論　文

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Study on structural changes of skin and subcutaneous tissue of foot by mild pressure in rats

ラット足部の軽微な圧迫に伴う皮膚および周囲組織の構造変化に関する研究

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Summary

Purpose of this study was to investigate effects of a mild pressure on skin and surrounding tissues histologically, as simulation of shoes unfitted for the size and the shape of the foot.

Forty-eight seven-week-old rats were used as materials and were divided into experimental group (EX) and control group (CO). Furthermore, EX was subdivided into three groups: 4-day group (EX4, CO4), 7-day group (EX7, CO7), and 14-day group (EX14, CO14) from the point of view of experimental period, respectively. Hind-paws of EX were wound with under wrap and elastic therapeutic tape in each experimental period. The fourth and fifth metatarsals and phalanges were extracted together with skin and surrounding tissues and they were observed histologically and immunohistologically.

It was noted macroscopically that body hair became short and furrows exited at the surface of the foot in EX14, but the body hair covered the whole foot in CO14. However, neither swelling nor rubor were recognized in any group of EX. Many mast cells were found at the dermis and the subcutaneous tissue in both groups. Their sizes in EX were larger than CO, and the matrixes around those cells were stained violet with toluidine blue staining. CD68-positive cells were hardly found at the dermis and the subcutaneous layer in each CO group. Many of those cells were observed in EX4. Those cells decreased from 7-day to 14-day, and the number of the cells in EX14 appeared to be the same as that in CO14. Thus, accumulations of the macrophages and mast cells were recognized at the subcutaneous tissue, despite of appearance of few swelling or rubor at the surface of body with the mild pressure to the foot. It was thought that the changes in the body surface didn't reflect those in tissues necessarily.

From the above, it was suggested that the mild pressure to the foot caused the structural changes of the dermis and the subcutaneous tissue, even if neither swelling nor rubor appeared at the surface of the skin.

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Keywords：pressure to foot, changes in body surface, inflammation

1. Introduction

It is known generally that skin shows hypertrophy and a callus appears when skin is pressed by living tool repeatedly for a long period[1]. The same conditions are also expected if shoes used ordinarily don't fit in size and shape. Moreover, it is thought that inflammation and structural changes could be brought about at tissue level, in that case. It has been shown that neutrophil and macrophages appear at inflamed site. Inflammation is activated by bioactive substance such as Histamin[2], prostaglandin[3], IL-6[4] that mast cells secrete. On the other hand, it has been reported that main inflammatory aspects don't appear at the body surface by mild pressure, but the death of muscle cells and accumulation of inflammatory cells are recognized by mild pressure, from the study using rats' hind limbs[5]. However, it hasn't been cleared what the mild pressure on skin and subcutaneous tissues affects. Then, this study aimed to investigate the effects of the mild pressure on the skin and subcutaneous tissues histologically, as simulation of unfit shoes.

2. Materials and Methods

2.1 Materials

Forty-eight seven-week-old rats were used as materials and were divided into an experimental group (EX) and a control group (CO). Furthermore, EX was subdivided into three groups: a 4-day group (EX4), a 7-day group (EX7), and a 14-day group (EX14) from the point of view of the experimental periods. (Fig.1)

2.2 Methods

2.2.1 Experiments

Hind-paws of EX were wound with under wrap (YONEX) and elastic therapeutic tape (TAKACHIHO MEDICAL) from distal side to proximal side under anesthesia. Their fingertips were uncovered for daily observation to prevent vascular insufficiency. After loosening the tapes, they were replaced with new ones. Because the degree of compression by the tapes influences the results, the experiments were conducted after pilot studies to standardize the way of wrapping, to prevent inflammation responses and to obtain stable results in the tissue structure. (Fig.2)

2.2.2 Sampling and fixation

Rats in each group were euthanized by carbondioxide gas inhalation after each experimental period. After confirming their death, the fourth and fifth metatarsals and phalanges were extracted together with skin and subcutaneous tissues. Those specimens were immersed and

fixed in 4% paraformaldehyde or Karnovsky fixation fluids containing 5% glutaraldehyde and 4% paraformaldehyde.

2.2.3 Macroscopic observation

Some specimens fixed by 4% paraformaldehyde were used for observing changes in skin surface macroscopically.

2.2.4 Histological and immunohistological analyses

The other specimens fixed by 4% paraformaldehyde were decalcified in 8% EDTA (ethylene diamine tetraacetic acid) at 4℃ for 4 weeks, and were dehydrated, cleared and embedded in paraffin.

Serial sections (thickness : 5μm) were cut by a microtome, and were stained by Hematoxylin-Eosin (HE) and toluidine blue staining methods and immunohistostaining method described below. Those sections were observed by a light microscope.

On the other hand, the specimens fixed by Karnovsky fluid were used with the following two observing methods. Some specimens were polymerized at 38, 45, 55 and 60℃, after dehydration, clearance and embedding in resin by ordinary methods. Those resin blocks were trimmed, grounded and etched, and they were stained by toluidine blue dye and then observed under the light microscope. The others were immersed in osmium tetraoxide, as post-fixation. They were dehydrated by alcohol and vacuum-dehydration apparatus, and platinum was evaporated on the surface of the specimens by vacuumed-evaporation apparatus. They were observed with a scanning electron microscope (SEM).

