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Quantitative Analysis of Extracellular Matrix Changes: Glycosaminoglycans, Collagen, and Elastin in Degenerated Ligamentum Flavum in Lumbar Spinal Canal Stenosis

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Qualitative changes in the extracellular matrix have been reported in thickened ligamentum flavum（LF）in lumbar spinal canal stenosis（LSCS）; however, no detailed quantitative reports are available to date. Degenerated and undegenerated LF were collected from patients who underwent decompression surgery for LSCS and lumbar disc herniation, respectively. Three cylindrical samples of 2.5 mm diameter were cut from one LF specimen, completely solubilized, and quantified glycosaminoglycan（GAG）, collagen（COL）, and elastin. The amounts of GAG, COL, and elastin in the LSCS group were significantly higher than those in the control group. Although the GAG was originally present only in trace amounts, GAG proportion, standardized by dry weight, was the highest increase rate. Besides component analysis, histologic analysis showed intraligamentous chondrometaplasia in the caudal portion of the degenerated LF. Increased GAG in LF may be a new therapeutic target in LSCS.

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INTRODUCTION

Lumbar spinal canal stenosis（LSCS）has a high prevalence in older adults and causes severe disability in daily life due to the neurologic symptoms caused by compression of nerve roots or cauda equina［1］. The current pharmacologic treatment for LSCS is primarily only symptomatic care. Except for surgery, no medication has yet been developed in practice to resolve spinal stenosis itself.

Degeneration with thickening of the ligamentum flavum（LF）in LSCS is a known major characteristic in stenotic conditions［1–4］, and several studies have been conducted to investigate the cause of this degeneration［5–9］. In this setting, the amounts of glycosaminoglycan（GAG）and collagen（COL）increase and the amount of elastin decreases. Although these previous reports have mainly focused on relative histologic comparisons, accurate quantification of the constituents is essential to elucidate these pathologic changes. No reports to date have accurately quantified GAG in LF. Although some reports actually quantified the amounts of COL or elastin, these quantifications were calculated from

partially dissolved tissues [10,11]. Mikawa *et al.* were the first to report in detail the COL and elastin content [12]; nevertheless, the study was not designed to compare degenerated LF with controls.

This study aimed to accurately measure the changes in GAG, COL, and elastin levels caused by degenerative thickening LF and to confirm whether these results were histologically consistent. We accurately measured the amounts of each in the degenerated LF and compared them with those in the control groups. Our simultaneous measurement results were obtained using an originally established method for quantification coupled with complete solubilization [13].

MATERIALS AND METHODS

Materials

Thermolysin (cat. no. 3504), desmosine (DES, cat. no. 191378) and isodesmosine (isoDES, cat. no. 191379), and ^{2}H (D)-labeled DES (d4-DES, cat. no. D296847) were obtained from the Peptide Institute (Osaka, Japan), MP Biomedicals (California, USA), and Toronto Research Chemicals (Toronto, Canada), respectively. All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Ligamentum Flavum

To obtain the LSCS samples, 8 thickened LF specimens were collected from 8 patients (5 men) who underwent endoscopic-assisted laminectomies for LSCS. The mean age was 72.3 years (63–83 years). For the control group, we analyzed 4 specimens with undegenerated LF from 4 patients (2 men, mean age 36.2 years (30–41 years)) those were removed during surgery for lumbar disc herniation. All LF were collected from L4/5 sites except one. Of these samples, 7 specimens (L4/5) in the LSCS group and 3 specimens (L4/5) in the control group were analyzed for components analysis. Histologic evaluation was also performed on a LSCS (L4/5) and control (L3/4) specimens.

Sampling for Component Analysis

Three cylindrical (2.5 mm in diameter) samples were cut out at 3 points from one LF specimen (Fig. 1). For quantification of each compornent, the samples were processed as described previously [13]. Briefly, freeze-dried samples (per mg) were incubated with thermolysin (550 PU, 60 μg) at 70℃ for 24 h in 0.1 mL of a solution (2.8 mM sodium acetate and 2.8 mM calcium acetate). Furthermore, 24 h digestion were performed with the same condition. After the digestion, the solubilized samples were centrifuged at 15,000 × g for 15 min.

