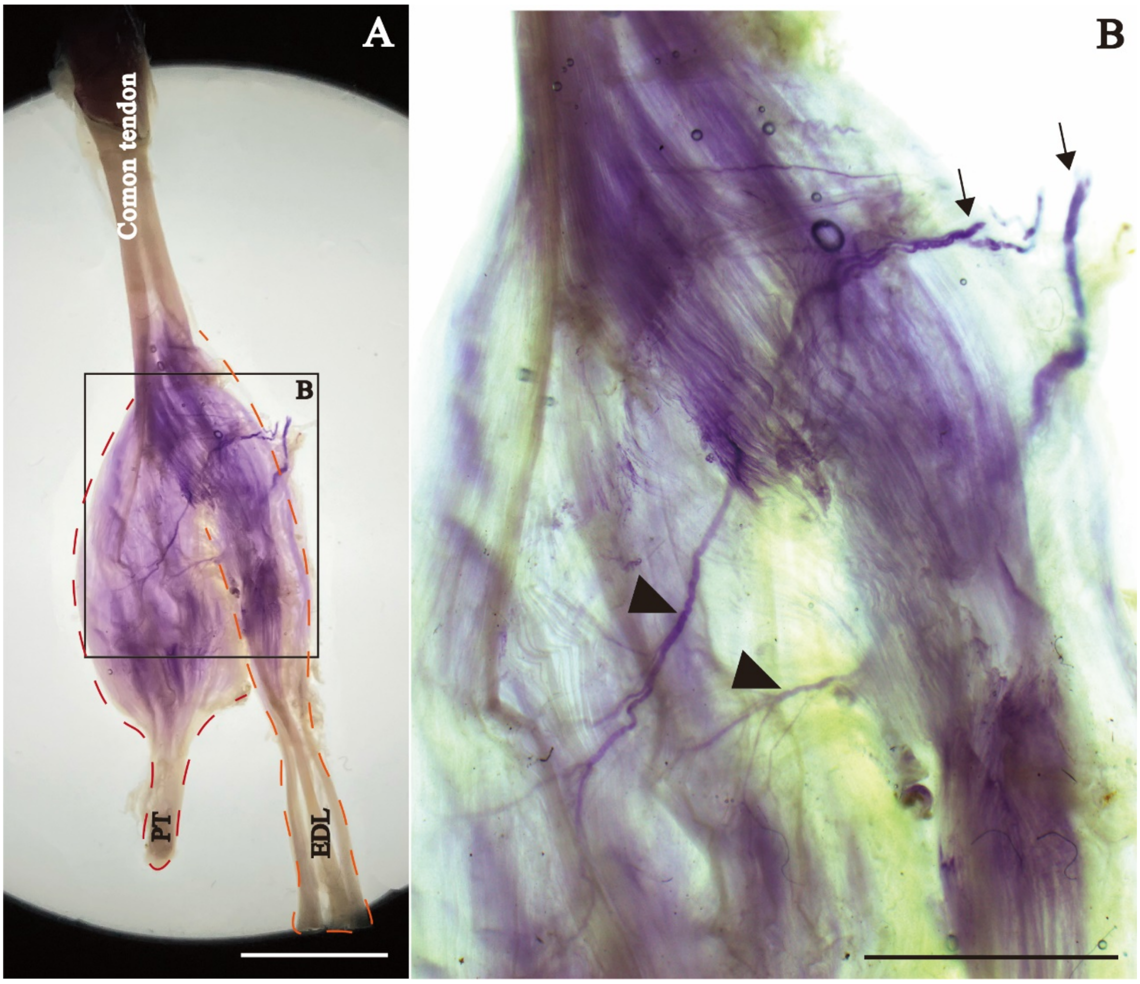


**Figure 4.** Photographs showing the innervation of the peroneus tertius in a fetal pig with Sihler's staining. The dotted lines indicate the borders of the structure. B, an enlarged view of the area enclosed by the square "B" in A (B). Arrowheads — the nerve branches to the PT;

Arrows — the nerve branches to the EDL; EDL — extensor digitorum longus muscle; PT — peroneus tertius muscle. Scale bar" (A) = 1.0 cm, (B) = 0.5 cm

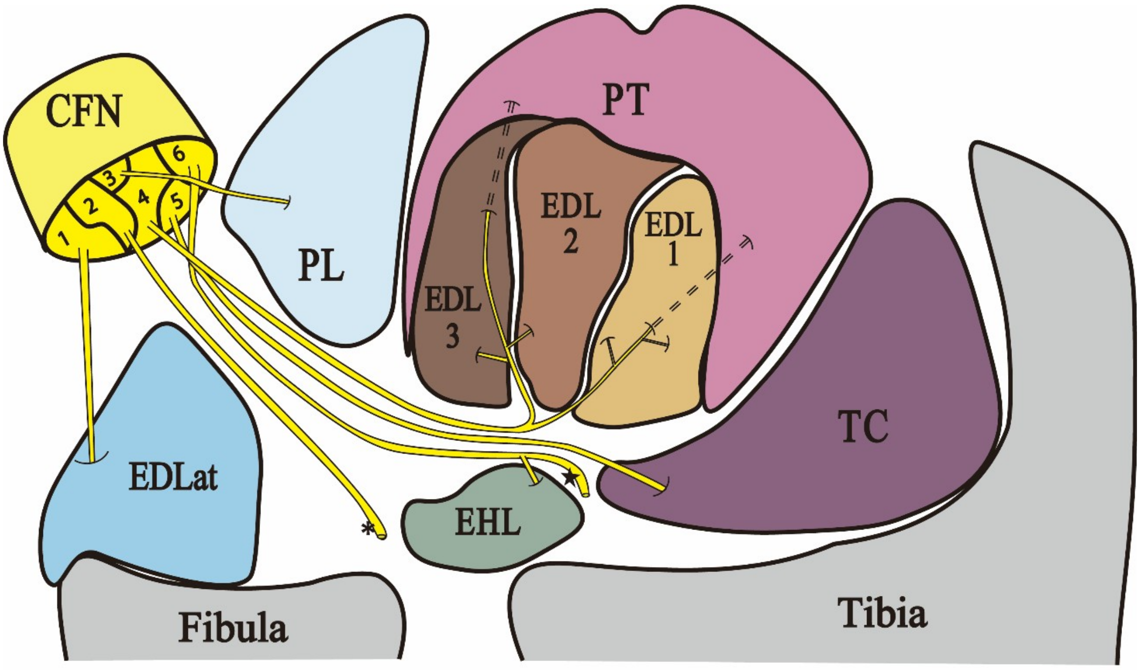


**Figure 5.** Schematic illustration showing the relationship between the nerve bundles and the branches to each muscle in figures 3 and 4. 1–6 — nerve bundles of the common fibular nerve at the gastrocnemius nerve bundles of the common fibular nerve at the gastrocnemius pierced, numbered in dorsal order on the nerve bundle; CFN — common fibular nerve; EDLat — extensor digitorum lateralis msucle; EDL1–3 — extensor digitorum longus muscle; EHL — extensor hallucis longus muscle; PL — peroneus longus muscle; PT — peroneus tertius muscle; TC — tibialis cranialis muscle; Asterisk — the nerve branch to dorsum of foot; Star — the nerve branch to extensor digitorum brevis muscle