Histochemical examination on principal collagen fibers in periodontal ligaments of ascorbic acid-deficient ODS-*od/od* rats

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Abstract

In this study, we aimed to clarify the role of ascorbic acid in collagen synthesis in periodontal ligaments using ODS/ShiJcl-*od/od* rats lacking L-gulonolactone oxidase. These rats cannot synthesize ascorbic acid *in vivo*. Eight weeks-old ODS/ShiJcl-*od/od* male rats were administered ascorbic acid solution at a concentration of 200 mg/dL (control group, n = 6) or ascorbic acid solution at concentration of 0.3 mg/dL (insufficient group, n = 12). Six rats of the insufficient group were then given with ascorbic acid solution at concentration of 200 mg/dL for additional three weeks (rescued group, n = 6), and then, their mandibles were histochemically examined. Consequently, the insufficient group specimens were seen to possess fewer collagen fibers, and silver impregnation revealed numerous fine, reticular fiber-like fibrils branching off from collagen in the periodontal ligaments. In control group, faint immunoreactivities for MMP2 and cathepsin H were seen in the periphery of blood vessels and throughout the ligament, respectively. In contrast, in the insufficient group, intense MMP2-immunoreactivity was observed to be associated with collagen fibrils in the periodontal ligaments, and cathepsin H-immunopositivity was seen in ligamentous cells. The rescued group showed abundant collagen fibers filling the periodontal ligament space. Under transmission electron microscopy, ligamentous fibroblasts incorporated collagen fibrils into tubular endosomes/lysosomes while simultaneously synthesizing collagen fibril bundles.

Thus, ascorbic acid insufficiency affected the immunolocalization of cathepsin H and MMP2; however, ligamentous fibroblasts appear to possess the potential to synthesize collagen fibers when supplied with ascorbic acid.

240 words

Introduction

Periodontal ligament is a typical dense fibrous connective tissue that secures teeth in the tooth sockets. It intervenes between the tooth and the alveolar bone and is important in physiological functions of the periodontal tissues, such as proprioception in response to mechanical stress, tooth support, blood supply, and tissue remodeling. The periodontal ligaments abundantly contain many extracellular matrix proteins: type I collagen, type III collagen, fibronectin, proteoglycans, periostin, and oxytalan fibers *i.e.,* pre-elastin type fibers [1-4]. Type I collagen fiber is the most abundant among the extracellular matrix constituents of periodontal ligaments. Although collagen fibers serve as a scaffold for mineralization in the bone, dentin, and cementum [5-8], the principal collagen fibers of the periodontal ligaments evade mineralization with a yet unknown mechanism. The bundles of collagen fibers are regularly arranged as specific groups in humans, *e.g.,* gingival, trans-septal, alveolar crest, horizontal, oblique, and apical fibers; this arrangement is strongly influenced by the mechanical stress provoked by the occlusal force. Presumably for adapting according to the strength and

direction of the mechanical force, collagen fibers in periodontal ligaments are always remodeled in a manner of fast turnover [1, 9-11]. In addition to serving as a dense connective tissue, the periodontal ligaments contain highly populated cellular constituents to perform a number of important functions, *e.g.,* the long-lasting masticatory stress, blood supply for nutrients, and nerve system for sensory. Collagen fibers apparently participate in these biological functions of periodontal ligament cells as well.

Ascorbic acid, also called vitamin C, is necessary for the hydroxylation of proline and lysine after the synthesis of primary amino acid α-chains of collagen. If ascorbic acid is insufficient, collagen with a stable triple helical structure is not formed, and therefore, the cross-link by which three α-chains are assembled together is not formed [12, 13]. Unlike humans, rats and mice can synthesize ascorbic acid *in vivo* through the enzymatic activity of L-gulonolactone oxidase. Rats with a congenital deficiency of L-gulonolactone oxidase, named Osteogenic Disorder Shionogi (ODS/ShiJcl-*od/od*) rats, were created in 1984 [14]. To synthesize normal collagen fibers, exogenous ascorbic acid-containing water/diet must be given to ODS-*od/od* rats [15]. Without ascorbic acid supplement, ODS-*od/od* rats cannot form a normal bone matrix after a few weeks, thus reducing urinary excretion of hydroxyproline or causing fractures [16, 17], and decreasing bone mechanical properties [17, 18]. We have previously observed many osteoblasts detached from trabecular bone surfaces, and fine, fragile fibrillar collagens without evident striation were found in ascorbic acid-deficient ODS-*od/od*

rats. These findings indicate that abnormal collagen fibrils are not able maintain osteoblast adherence with them [19].

To provide better understanding of the biological function of ascorbic acid on collagen in periodontal ligaments, in this study, we have attempted to examine the histological alteration of the periodontal ligaments using ODS-*od/od* rats fed with ascorbic acid-insufficient diet.

Materials and methods

Animals and experimental design

Eighteen 8-week-old ODS/ShiJcl-*od/od* male rats purchased from CLEA Japan Inc. (Tokyo, Japan) were fed with an ascorbic acid-sufficient diet (control group: n = 6) or an ascorbic acid-insufficient diet (insufficient group: n = 12) until 13 weeks of age, as reported previously [19]. Ascorbic acid solution at a concentration of 200 mg/dL or 0.3 mg/dL was given via drinking water to the control group or insufficient group, respectively. After 3 weeks administration of ascorbic acid solution, six rats of the insufficient group were given ascorbic acid solution at concentration of 200 mg/dL (rescued group: n = 6) for additional 3 weeks. The experimental procedures were performed in compliance with the institutional guidelines for care and handling of experimental animals with the approval of the Eisai ethics committee.

Tissue preparation

Rats of the control group, insufficient group, and rescued group were anesthetized with an intraperitoneal injection of pentobarbital sodium and perfused with 4% paraformaldehyde diluted in 0.1-M phosphate buffer (pH 7.4) through the cardiac left ventricle [5, 20, 21]. Mandibles were immediately removed and immersed in the same fixative for 24 h at 4°C. The specimens were decalcified with 10% ethylenediamine tetraacetic disodium salt (EDTA-2Na) solution for 1 month at 4°C. The decalcified specimens were dehydrated in ascending alcohol solutions before paraffin embedding. Sagittal paraffin sections parallel to the mandibles were made, and histochemistry of the first molars and the periodontal tissues was examined. For transmission electron microscopy (TEM) observations, as reported recently [21], some specimens from the rescued group were decalcified with 5% EDTA-2Na for 2 months at 4°C and post-fixed with 1% osmium tetraoxide with 0.1 M cacodylate buffer for 4 h at 4°C; then, they were dehydrated in ascending acetone solutions and embedded in epoxy resin (Epon 812, Taab, Berkshire, UK). Ultrathin sections were prepared with an ultramicrotome and then stained with tannic acid, uranyl acetate, and lead citrate for TEM examination (Hitachi H-7100 Hitachi Co. Ltd, Tokyo, Japan) at 80 kV.