Measurement of GAG Amount

To measure the GAG amount, 150 μL of the above supernatants were precipitated with 1350 μL of ethanol (final 90%) at 4℃ overnight and centrifuged at 15,000 × g for 15 min. Precipitates were reconstituted with 30 μL of water and measured GAG with 1,9-dimethylmethylene blue using the Blyscan GAG assay kit (Biocolor, Carrickfergus, UK) [14]. By this method, all GAGs with sulfate groups can be detected.

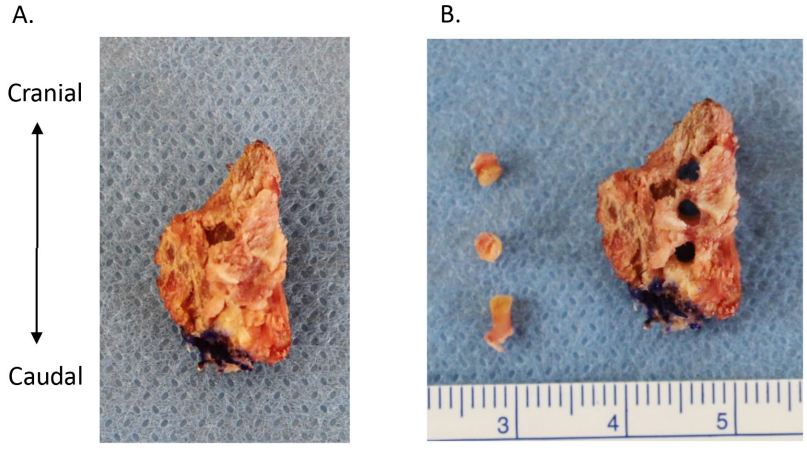

Fig. 1. Cylindrical sampling to measure the amount of components
The caudal side of the ligamentum flavum (LF) is marked in purple. Three cylindrical samples were cut out into 2.5 mm in diameter from one LF specimen and were placed horizontally. A shows before cutting out; B shows after cutting out the lumbar spinal canal stenosis specimen.

Acid Hydrolysis

Five μL of the supernatants of the above solubilized solution were added to 11.5 uL of 8.6 N hydrochloric acid (HCl) solution. The mixture (final 16.5 uL of 6N HCl solutions) were hydrolyzed at 110℃ for 48 h in the gas phase with a PicoTag system (Waters, Massachusetts, USA). The acid hydrolysates were dried and dissolved in 25 μL of water. With the reconstructed solution, the amounts of hydroxyproline (Hyp) and desmosine (DES) and isodesmosine (isoDES) were measured.

Estimation of COL Amount

Hyp is a unique amino acid to COL, and is used as an indicator to estimate COL amount. The Hyp amount was measured as described previously [15], with some modifications. Briefly, 10 μL of the above reconstructed solutions were incubated with 0.2 mL of 1.4% chloramine T, 10% *n*-propanol, and 0.5 M sodium acetate (pH 6.0) at room temperature for 25 min. Subsequently, 0.2 mL of Ehrlich solution comprising 1 M *p*-dimethylaminobenzaldehyde in 70% *n*-propanol and 20% perchloric acid were added, and the solution were incubated at 65℃ for 20 min. Absorbance was then measured at 550 nm, and the Hyp amount was determined. We calculated the amount of COL, excluding elastin-derived Hyp, on the basis of the report that Hyp in COL and elastin are 12.3% [16] and 1.7% [12], respectively.

Estimation of Elastin Amount

Both DES and isoDES are unique crosslinks to elastin and are used as indicators to estimate elastin amount. The DES and isoDES amounts were measured as described previously [17,18], with some modifications. Briefly, 13 μL of the above reconstructed solution and 52 μL of 12.5 μM internal standard d4-DES were mixed and filtrated with Minisart RC4 (Sartorius, Stonehouse, UK). Then, 5 μL of this mixture was used for liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis.