2.2.5 Immunohistostainng using anti-CD68 antibody

We performed immunohistostaining with methods described below. The sections fixed in 4% parafolmaldehyde were deparaffinized, quenched with endogenous peroxidase by 1% H_2O_2 diluted in methanol for 30 minutes at room temperature (RT) and incubated in 0.05% protease for 15 minutes at RT for antigen retrieval. Then, primary antibody (CD68 monoclonal antibody: sc-59103 ; Santa Cruz) diluted in PBS containing 1% bovine serum albumin (BSA, 1 : 100) was applied to the sections at 4℃ overnight after blocking in 3% BSA, then secondary antibody was added to them for one hour. The sections were incubated in diaminobenzindine (DAB) and counterstained with Mayer's Hematoxylin for microscopic observation.

3. Results

From observing the foot surface macroscopically, it was noted that body hair became short and furrows exited in EX14, but the body hair covered the whole foot in CO14. However, neither

swelling nor rubor were recognized in any group of EX. (Fig.3)

When observing longitudinal ground sections that were stained by toluidine blue staining, many shallow furrows were seen on the skin surface in the fourth and fifth phalanges of CO. A thick stratum corneum that was stained dark blue existed at the surface of the epidermis, but the dermis was stained pale blue and was thicker than the stratum corneum.

By this staining method, the bone matrix in the cortical bone of the fourth phalanges showed metachromasy and was stained violet and the calcified cartilage matrix stained dark blue existed in the deep region of those bone matrix in CO. The calcified cartilage matrix that showed the same staining characteristics as CO were also recognized at the deep region of the bone matrixes in EX. (Fig.4) The bone surface was smooth in each group and little difference in structural characteristics of cancellous bone was also recognized between CO and EX, even when observing magnified images by SEM. (Fig.5)

TRAP positive cells were observed in the periosteum, but they didn't exist at the surface of the cortical bone. Bone resorption grooves were hardly seen at that surface, and there were many osteoblasts or bone lining cells. (Fig.6)

Loose matrix fibers were arranged in parallel to the body surface at the dermis and subcutaneous tissue on the surface of the proximal phalanx in CO4, but in EX, density of the matrix fibers was lower than CO4 and those fibers were arranged irregularly. The densities and arrangements of the matrix fibers of CO7 and CO14 were the same as CO4. Those fibers increased acutely in EX7, but decreased in EX14, and as a result, showed at the same level of CO14. So, the density of the matrix fibers reached the highest level in EX7, and the density of cells was also the highest in the dermis in EX7. (Fig.7)

Many cells that showed metachromasia and were stained violet with toluidine blue staining, that is, many mast cells were found at the dermis and the subcutaneous tissue in each group of CO. The matrix around cells was also stained violet in some of those cells. The cells in each group of EX were remarkably larger compared to those of CO. Cytoplasm of the cells in EX often showed a high staining level and was stained dark violet. Furthermore, it was recognized, in EX4 especially, that many of the cells had lesser granules around pale-stained nuclei, and the matrixes around those cells were stained the same color of granules. Those characteristics concerned to cell staining and granule accumulation in EX4 weren't clear in EX7 and EX14. (Fig.8)

CD68-positive cells were hardly found at the dermis and the subcutaneous tissue in each CO group. Many of those cells were observed in EX4. Those cells decreased from 7-day to 14-day in EX, and the number of the cells in EX14 appeared to be the same as those in CO14. (Fig.9)

4. Discussion

It was investigated that the changes in the body surface and the tissue with pressure on the foot

in this study. It has been reported that hypertrophy of skin like a callus appeared by repetitive pressure for a long-term[1]. On the other hand, lesion like bed sore extends to deep area of skin, when more pressure was given to local site of body by long-term bed rest[6]. The tape was wound loosely onto the foot of rats, as simulation of unfit shoes. As a result, little decrease of body hair of the foot was recognized, but no inflammatory symptoms such as swelling and rubor were observed at the skin surface. Moreover, osteoclasts attaching to bone surface and Howship's grooves weren't found in any group. Then, it was thought that the pressure corresponded to that causing callus, from the facts that the inflammatory symptoms at the skin surface and the bone resorbing weren't observed.

The density of the matrix fibers decreased and their arrangements were also going to be irregular in the subcutaneous tissue, after four days of the experiment. They increased drastically after seven days of that, and decreased again to the same level of CO. Many macrophages were also observed after four days of that. It was thought that decrease in density of the matrix fibers was caused by phagocytosis of those many macrophages for remodeling tissue after four days of the experiment, and many fibers were produced accurately after seven days of that. Furthermore, it was speculated that unnecessary fibers were removed and were rearranged after fourteen days of that.

Neutrophils, dendritic cells and macrophages appear at inflammatory site, and they phagocytose bacteria and viruses in the case of infection. Those cells phagocytosed debris of necrotic cells, in the case that tissues were destroyed by physical or chemical stimulations. It has reported that the macrophages of brain and Kupper's cells of liver increased by administrating LPS to the experimental animals[7, 8]. Then, the macrophages play important role of repair the tissue destroyed in the inflammation.

The macrophages were derived from monocytes in bone marrow, differentiated in the tissue, and acquired active phagocytic ability. They expressed antigens in cell membrane according to each differentiation stage. CD68 wasn't expressed in the macrophages specifically, and was also expressed in the osteoclasts and the fibroblasts[9, 10]. The macrophages were identified using CD68 antibody in this study, because this antibody was often used for identification of the macrophages[11, 12, 13, 14].