Immunohistochemistry for ED1, type I collagen cathepsin K, cathepsin H, and matrix metalloproteinase (MMP)2

Dewaxed histological sections were pretreated with 0.1% hydrogen peroxide for 15 min for endogenous peroxidase inhibition. After pre-incubation with 1% bovine serum albumin (BSA: Serologicals Proteins Inc. Kankakee, IL)–PBS for 30 min at room temperature (RT), the sections were incubated with 1) rabbit polyclonal antibody against type I collagen (Cosmo Bio, Co., Ltd., Tokyo, Japan) at 1:200 for 1 h at RT , 2) rabbit anti-cathepsin K (Daiichi Fine Chemical Co., Ltd., Toyama, Japan) at 1:200 for 2 h at RT , or 3) rabbit antibody against human/mouse cathepsin H (Protein Tech Group, Inc. Campbell Park Dr. Chicago, IL) at 1:100 dilution for 1 h at RT. Then, the treated sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (DakoCytomation, Glostrup, Denmark). To detect MMP2 and rat CD68 (clone: ED1), the dewaxed sections treated with 1% BSA–PBS were incubated with mouse monoclonal IgG against human MMP2 (Daiichi Fine Chemical Co., Ltd., Toyama, Japan) or mouse monoclonal antibody against rat CD68 (ED1: AbD Serotec, Oxford, UK) at 1:100 dilution for 1 h at RT. Then, the sections were incubated with HRP-conjugated rabbit anti-mouse IgG (Bethyl Laboratories, Inc., Montgomery, TX). For visualization of all immunoreactions, diaminobenzidine tetrahydrochloride was used as a substrate. All sections were counterstained with methyl green and observed under a light microscope (Eclipse E800, Nikon Instruments Inc. Tokyo, Japan). For negative control experiments, normal rabbit serum or mouse serum was used, following incubation with the HRP-conjugated secondary antibodies.

Enzyme histochemistry for acid phosphatase

To detect acid phosphatase activity, as previously reported [22], histological sections were incubated with a mixture of 2.5 mg of naphthol AS-BI phosphate (Aldrich-Sigma, St. Louis, MO) and 18 mg of red violet LB salt (Sigma) diluted in 30 mL of 0.1 M sodium acetate buffer (pH 5.0) for 15 min at 37°C.

Silver impregnation for detection of reticular fibers

We performed silver impregnation using Watanabe's protocol [23]. Briefly, the dewaxed sections were soaked in a 0.5% potassium permanganate solution or 5–15 min at RT. After rinsing with distilled water, the sections were dipped in a 2% oxalic acid solution until they became transparent. The reaction was enhanced by an aqueous solution containing 0.01% ammonium nitrate, 0.005% sodium hydroxide, and 0.006% silver nitrate for 15 min at RT after additional rinsing. After quick rinsing with 95% alcohol solution, they were treated with 2% iron alum solution + 1% formalin and were then rinsed with distilled water. Sections were subsequently treated with 0.2% gold chloride and 2% oxalic acid for reduction. Finally, after rinsing with distilled water, they were fixed in 0.5% sodium thiosulfate.

Quantification of the area of collagen fibers in the periodontal ligaments and the numbers of ligament cells, and statistical analyses

The periodontal ligament between the interradicular alveolar bone and the distal root of the mandibular first molar was used for analyses. The area surrounding the alveolar bone and cementum located 600 μm under the alveolar crest was selected as the region of interest (ROI). Collagen fibers and ligamentous cells were estimated by Azan staining. All statistical analyses were assessed by Student *t*-test. All values are presented as mean ± standard deviation, and $p < 0.05$ was considered statistically significant.

Results

Periodontal ligaments of the control and ascorbic acid-insufficient groups

Azan staining showed thick collagen fibers in the periodontal ligaments of the control first molars, which ran parallel to each other connecting the alveolar part and facing the cementum **(Fig. 1a and c)**. Conversely, the ascorbic acid-insufficient periodontal ligaments showed fewer collagen fibers, but the cellularity of ligament cells remained unchanged from that of the control group **(Fig. 1b and d)**. The collagen fibers appeared to be scarce in the insufficient group **(Fig. 1d)**. Statistical analyses of the area of collagen fibers and cell numbers in the ROI revealed the markedly reduced index of collagen fibers (55.63 ± 1.90 vs. 8.93 ± 0.41, $P < 0.001$) but not the ligament cell numbers (227.81 ± 5.71 vs. 219.17 ± 5.57, $P > 0.001$) in the ascorbic acid-insufficient specimens **(Fig. 1e)**.

When silver impregnation was examined, the periodontal ligaments of the control group showed thick collagenous fibers that were not stained with silver impregnation **(Fig. 2a)**. However, in the ascorbic acid-insufficient periodontal ligaments, the amount of collagen fibers was extremely reduced **(Fig. 2b)**. When compared with the collagen fibers in the control group, many collagen fibers extending form the cementum were short in the ascorbic acid – insufficient periodontal ligaments. **(Compare Fig. 2c and d)**. On observing at a higher magnification, collagen fibers appeared to branch off into silver staining-positive fine fibrils resembling reticular fibers, while the periodontal ligaments of the control group specimens showed continuous thick collagen fibers with a certain width maintained throughout **(Compare Fig. 2e and f)**. The tips of the reticular fiber-like fibrils further branched off in all directions and showed no connectivity. Unlike principal collagen fibers, blood vessels were surrounded by silver staining-positive fibers in both control and ascorbic acid-insufficient groups **(Fig. 2g and h)**.