The system consisted of a Shimadzu HPLC system coupled to a triple quadrupole mass spectrometer equipped with an electrospray ionization source (Nexera X2, LCMS-8030, Shimadzu Co., Kyoto, Japan). The analysis was performed in the selected reaction monitoring (SRM) mode using an Intrada amino acid column (3 μm, 50 × 3 mm, Imtakt, Kyoto, Japan). The mobile phases were 100 mM ammonium formate (solvent A) and 100% acetonitrile (solvent B). Separation was performed using a binary gradient system at a flow rate of 0.6 mL/min. The gradient program was as follows: 0 min, 70% B; 1 min, 70% B; 5.9 min, 1% B; 7.9 min, 1% B; 8.4 min, 70% B; and 11 min, 70% B. Because DES and isoDES have the same SRM transition m/z 526.25 > 481.2 and cannot differ in their elution time, it is impossible to distinguish between them. Thus, we denoted the sum of DES and isoDES as DES. The SRM transition of d4-DES was m/z 530.25 > 485.25. Data acquisition and analyses were performed using LabSolutions software (version 5.60SP2, Shimadzu). The amount of elastin was estimated based on a report that elastin from human LF contains 1.2% DES by dry weight [12].

Statistical Analysis for Measurement of Each Component

A comparison between the LSCS and control groups was performed using Mann-Whitney U test with the free software R (version 3.3.1).

Histologic Analysis

The harvested tissues were soaked using ALTFiX (Falma Co., Tokyo, Japan). The tissues decalcifed with K-CX (No. CS-5151, Lot. No. K457054, Falma Co.). The samples were dehydrated and embedded in paraffin. In both groups, all sections were cut in the sagittal plane at 6 μm thickness. Sectioned samples were deparaffinized and stained by toluidine blue for sulfated GAG and by Elastic Stain Kit (using the modified Verhoeff method) for elastin fiber (black) (ScyTek Laboratories, Utah, USA).

The sections were activated by hyaluronidase (No 56177-10, Sigma-Aldrich Japan K.K., Tokyo, Japan) and reacted with mouse anti-human type I and type II COL monoclonal antibodies (×100, anti-hCL I and anti-hCL II, respectivety, Daiichi Fine Chemical CO. Ltd., Toyama, Japan). They were detected by Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA), and visualized by ImmPACT

DAB (Vector Laboratories). The tissue sections were captured by DS-Ri1 CCD camera (Nikon co.) through ECLIPSE80i microscope (Nikon co.) and were imaged by NIS-Elements Documentation Ver. 3.22 software (Nikon co.).

RESULTS

Measurement of GAG, COL, and Elastin Amounts

Fig. 2 shows the amounts of GAG, COL, and elastin in each of the cylindrical samples collected. In the LSCS and control groups, the amounts of GAG were 27.0 and 9.4 μg (Fig. 2A), those of COL were 1473 and 530 μg (Fig. 2B), and those of elastin were 822 and 408 μg (Fig. 2C), respectively. These amounts were 2.9, 2.8, and 2.0 times higher in the LSCS group than the control group, respectively.

For the generalization of these results, the compositional proportion was calculated on the basis of the dry weight of the collected tissue, and Fig. 3 shows those of each component. In the LSCS and control groups, the proportions of GAG were calculated as 8.3 and 3.7 μg/dry tissue (mg) (Fig. 3A), those of COL were 474 and 435 μg/dry tissue (mg) (Fig. 3B), and those of elastin were 268 and 326 μg/dry tissue (mg) (Fig. 3C), respectively. The proportions of them were 2.2, 1.1, and 0.8 times higher than in the control group, respectively.

Histologic Analysis

The LF of LSCS (Fig. 4A–D) and control (Fig. 4E–H) groups were analyzed histologically in the sagittal plane. The black arrow indicates a region of chondrometaplasia nearby caudal insertions in the LSCS section. In that region, GAG was strongly stained (Fig. 4A) and a slight increase in COL2 was observed (Fig. 4C). By contrast, COL1 was stained in the cranio-mid area of the ligament (Fig. 4B). Elastin stained strongly and homogeneously in