The inflammatory symptoms like swelling, rubor, and decline of pain threshold appear with bruise or pressing skin, generally, and they are induced by secretion from mast cells[15]. It has reported that neutrophils and macrophages appeared at affected site, in the study that artificial inflammation had been induced[16]. Thus, the inflammatory response is brought about by physical and chemical stimulations, then, histamine[2], prostaglandin[3] and IL-6 that the mast cells secrete induce the inflammatory symptoms.

The cells that had many granules showing metachromasy were found in the subcutaneous tissue, as observing decalcified paraffin sections stained by toluidine blue. It has been showed that

the cells indicating those staining characteristics were mast cells[17].

In this study, the mast cells in EX were larger than in CO. The cells had pale nuclei and the granules that were stained violet, moreover the matrix around the cells showed metachromasy, in EX4. It was thought that the cells in EX were large size because they had many granules, and on the other hand, the existence of the nucleus was clear because they had already released the granules. In fact, the matrix stained violet and it was speculated the granules that this meant the mast cells secreted the granules actively, in EX4. After that, the reaction caused by mild pressure reached a peak because those cells that indicated such a characteristics decreased gradually from 7-day to 14-day in EX.

Nanakawa et al.[5] has reported, by adding a light pressure to hind-limb of mouse, that necrosis of muscle and the accumulation of inflammatory cells were recognized, in spite of finding no chief inflammatory symptoms. In this study, no changes were found at the body surface, but increases in the mast cells and the macrophages were recognized at the dermis and the subcutaneous tissue, by pressing on the foot by the elastic therapeutic tape for 4, 7 or 14 days. It was thought that the dermis and the subcutaneous tissue had a higher sensitivity to the stimulation from an external environment than the epidermis, and at the same time, it showed high resistant ability to the stimulation.

As above, increases of the mast cells and the macrophages were recognized at the dermis and the subcutaneous tissue, in spite of finding no changes of the body surface, in this study. It was thought, from this data, that the changes in the body surface accompanied by pressure didn't necessarily reflect those in tissue.

The number of the mast cells and the macrophages reached peak level at the end of 4-day experiment, and they decreased afterwards, by the pressure on the foot in this study. The changes in those cells during the experimental periods weren't able to understand because their changes were observed at the 4-day experimental period, the earliest observing point of this study. However, the results of this study, at least, meant that the appearance of the macrophage reached the highest level by the end of 4-day experiment, and they decreased to the same level as control when the pressure continued for two weeks after that. The reason why the inflammation continued wasn't able to be cleared in this study, and this was future issues.

5. Conclusion

It was suggested that the mild pressure to the foot caused the structural changes of the dermis and the subcutaneous tissue, even if neither swelling nor rubor appeared at the surface of skin.

Acknowledgement

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Committee of Animal Experiment and Ethics

This study was approved by the Ethical Committee for the research of the Faculty of Human Life Design and by the Animal Care and Use Committee, Toyo University.

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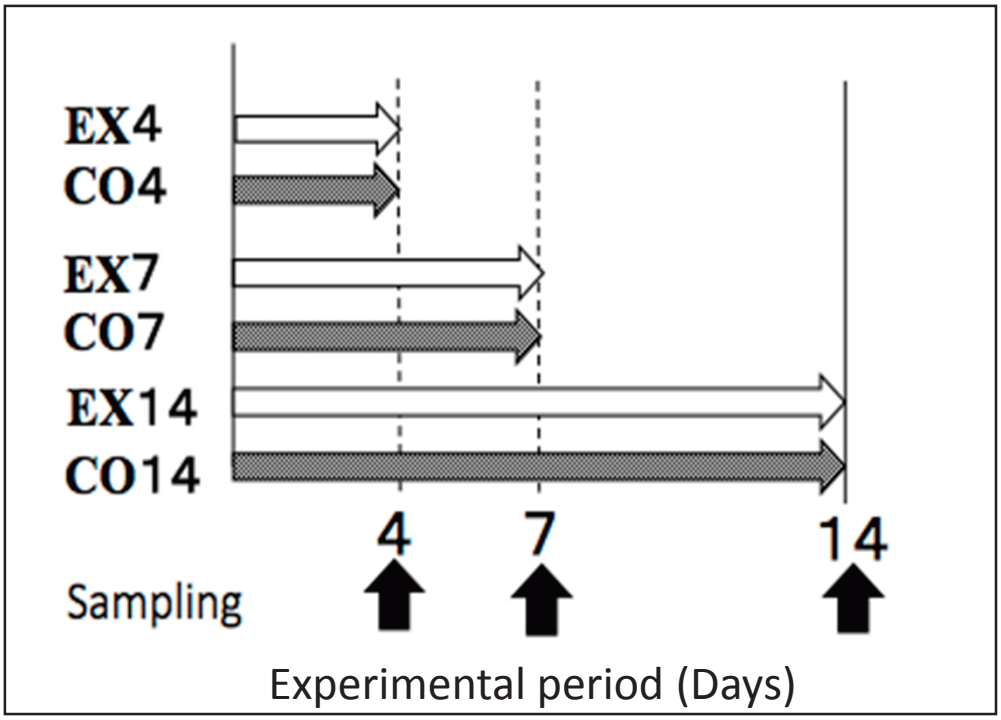

Fig.1 Protocol of experiment.
After each experimental period, rats were euthanized and specimens were removed.