Histochemical examination on the periodontal ligaments of the control and the ascorbic acid-insufficient groups

Consistent with histological observations, the amount of type I collagen-immunopositive periodontal ligaments was markedly reduced in the ascorbic acid-insufficient group **(Fig. 3a and e)**. However, the immunoreactivity of type I collagen was not different in dentin and the

alveolar bone of the control and insufficient groups. We have attempted to examine the distribution of osteoclasts/macrophages using ED1-immunohistochemistry and acid phosphatase enzyme histochemistry **(Fig. 3b, c, f, and g)**. Consequently, ED1/acid phosphatase histochemistry revealed that multinucleated osteoclasts were predominant rather than macrophages in the control periodontal ligaments **(Fig. 3b, c)**. Conversely, periodontal ligaments had more macrophages in the ascorbic acid-insufficient group specimens than in the control group specimens **(Fig. 3f, g)**; however, both control and ascorbic acid-insufficient group specimens had comparable numbers of cathepsin K-immunoreactive osteoclasts in periodontal ligaments **(Compare Fig. 3d, h)**. Consistent with histochemical findings, statistical analyses showed significantly more ED1-positive mononuclear cells (75.00 ± 5.93 vs. 100.33 + 8.45, $P < 0.005$) and mononuclear acid phosphatase-reactive cells (82.67 $\pm$ 6.55 vs. 110.83 + 7.94, $P < 0.005$) in the ascorbic acid-insufficient group than in the control group **(Fig. 3i)**.

To localize the proteolytic enzymes, we investigated the levels of MMP2 and cathepsin H, which have been reported to be abundantly expressed. MMP2 was observed predominantly proximal to blood vessels of the control periodontal ligaments **(Fig. 4a)**, whereas it was intensely associated with extracellular fibers in the ascorbic acid-insufficient periodontal ligaments **(Fig. 4b)**. In contrast to MMP2, cathepsin H was evenly and faintly present throughout the control periodontal ligaments **(Fig. 4c)**. However, cathepsin H immunoreactivity was unevenly seen in the ascorbic acid-insufficient periodontal ligament.

(Fig. 4d). When observing at a higher magnification, double staining of cathepsin H and acid phosphatase demonstrated cathepsin H-immunoreactivity in many periodontal ligament cells but not in acid phosphatase-positive macrophages/osteoclasts **(Fig. 4e)**.

Periodontal ligaments in the rescued group

We further examined if ascorbic acid supplementation could rescue the malformed periodontal ligaments caused by ascorbic acid insufficiency. As shown in **Fig. 5**, after three weeks, the ascorbic acid supplementation rescued the collagen fibers to a normal state, *i.e.,* featuring thick bundles of collagen fibers filling the periodontal ligament space **(Fig. 5a and b)**. Unlike the ascorbic acid-insufficient group, few cathepsin H-immunoreactive cells were scattered in the periodontal ligaments of the rescued group **(Fig. 5c)**. To identify the cell types involved in remodeling of collagen fibers in periodontal ligaments, we observed the ascorbic acid-supplemented periodontal ligaments under TEM. It was seen that thick bundles of collagen fibers had filled the periodontal ligament space, including ligamentous fibroblasts **(Fig. 5d)**. These collagen fibers formed stout bundles, which appeared to extend from the cell membranes of ligamentous fibroblasts **(Fig. 5e)**. However, some isolated fibers could be seen in the cytoplasm of fibroblasts. At a higher magnification, ligamentous fibroblasts were seen to possess many vesicles and secondary lysosomes, and they also incorporated one or two tannic acid-positive collagen fibrils into tubular endosomes/lysosomes **(Fig. 5f and g)**.

Discussion

In the previous study, many investigators have investigated the biological function of ascorbic acid in many tissues, including bone and teeth by using rodent model [15-18, 24-35].

Our histological observations in this study evidently demonstrate markedly reduced amounts of collagen fibers in ascorbic acid-deficient rats; further, the collagen fibers branched off into fine fibrils resembling reticular fibers immediately after extending from the cementum. Furthermore, we observed that these fine fibrils did not connect with each other, implying the attenuated ability to resist against occlusal forces, the most prominent mechanical stress. In general, collagen fiber deformity must lead to fragile architecture of bone and teeth. Miyajima et al. [31] reported that the periodontal ligaments of ODS/ShiJcl-*od/od* rats were narrower than those of the control rats. Ascorbic acid deficiency in ODS/ShiJcl-*od/od* rats causes marked bone loss and reduced bone formation, which consequently leads to a greater reduction of biomechanics without causing macro-architectural changes [15]. Hara and Akiyama [17] suggested that the level of collagen and abnormalities of hydroxylation are involved in the ascorbic acid deficiency-related reduction in mechanical properties of the bone. Considering these reports, extremely reduced collagen synthesis in ascorbic acid-insufficient periodontal ligaments may compromise the mechanical properties. Although it is anticipated, we have

demonstrated in this study that without enough ascorbic acid, it is impossible to maintain thick collagen fibers running parallel to each other.

We have postulated that fine fibril-resembling reticular fibers induced by ascorbic acid insufficiency would be digested by macrophages or ligamentous fibroblasts. Ascorbic acid-insufficient periodontal ligaments showed a tendency of relatively increased numbers of macrophages. However, in our observation, MMP2 and cathepsin H were seen mainly in ligamentous cells rather than in macrophages. As shown in **Fig. 5**, ligamentous fibroblasts incorporated fine collagen fibrils, which possessed developed lysosomes inside the cytoplasm. Notably, fibroblasts newly synthesize collagen fibrils but simultaneously engulf the collagen fibrils **(See Fig. 5f)**. This suggests that ligamentous fibroblasts remodel principal collagen fibers without cooperating scavenger macrophages.

In the rescue experiment, the amount of collagen fibers filled in the periodontal ligament space after three weeks of ascorbic acid supplement, and ligamentous fibroblasts already began to remodel the collagen fibers. These findings seem to be in agreement with previous literatures describing that measurements of isotope-labeled proline uptake in the periodontal ligament have indicated a high turnover of collagen with a half-life varying between 3 and 23 days in young rats [36]. Another possible reason for the immediate recovery of the collagen fibers by ascorbic acid supplementation may be that cellularity of periodontal

ligaments was not decreased in the ascorbic acid-insufficient group (**Fig.1e)**. This implies that periodontal ligament cells may still have the potential to synthesize collagen even under ascorbic acid-insufficiency. Indeed, our previous examination of ascorbic acid-insufficient bones demonstrated that although many osteoblasts were present, most of them were detached from the bone surface [19]. Taken together, the turnover rate of collagen in the periodontal ligaments is estimated to be faster, and the supplementation of ascorbic acid could immediately rescue the malformed collagen to a normal state in periodontal ligaments.