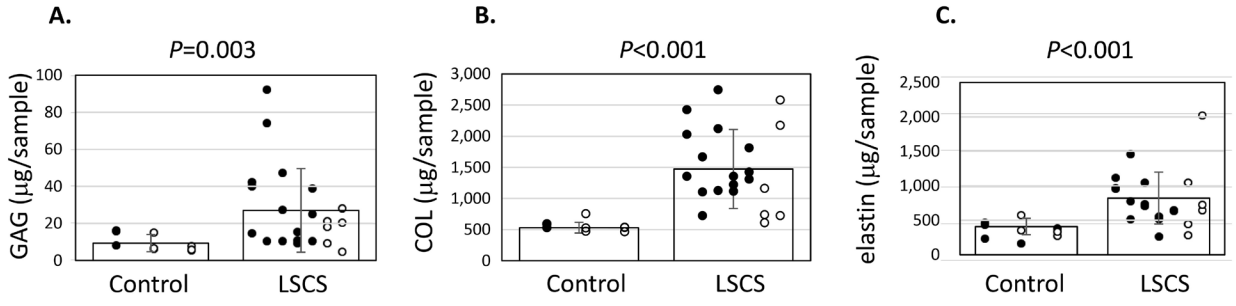

Fig. 2. Comparison of component amounts (each cylindrical sample) between the LSCS and control groups
The amounts of glycosaminoglycan (GAG) (A), collagen (COL) (B), and elastin (C) are shown. For the lumbar spinal canal stenosis (LSCS) and control groups, the mean and standard deviation are shown as bar graphs. The *P* values between the LSCS and control groups are shown. The raw data of three sides (the cranial, center, and caudal sides) for each person (male (●) and female (○)) are plotted vertically.

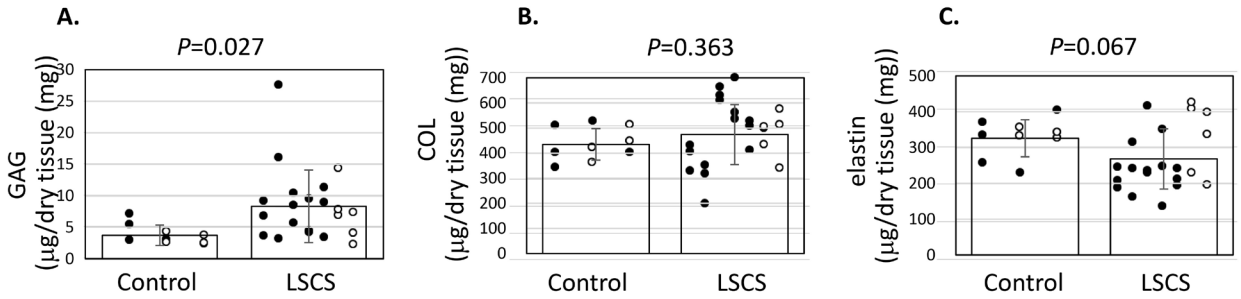

Fig. 3. Comparison of compositional proportion (dry weight) between the LSCS and control groups
The prportions of glycosaminoglycan (GAG) (A), collagen (COL) (B), and elastin (C) in dry tissue are shown. For the lumbar spinal canal stenosis (LSCS) and control groups, the mean and standard deviation are shown as bar graphs. The *P* values between the LSCS and control groups are shown. The raw data of three sides (the cranial, center, and caudal sides) for each person (male (●) and female (○))are plotted vertically.