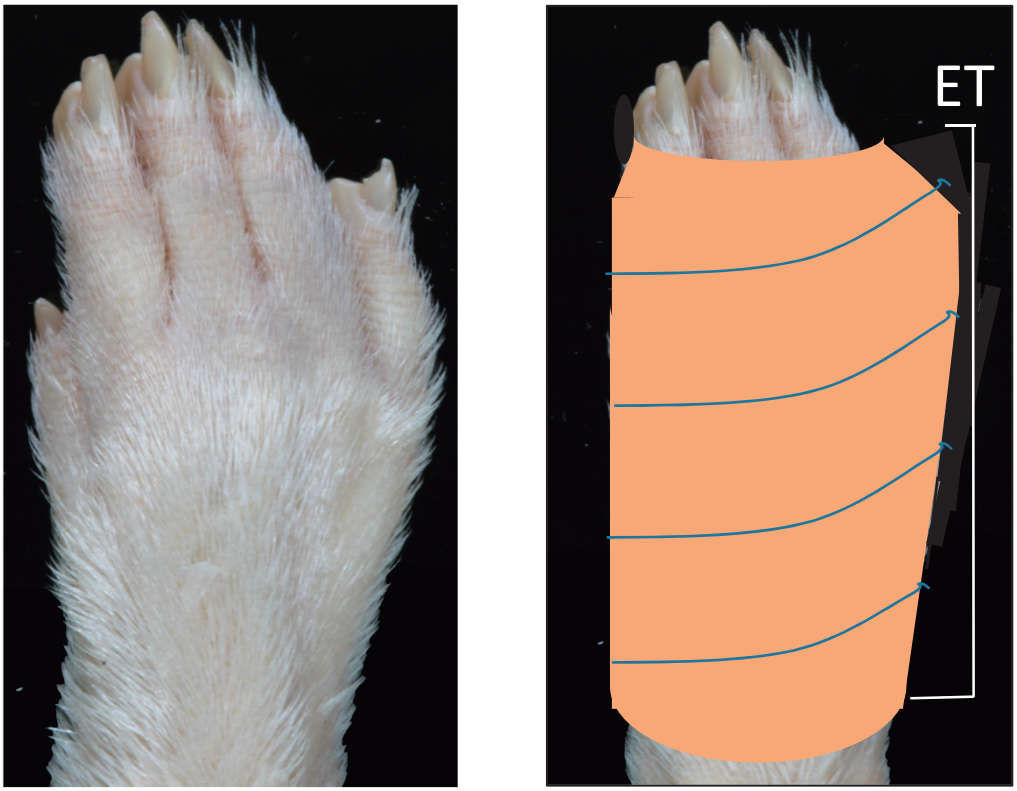

Fig.2 Method of winding tape around foot.
Left：the surface of the foot of rats
Right：the elastic therapeutic tape（ET）wound onto foot

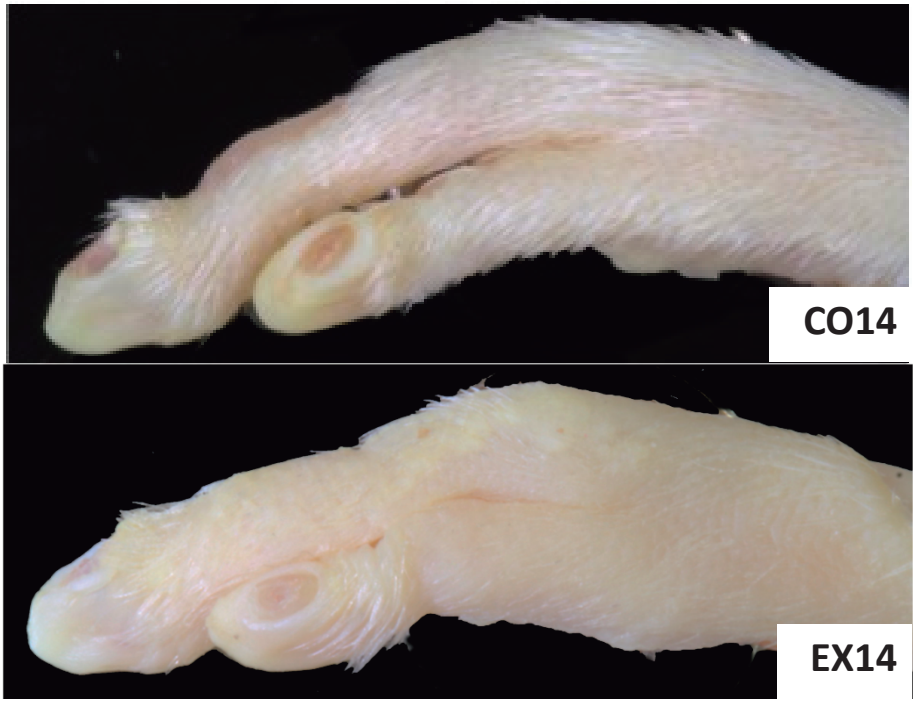

Fig.3 Macroscopic observations of the feet of CO14 and EX14.
Little Inflammation responses such as swelling and rubor were observed macroscopically in each group.