Among the various proteolytic enzymes, MMP1, 2, and 9, and tissue inhibitor of metalloproteinases (TIMP) 1 and 2 are the major proteinases and their inhibitors in periodontal ligaments, respectively [37]. In our previous study, orthodontic tooth movement induced by the Waldo method showed markedly reduced immunoreactivity of MMP2 in the compressed side of the periodontal ligaments [38]. When periodontal ligament cells were cultured on flexible-bottom culture plates while being subjected to cyclic mechanical stretching, MMP2/TIMP2 expression was elevated [39]. Fluid shear stress may have increased the expression of MMP1, 2, and TIMP1 [37]. Furthermore, several researchers reported that vascular endothelial cells produce MMP2 and MMP9 to digest the vascular basement membrane composed of type IV collagen, thus enabling the subsequent angiogenesis [40]. This suggests that MMP2 plays a pivotal role in the tissue remodeling surrounding blood vessels as

well as angiogenesis in the periodontal ligaments. When Myokai et al. isolated the mechanical stress-induced genes from human periodontal ligament cells, cathepsin H gene, in addition to MMP2, was markedly induced [41]. In agreement with the findings of Myokai et al., we observed intense immunoreactivity of cathepsin H in the ligamentous cells as shown in **Fig. 4e**. Our TEM observation shown in **Fig. 5** shows the incorporation of collagen fibrils in tubular endosome/lysosomes. Cathepsin H is a lysosomal cysteine proteinase important in the overall degradation of lysosomal proteins, possessing the activity of endopeptidase [42]. Therefore, ligamentous fibroblasts may contain abundant cathepsin H to digest the excess amounts of fine fibril and may contain collagen fibers for remodeling of the principal collage fibers. Taken together, the altered distribution of MMP2 and cathepsin H provoked by ascorbic acid insufficiency appears to reflect the digestion of extracellular fine fibrils caused by ascorbic acid insufficiency.

There are several reports that ascorbic acid insufficiency/deficiency affects not only collagen synthesis but also cellular activities and gene expression. For instance, Fatemeh et al. reported substantial reductions in the concentrations of alkaline phosphatase (ALP) and mRNAs for osteocalcin and type I collagen, while the concentration of osteopontin mRNA increased. They postulated that decreased expression of collagen, ALP, and osteocalcin could explain the bone defects in scurvy [43]. Ishikawa et al. have reported that both ALP activity and type I

collagen production were upregulated when periodontal ligament cells were cultured in the presence of ascorbic acid, suggesting that ascorbic acid promotes the osteoblastic differentiation of periodontal ligament cells [44].

However, it is difficult to define the accurate mechanisms associated with the the biological function of ascorbic acid. For example, the ascorbic acid insufficiency in our study resulted in reduced amounts of collagen fibers, but instead, the deposition of fine fibrillary constituents accompanied with increased MMP2-immunoreactivity as shown in **Fig. 4**. However, MMP degradation of the extracellular matrix affects cellular behavior through changes in integrin–cell binding by releasing growth factors harbored in the extracellular matrix and generating the degradation products from extracellular matrix among other ways [45]. For instance, MMP2 degradation of type I collagen can reveal a previously inaccessible cryptic binding site that binds with the αvβ3 integrin. Thus, ascorbic acid may not only directly affect collagen synthesis but also regulate cellular activity through the signaling network of cell-to-matrix adhesion. Further examination is necessary for clarifying this issue in near future.

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Competing Interests

Eisai, Co. Ltd provided funding for this study. Kuniko Hara, Masatoshi Kobayashi, Yasuhiro Akiyama are the employees of Eisai, Co. Ltd, Tokyo, Japan.

Author Contributions

TH is the first author of this work and prepared paraffin sections and performed histochemical analyses. MA, QZ, and TY performed animal experiments, including fixation of animals and paraffin embedding; YM and AY conducted statistical analysis associated with quantifying the area of collagen fibers and the numbers of ligamentous cells positive for ED1, acid phosphatase, and cathepsin K. TY, HY, and HH assisted in the preparation of paraffin sections. YM, MS, TM, PHLF, ML, and NA participated in the discussion and preparation of the manuscript. KH, MK, and YA kept ODS/ShiJcl-*od/od* by giving ascorbic acid, and TH is the principal investigator of this research project, organizing collaborators and conceptualized this experiment. All the above authors have read and approved the final manuscript.

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Figure Legends

Fig. 1

Histological aspects of the periodontal ligament in the control and ascorbic acid-insufficient groups

The control periodontal ligament comprises thick collagen fibers which are regularly arranged in the periodontal space **(Figs. 1a, b)**. The ascorbic acid-insufficient group shows fewer collagen fibers in the periodontal ligament compared to the control group; however, the ligament cell numbers remain comparable between groups **(Figs. 1c, d)**. **Fig.1e** shows the statistical analyses of the ratio of collagen fiber area and the number of ligament cells in the control and ascorbic acid-insufficient groups.

Bars: a, b: 500 μm; c, d: 20 μm

Fig. 2

Silver impregnations in the control and ascorbic acid-insufficient periodontal ligaments

Silver impregnation demonstrates the reduction of collagen fibers and reveales reticular fiber-like fine fibrils branching from the collagen fibers in the ascorbic acid-insufficient group **(Figs. 2b, d, f)**; conversely, the control group reveals thick collagenous fibers in the periodontal space **(Figs. 2 a, c, e)**. The silver impregnation-positive reticular fibers are located at the periphery of the blood vessels in both control and ascorbic acid-insufficient groups **(Figs. 2g and h)**.

Bars: a, b: 50 μm; c, d, g, h: 30 μm; e, f: 10 μm

Fig. 3

Histological alteration of the periodontal ligaments, osteoclasts, and macrophages in the control and ascorbic acid-insufficient groups

Type I collagen-immunoreactive periodonatal ligaments are markedly diminished in the ascorbic acid-insufficient group compared to the control group **(Figs. 3a and e)**. The number of acid phosphatase/ED1-positive macrophages is significantly increased in the periodontal ligament of the ascorbic acid-insufficient group than of the control group **(Figs. 3b, c, f, g, i)**; however, there are no significant difference in the number of cathepsin K immunoreactive-osteoclasts between control and ascorbic acid-insufficient groups **(Figs. 3d, h, i)**.