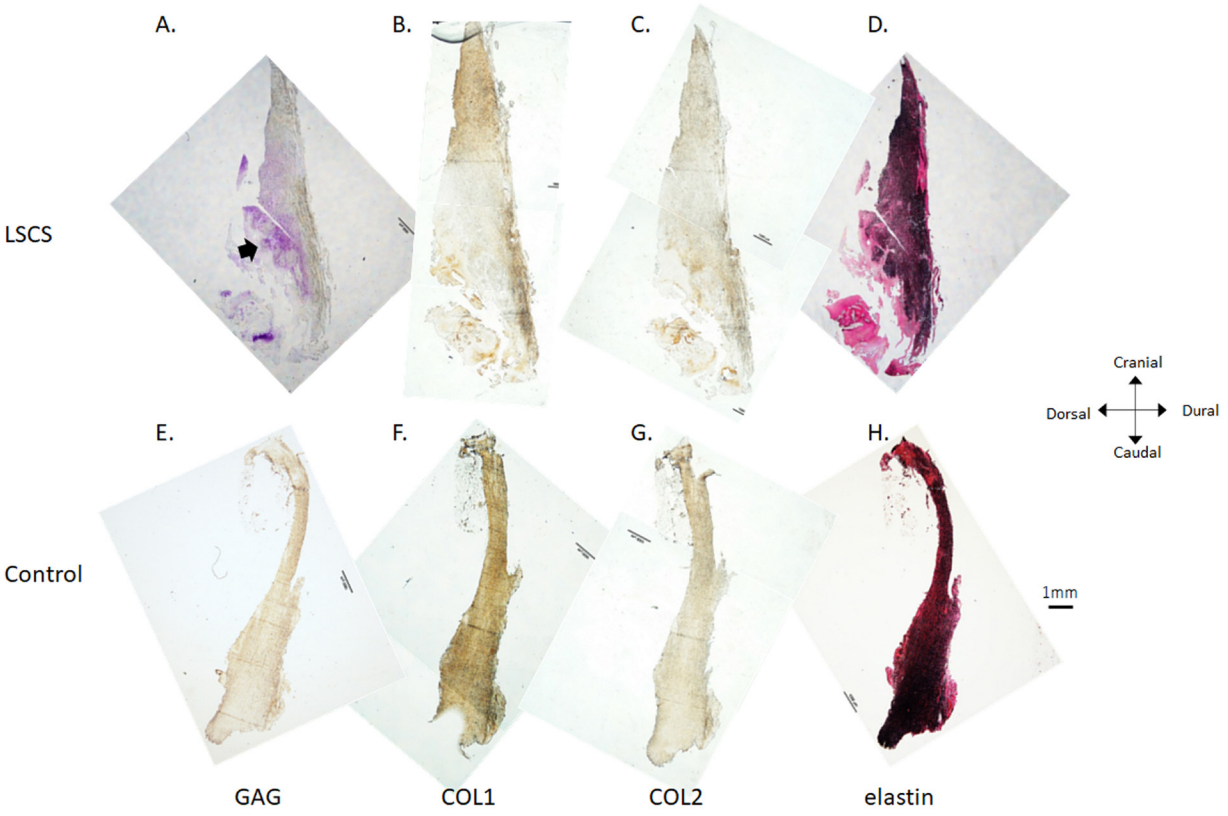

Fig. 4. Histologic analysis of each component (sagittal section)
Lumbar spinal canal stenosis (LSCS) (A–D) and control (E–H) sections were analyzed histologically in the sagittal sections, with glycosaminoglycan (GAG) (A, E) and elastin (D, H) visualized by toluidine blue and Verhoeff staining, respectively. The localization of collagen (COL)1 (B, F) and COL2 (C, G) were detected via immunohistochemistry. The black arrow indicates a region of chondrometaplasia.

the control ligament (Fig. 4H), whereas it stained sparsely throughout the degenerated ligament (Fig. 4D).

DISCUSSION

It is known that the LF thickens with age, and samples from young patients without thickening are generally used as control samples for LSCS. Although it is necessary to examine whether there is a difference in composition between thickening due to aging and thickening due to LSCS, in the present study, we purposely used the LF removed from a young patient with a different disease as a control in order to compare it with an undenatured ligament.

Comparing the mean values of GAG, COL, and elastin amount in the LSCS group to the control group, all amounts were significantly higher (Fig. 2). The increase in GAG was the most remarkable among them. In the present study, we analyzed cylindrical samples with the same surface area. This analysis revealed the quantitative differences that were actually induced in the degenerated ligament. The differences resulted not only from the compositional differences of the ligament degeneration but also from the thickening itself (Fig. 1B).

By contrast, the component proportion of dry weight could standardize the degree of thickening, indicating a purely compositional difference. The mean value of the GAG proportion in the LSCS group was significantly higher than that in the control group (Fig. 3A). The proportion of COL tended to be higher (Fig. 3B), although that of elastin tended to be lower (Fig. 3C). Previous tissue staining studies have shown that the amount of elastin is reduced in LSCS [2–8]. The present results indicate that the decrease in elastin does not mean that degradation is accelerated, but that the relative amount of elastin to non-water molecules is reduced as a result of degeneration accompanied by thickening.

Both the amount and proportion of GAG were higher in the LSCS group. However, GAG was originally found to be present only in trace amounts and was markedly increased by degeneration. Histologically, GAG was detected on the caudal side of the degenerated area in the LSCS ligament. This report is the first to provide a detailed quantitative evaluation of the increase in GAG in the degenerated LF.