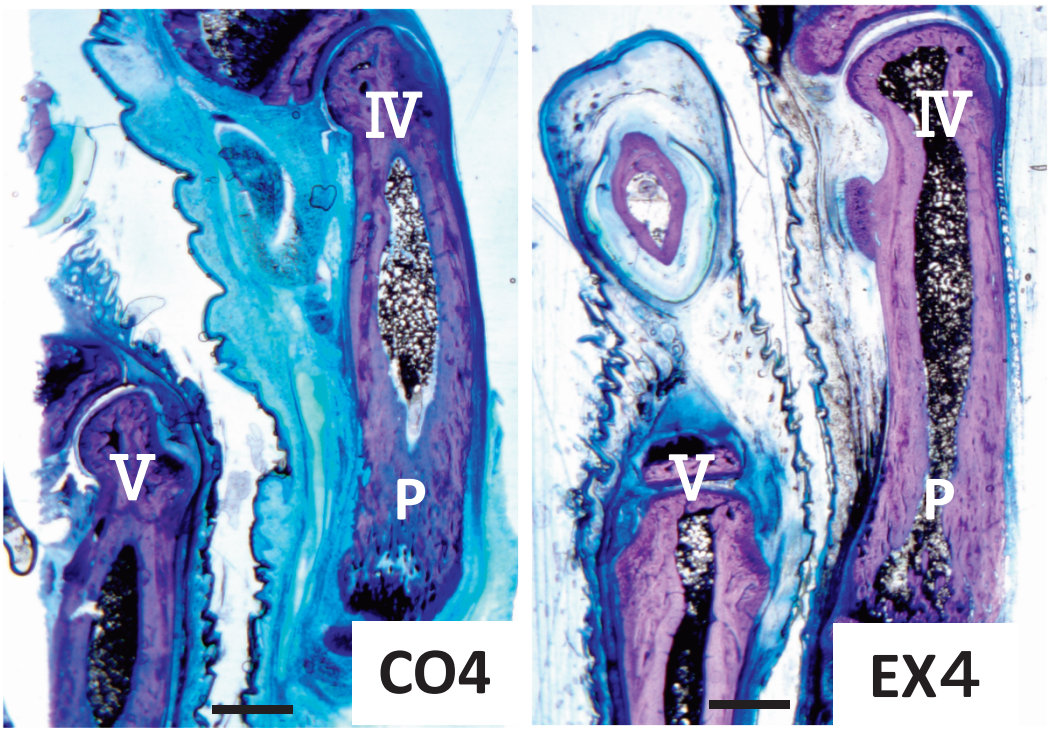

Fig.4 Histological observations of fourth and fifth phalanges of CO4 and EX4.
Morphological differences were not confirmed between two groups.
IV : fourth phalanx, V : fifth phalanx, P : proximal phalanx bone, bar : 1mm

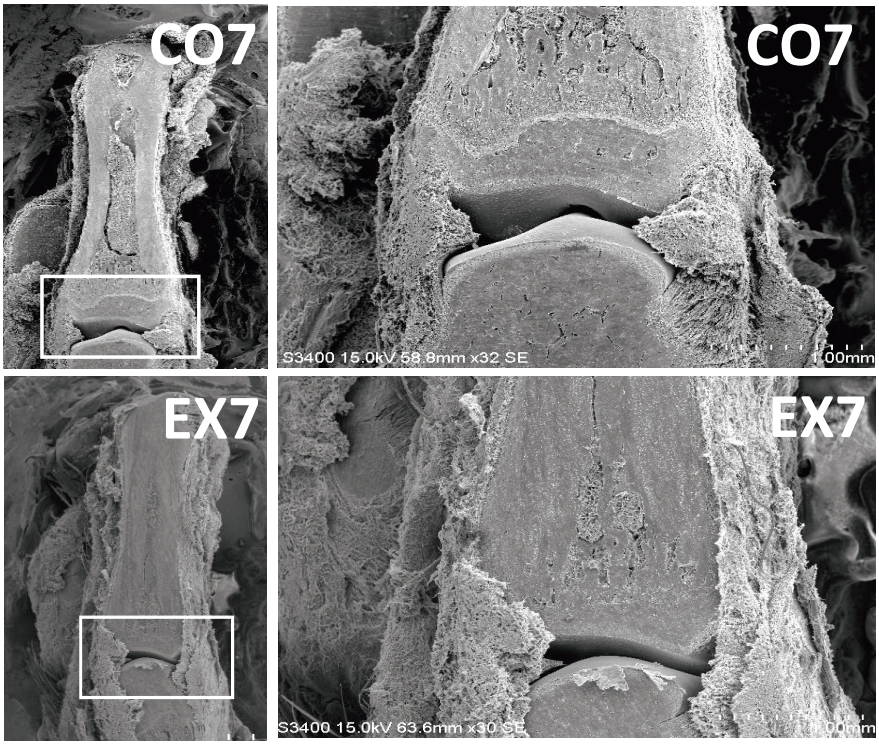

Fig.5 SEM images of fourth proximal phalangeal bones.
Little difference in shape and structure of cortical and cancellous bone was observed between two groups.
Left：low magnified images in CO7 and EX7（x10）
Right：magnified images of squares of left images（x30）