Bars: a, b, d-f, h: 20 μm; c, g: 10 μm

Fig. 4

The distribution of MMP-2, cathepsin H, and acid phosphatase in the periodontal ligament of control and ascorbic acid-insufficient groups

Immunohistochemistry demonstrates MMP2-positive fibers (arrows in b) in the ascorbic acid-insufficient periodontal ligaments (Fig. 4b), whereas MMP2-immunoreactivity are seen proximal to blood vessels in the control group (See arrows, Fig. 4a). Cathepsin H-immunopositivity is unevenly observed in the periodontal ligament of the ascorbic acid-insufficient group (Fig. 4d). However, cathepsin H is evenly and faintly seen throughout the periodontal ligament of the control group (Fig. 4c). Double detection of cathepsin H and acid phosphatase shows cathepsin H-positive/acid phosphatase-negative periodontal ligament cells (arrows) and cathepsin H-negative/acid phosphatase-positive macrophages/osteoclasts (arrowheads) in the ascorbic acid-insufficient group (Fig. 4e).

Bars: a, b, e: 10 μm; c, d: 20 μm

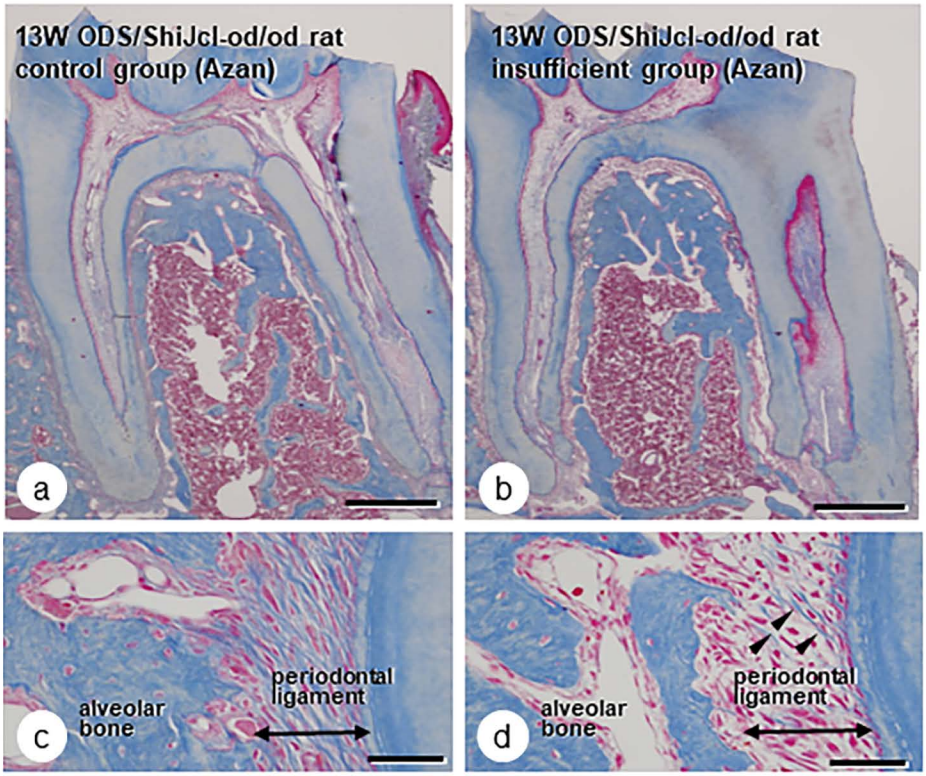
Fig. 5

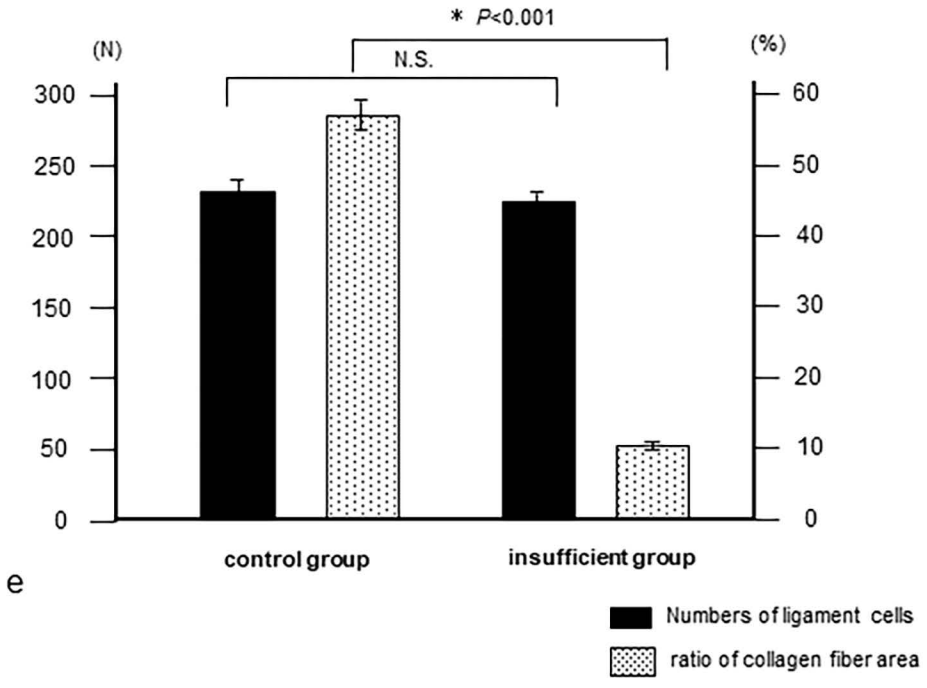
Histological aspects of periodontal ligaments in the rescued group

The rescued group demonstrates dense collagen fibers and slight cathepsin H-positive periodontal ligament cells in the periodontal ligament space **(Figs. 5a–c).** In the corresponding area, thick bundles of collagen fibers are extended from the cell membranes of ligamentous fibroblasts which possess many vesicles, secondary lysosomes, and tubular

endosomes/lysosomes incorporated a few collagen fibrils **(Figs. 5d–g)**.