To calculate the COL amount, the Hyp amount was measured. Some studies have reported the Hyp content in COL to be 12.3% [16], or roughly 10% [19]. Elastin is also said to contain 1.1%–3.6% Hyp [12, 20–22], with 1.7% [12] reported from LF-derived elastin. To calculate the COL amount in LF with high elastin content, elastin-derived Hyp should be excluded. In this study, we calculated the COL amount on the basis of the report that the contents of Hyp in COL and elastin are 12.3% [16] and 1.7% [12], respectively. By this conversion rate, the COL contents in dry tissue were calculated as 47.4% and 43.5% (Fig. 3B) in the LSCS and control groups, respectively. The COL amount in LF excluding elastin-derived Hyp has been calculated for the first time via this study. This amount is considered to represent mostly type I collagen based on the results of Fig. 4B, C, F, and G. The COL accounts for 20% of LF which from a previous histologic report [3], showed a large difference from this study. The previous reported COL content as 31.1% [12] and 41.7% [23] were low, although they contain elastin-derived Hyp. The LF, which contains a large amount of COL and elastin, has been known to be difficult to solubilize completely [24]. Thus, they might have been underestimated because of the incomplete solubilization.

This is the first report to estimate elastin amount in the LF by measuring DES with LC-MS/MS method. Previously, elastin amount was measured by amino acid composition analysis [12, 20–22] or HPLC analysis [25,26]. We applied the LC-MS/MS method [17,18] in the present study. Although the DES content in elastin has been reported to be 1.05%–4.2% [12, 20–22, 25, 26], we have used a value of 1.2% [12] in human LF. By this conversion rate, the elastin proportion in dry tissue were calculated as 26.8% and 32.6% (Fig. 3C) in the LSCS and control groups, respectively. The reports that elastin accounts for 75% [5] or 80% [3] of LF were results from tissue staining. The value of 46.7% was calculated by another solubilization method using a large amount of tissue from many cases [12]. Compared with these values, the results of the present study are more reliable because of full solubilization method and analyzation from small sites; hence, it can be concluded that the absolute elastin amount is higher (Fig. 2), but the relative elastin proportion is lower in LSCS (Fig. 3).

In the control group, the coefficients of variation calculated from the standard deviation and mean values of GAG, COL, and elastin proportion were 0.43, 0.14, and 0.16, respectively, showing little variation. By contrast, in the LSCS group, the coefficients of variation were 0.69, 0.25, and 0.31, respectively, which were particularly high for GAG (Fig. 3). The variation may have been due to individual differences as well as differences in sampling sites within the same LF. It is known that even within a single LF, there are regions with different compositions [5,7]. The particularly large inter-sample differences in the LSCS group (Fig. 2, 3) may have reflected the heterogeneous distribution of composition (Fig. 4); nevertheless, a detailed site-specific quantitative analysis was not performed in this study. Further site-specific studies from small fragments of multiple sites may help to elucidate the disease progression mechanism. The differences of the constituent proportion in this study are consistent with previous reports [2, 4, 8, 9] and our histologic results (Fig. 4A–D) that showed chondrometaplasia near the caudal attachment of the degenerated LF. This study did not assess the degree of thickening directly or mechanical properties of the degenerated ligaments, and further detailed analysis is required.

The results of this study showed that the most increased component with degeneration was GAG. As GAG is increased in degenerated ligaments, it may be a potential new therapeutic target. Degradation and reduction of GAG in degenerated ligaments may contribute to the development of novel drug therapies for LSCS.

CONCLUSION

The results of this study indicate that quantitative analysis of the GAG, COL, and elastin content of degenerated LF revealed the highest proportional change of GAG content compared with controls. This result may be explained by histologic findings that showed chondrometaplasia near the caudal attachment of the degenerated LF.

Ethical approval and informed consent

This study was approved in advance by the Medical Research Ethics Committee of Shimane University Faculty of Medicine（approval number: 20110831-2）. Written informed consent for study participation and publication was obtained from all participants in this study.

Author Contributions

M.K. designed the study; H.O., M.K-M., A.M., and M.A. performed the experiments; M.K., H.O., and M.K-M. analyzed the data; M.K., and M.H. supervised the experiments; M.K., T.M., and S.N. performed surgery and took samples; The manuscript was drafted by M.K., H.O., M.K-M., and M.H. and critical revision for intellectual content was performed by Y.U. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare there are no conflicts of interest.

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