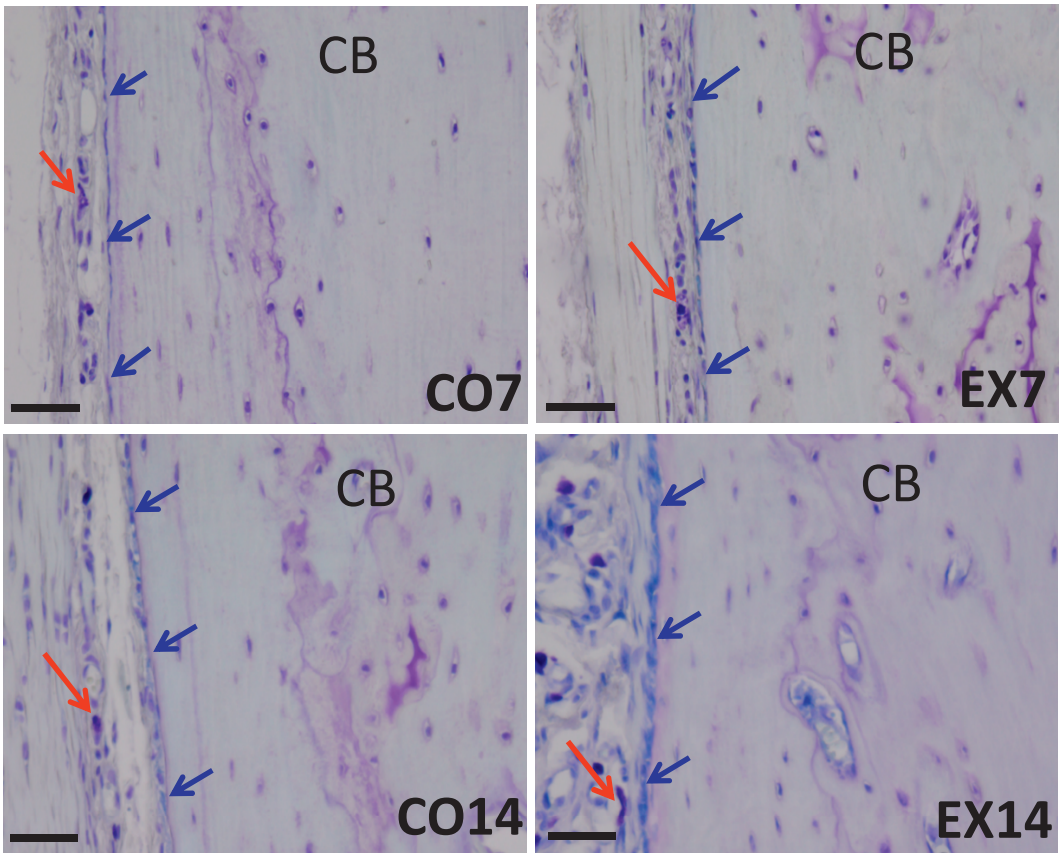

Fig.6 Localizations of TRAP-positive cells in diaphyses of fourth proximal phalangeal bones.
TRAP-positive cells existed in the periosteum but not at the bone surface.
CB : cortical bone
red arrows : TRAP-positive cells
blue arrows : bone surface
bar : 20μm