Bars: a: 20 μm; b, c: 10 μm; d: 5 μm; e: 1 μm; f: 0.5μm


13W ODS/ShiJcl-od/od rat
control group (Azan)
13W ODS/ShiJcl-od/od rat
insufficient group (Azan)
a
b
c
d
alveolar
bone
periodontal
ligament
alveolar
bone
periodontal
ligament


* P<0.001
N.S.
(N)
(%)
300
250
200
150
100
50
0
60
50
40
30
20
10
0
control group
insufficient group
Numbers of ligament cells
ratio of collagen fiber area

e

Fig.1

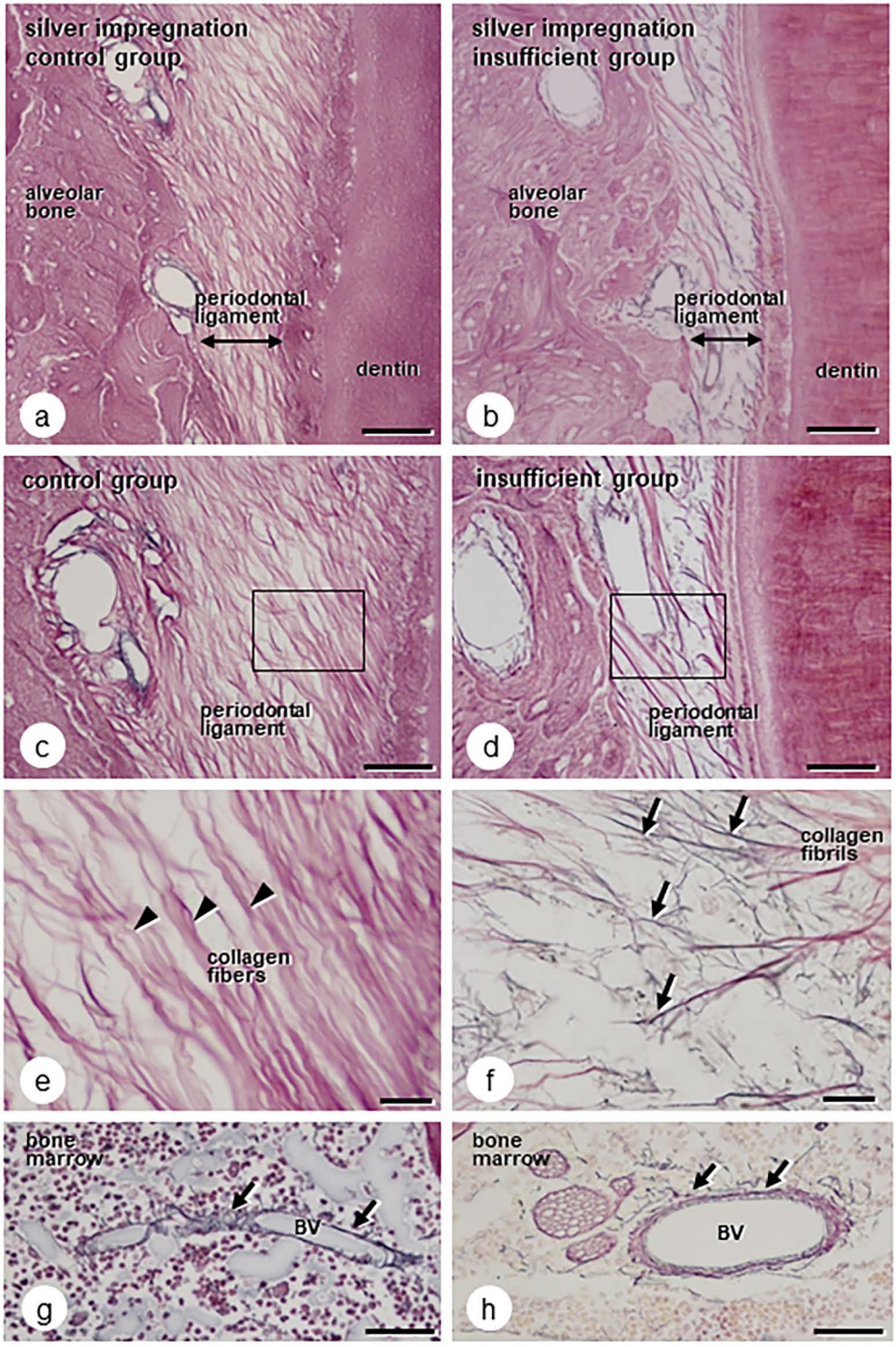
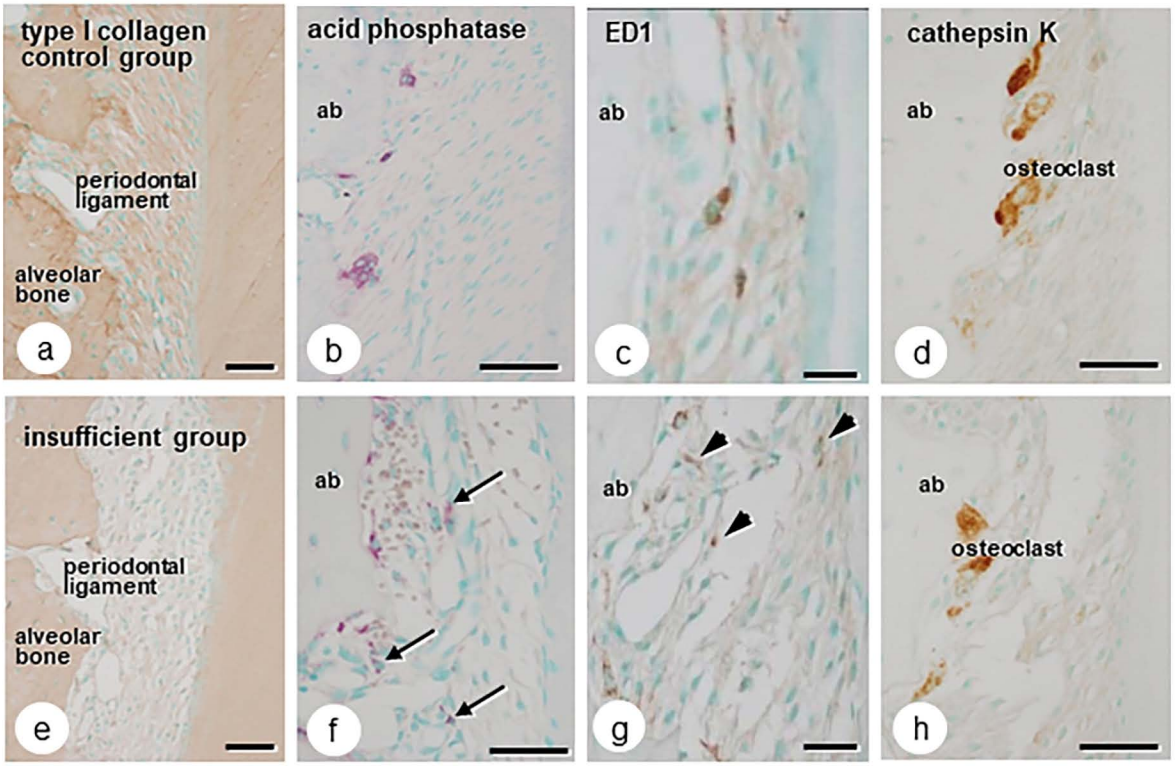
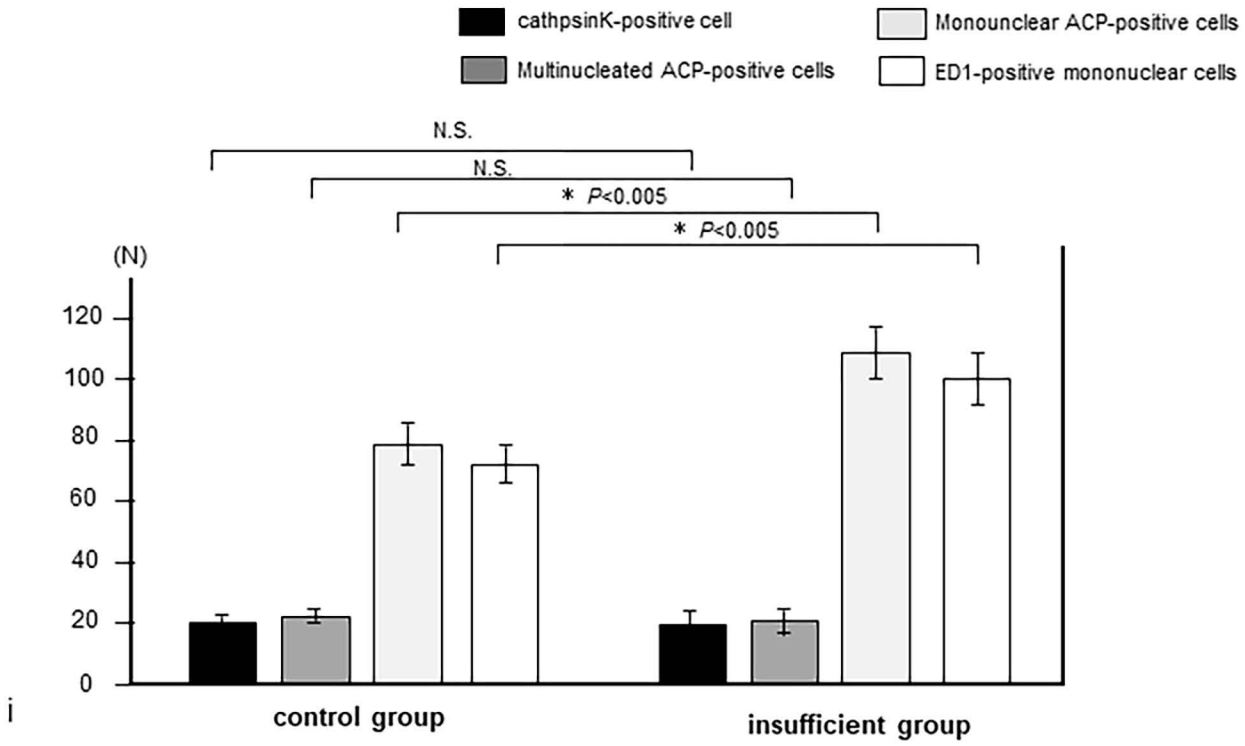