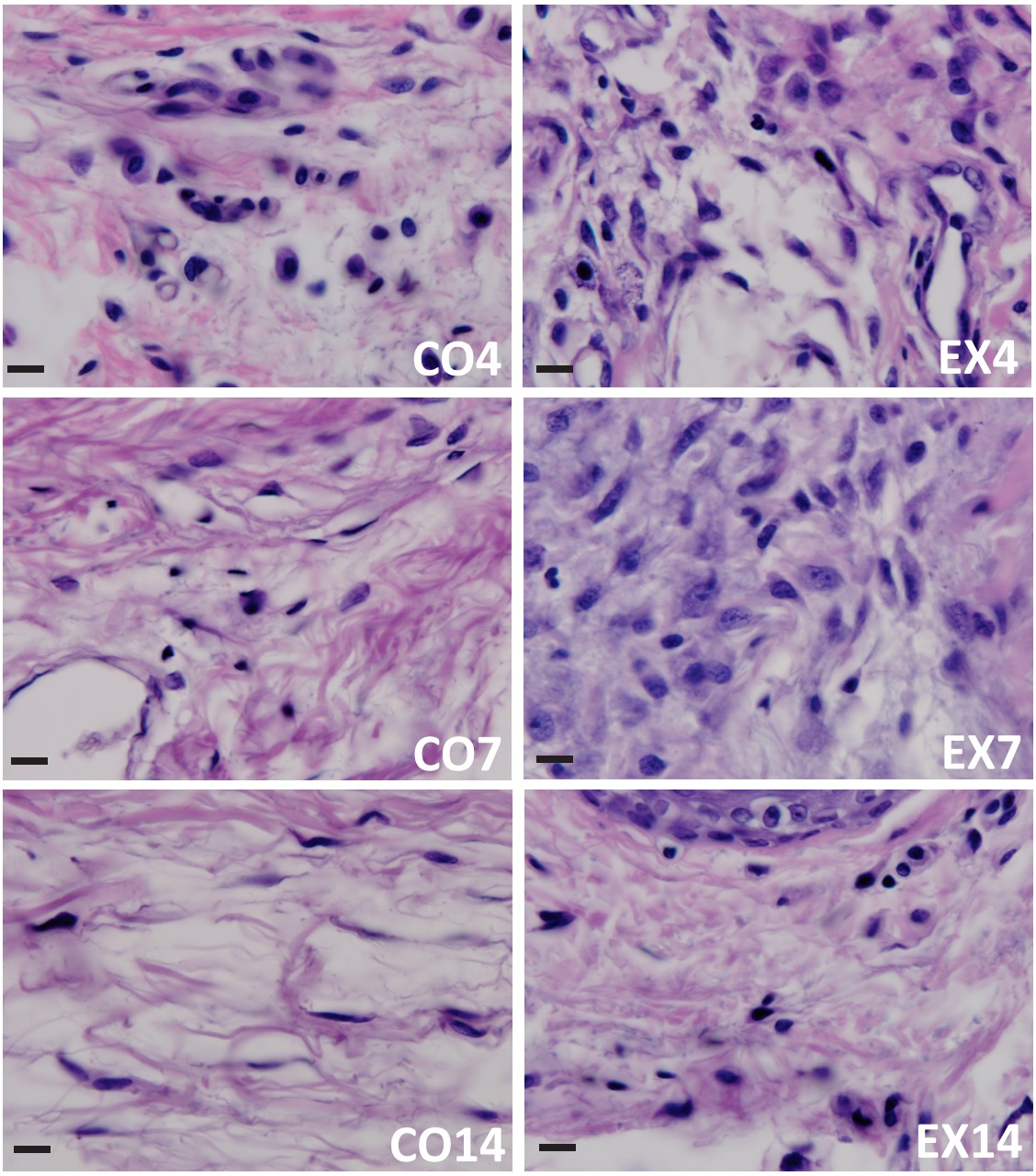

Fig.7 Comparison of densities of cells and matrix fibers in dermis and subcutaneous tissue of both groups.
Densities of cells and matrix fibers indicated the highest level in EX7.
bar：10μm

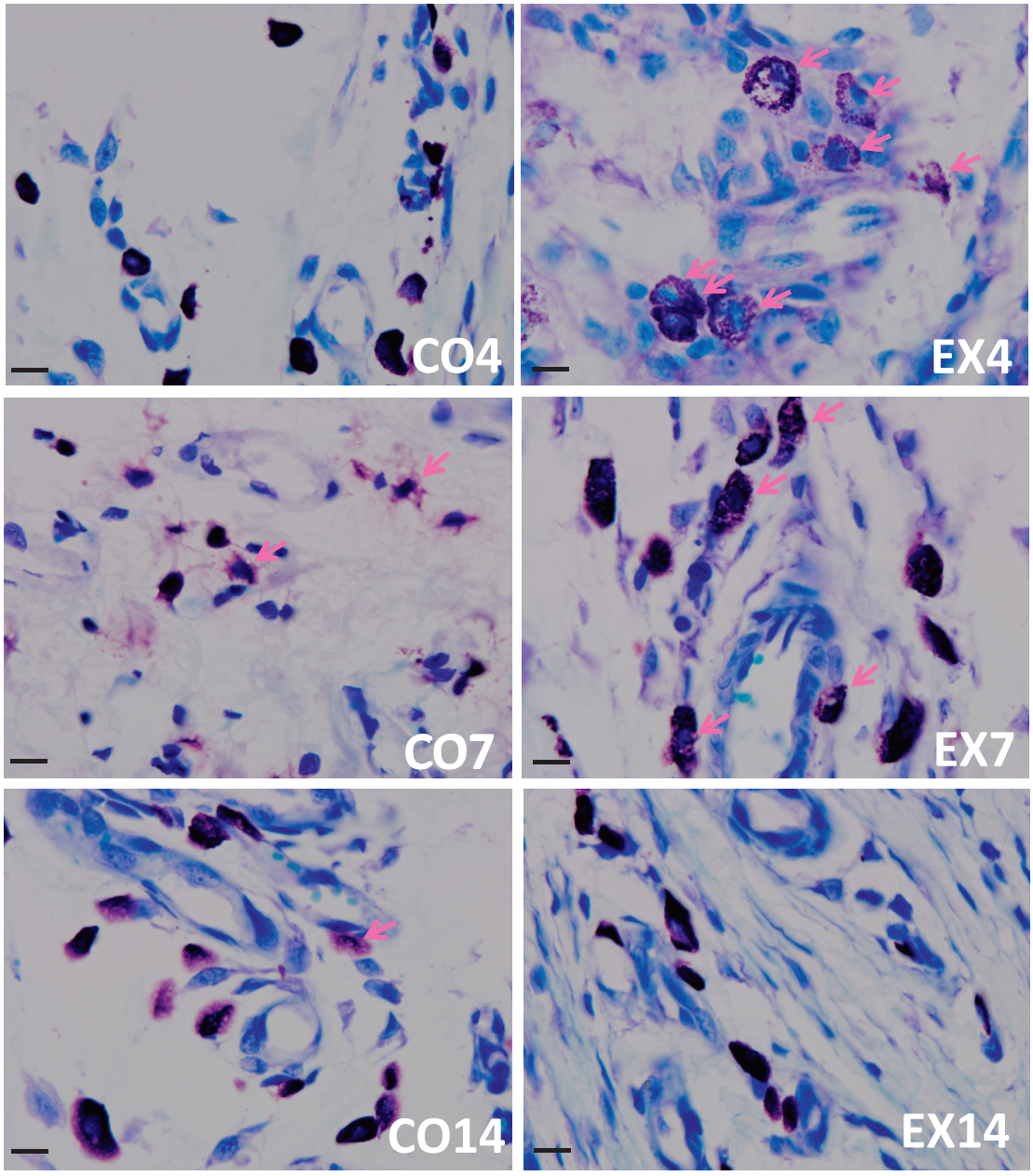

Fig.8 High magnification of mast cells in subcutaneous tissue.
Mast cells in EX were larger than the ones in CO. Arrows show degranulation from those cells.
bar : 10μm

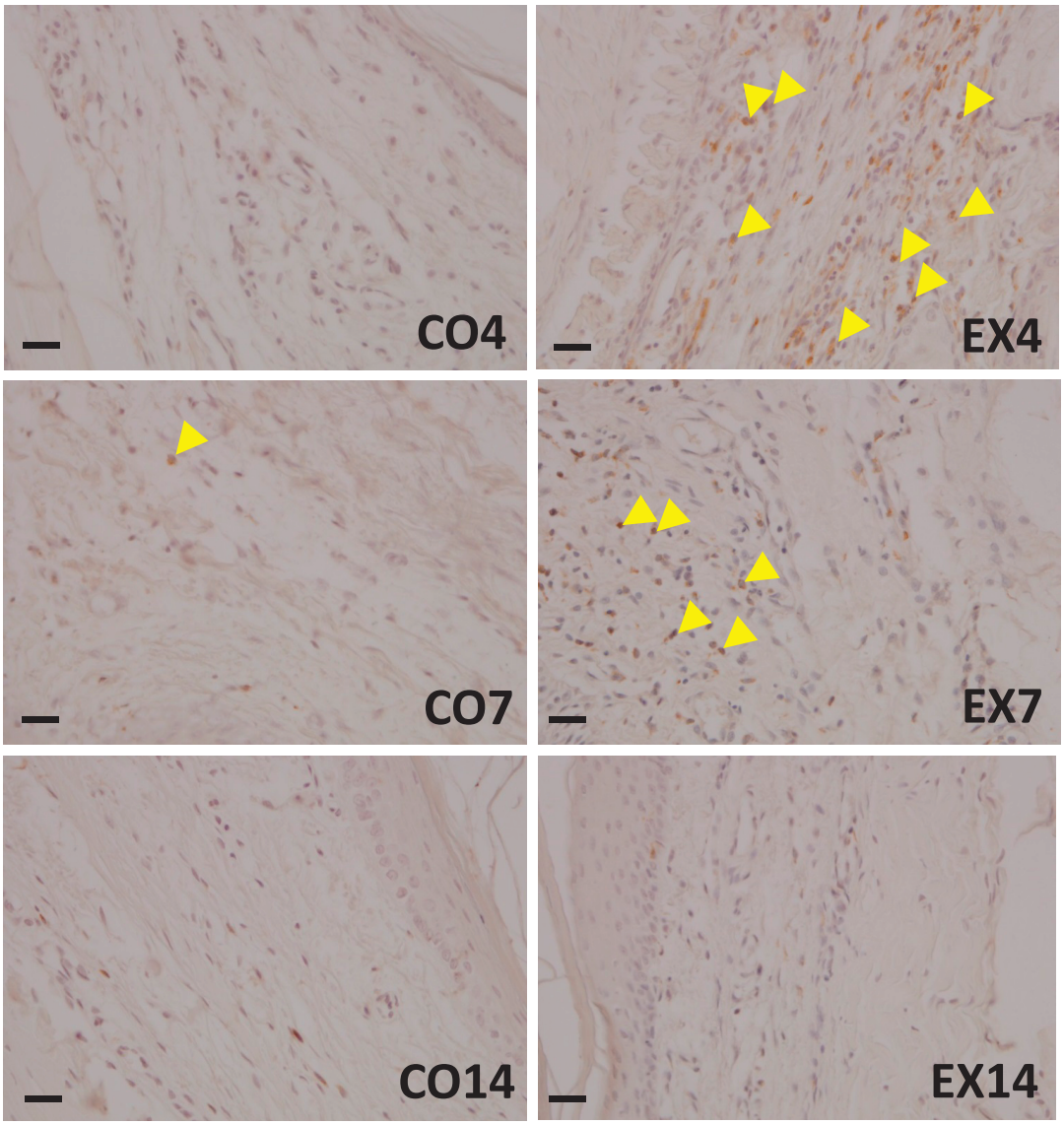

Fig.9 Appearance of CD68-positive cells at skin around fourth phalangeal bones.
CD68-positive cells (yellow arrow heads) were increased in EX4 and EX7, but decreased in EX14.
bar : 10μm

ラット足部の軽微な圧迫に伴う皮膚および周囲組織の構造変化に関する研究

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要旨

【目的】 本研究は、足部圧迫による骨および周囲組織への影響に関する基礎的研究として、ラットを用いて後肢足部の圧迫に伴う第４指骨と皮膚における構造変化について検討することを目的とした。

【材料および方法】 材料として７週齢のラット48匹を用い、それらを実験群EXと対照群COに分け、さらにEXは実験期間別に４日群、７日群および14日群（EX4、EX7、EX14）に分類した。EXには後肢足部の外側からテープで固定した。各実験期間の終了後、両群から第４・５指の中足骨および指骨を周囲組織と共に一塊として摘出し、肉眼的および組織学的に観察した。なお、本実験ではテープによる圧迫の強さが結果に大きく影響するため、予備実験において足部の圧迫がほぼ一定になるように繰り返し練習した。装着した翌日に腫脹、発赤等がみられず、また、組織構造にも毎回同様な結果が得られるになった後に本実験を実施した。

【結果】 いずれの群においても腫脹、発赤等の炎症症状はみられなかった。第４指骨のトルイジンブルー染色切片およびCD68の免疫染色切片をみると、肥満細胞、マクロファージはともにEX4、EX7ではCOより多く存在するが、EX14では減少した。TRAP染色切片を見ると、EXの骨幹には破骨細胞は出現しなかった。

【結論】 軽度の足部圧迫では、体表には腫脹、発赤のような炎症症状が見られないが、皮下では炎症性細胞の増加がみられており、体表の状況と組織構造の変化は必ずしも一致しないことが理解された。

キーワード：足部圧迫　皮膚　骨構造

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