silver impregnation
control group
alveolar
bone
periodontal
ligament
dentin
a
silver impregnation
insufficient group
alveolar
bone
periodontal
ligament
dentin
b
control group
periodontal
ligament
c
insufficient group
periodontal
ligament
d
collagen
fibers
e
collagen
fibrils
f
bone
marrow
BV
g
bone
marrow
BV
h

Fig.2


type I collagen
control group
periodontal
ligament
alveolar
bone
a
acid phosphatase
ab
b
ED1
ab
c
cathepsin K
ab
osteoclast
d
insufficient group
periodontal
ligament
alveolar
bone
e
ab
f
ab
g
ab
osteoclast
h


cathpsinK-positive cell
Monounclear ACP-positive cells
Multinucleated ACP-positive cells
ED1-positive mononuclear cells
N.S.
N.S.
* P<0.005
* P<0.005
(N)
120
100
80
60
40
20
0
control group
insufficient group

i

Fig.3

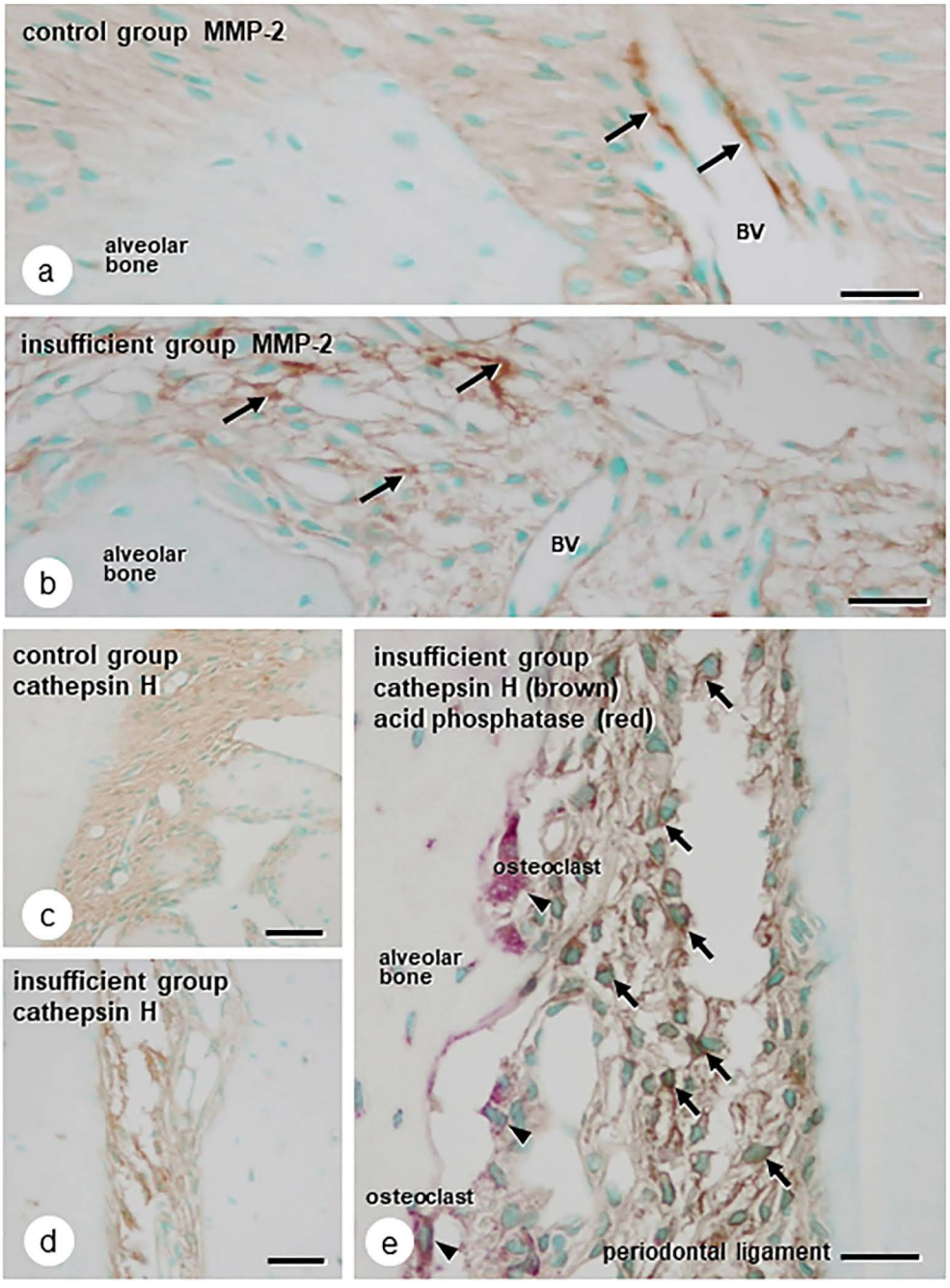

control group MMP-2
alveolar bone
BV
a
insufficient group MMP-2
alveolar bone
BV
b
control group cathepsin H
c
insufficient group cathepsin H
d
insufficient group cathepsin H (brown) acid phosphatase (red)
osteoclast
alveolar bone
osteoclast
e
periodontal ligament

Fig.4

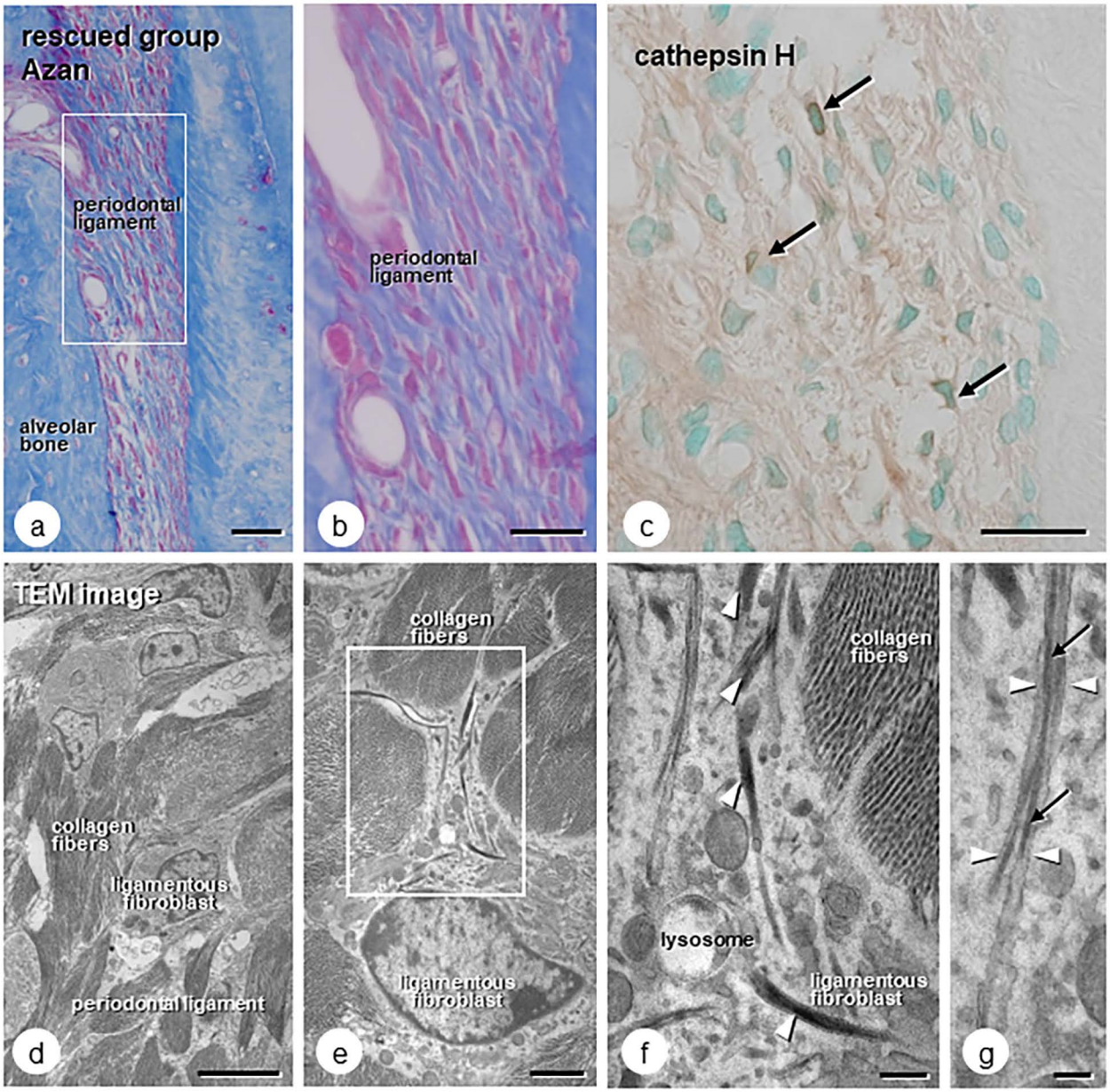

rescued group
Azan
periodontal ligament
alveolar bone
a
periodontal ligament
b
cathepsin H
c
TEM image
collagen fibers
ligamentous fibroblast
periodontal ligament
d
collagen fibers
ligamentous fibroblast
e
collagen fibers
lysosome
ligamentous fibroblast
f
g

Fig.5