Journal of Biosciences and Medicines, 2020, 8, 20-31
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Age-Related Changes of Procollagen Alpha Polypeptide in Vascular Remodeling in Rat Vascular Smooth Muscle Cell

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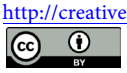
How to cite this paper: Yu, M.M., Zhan, H.C., Song, D.L. and Gai, W.L. (2020) Age-Related Changes of Procollagen Alpha Polypeptide in Vascular Remodeling in Rat Vascular Smooth Muscle Cell. *Journal of Biosciences and Medicines*, **8**, 20-31.
https://doi.org/10.4236/jbm.2020.812003

Received: October 19, 2020
Accepted: December 11, 2020
Published: December 14, 2020



Abstract

The current study revealed that increased synthesis and secretion of collagen types I and III play major roles in arterial wall remodeling, aneurysm formation, and atherosclerotic cap stability. The aim is to investigate the age-related changes of the procollagen alpha polypeptide gene mRNA and protein expression in the vascular smooth muscle cells (VSMCs) in rats, as well as the possible underlying mechanisms. We tested *in vitro* culture of VSMC from the thoracoabdominal aorta in neonate and 9-month-old healthy male Wistar rats; procollagen alpha polypeptide mRNA and procollagen alpha polypeptide protein expression were detected, using RT-PCR, VG staining, Western blot and ELISA methods. Semi-quantitative analysis displayed that, in the real-time reverse transcription polymerase chain reaction (RT-PCR), the type I collagen α polypeptide chain mRNA increased in the adult group, but not significantly ($P = 0.05$). Further, there was no significant difference between the two groups of type III collagen α polypeptide chain mRNA ($P > 0.05$). Both the type I and type III procollagen alpha polypeptide protein expression were increased significantly in the older group as compared with the young group ($P < 0.05$). This phenomenon mainly lies in the fact that the regulatory pathway on age-related changes of procollagen alpha polypeptides may be one of the molecular mechanisms in vascular remodeling during aging.

Keywords

Age-Related, Vascular Smooth Muscle Cell, Procollagen Alpha Polypeptide, Vascular Remodeling

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1. Introduction

Vascular remodeling is related to various vascular disorders such as atherosclerosis, in-stent restenosis, vein graft disease, and transplantation-associated vasculopathy [1] [2]. Vascular remodeling refers to structural changes in the arterial wall, such as arterial enlargement of shrinkage [3], which occurs during sustained bloodflow changes [4] [5] and during secretion of the dysfunctional endothelial cells [6] [7]. We previously reported that aging is an independent cardiovascular risk factor affecting vascular remodeling [8] [9]. However, the molecular mechanisms underlying the process of aging affecting vascular remodeling are far from clear. The current study revealed that the phenotypic modulation and abnormal proliferation of vascular smooth muscle cells (VSMCs) play an important role in the development of age-related vascular remodeling [10] [11] [12]. VSMCs can synthesize and secrete extracellular matrix proteins, including collagen, elastin, and proteoglycans. In addition, VSMCs also are responsible for the structural characteristics of the vessel wall, including growth, development, remodeling, and repair [13]. Furthermore, the proliferation and migration of VSMCs are core processes in vascular remodeling [14]. VSMCs can synthesize and secrete collagen, which is the most abundant component of the extracellular matrix in arteries. This study was designed to observe the changes of procollagen gene transcription and protein expression *in vitro*, in order to investigate age-related effect on the procollagen alpha polypeptide gene mRNA and protein expression in VSMCs and the molecular mechanism may lead to this phenomenon.

The balance, stability, and compliance of the vascular wall are dependent on the contribution of two proteins-collagen and elastin, and aging is associated with profound changes in the properties of elastin and the stimulation of collagen synthesis [6]. An increase in collagen in arterial walls results in the changes to the vessel wall structure, which can then lead to increased arterial stiffness, a major contributor to the development of atherosclerosis, and consequently, cardiovascular disease [15] [16] [17]. The aging process is associated with quantitative and qualitative alterations of collagen and elastin fiber [18] [19]. One possible structural modification is collagen reorientation [20].

The collagen biosynthesis process is complex, involving numerous intracellular and extracellular steps. The first event following synthesis of procollagen α chains on the ribosome is their import into the rough endoplasmic reticulum [21]. There, they undergo a series of post-translational modifications, resulting in the assembly of procollagen molecules [22] (**Figure 1**).

Assembly and secretion processes of collagen have the same pattern as other multimeric proteins (**Figure 2**). The required amino acids are absorbed by cells to synthesize peptides, including proline, lysine and glycine. Next, these amino acids enter the process of translation in accordance with the specific collagen mRNA nucleotide sequence on the ribosome of the rough endoplasmic reticulum. Proline and lysine are hydroxylated through hydroxylase action. The three procollagen alpha polypeptide chains are then polymerized into rope-shape

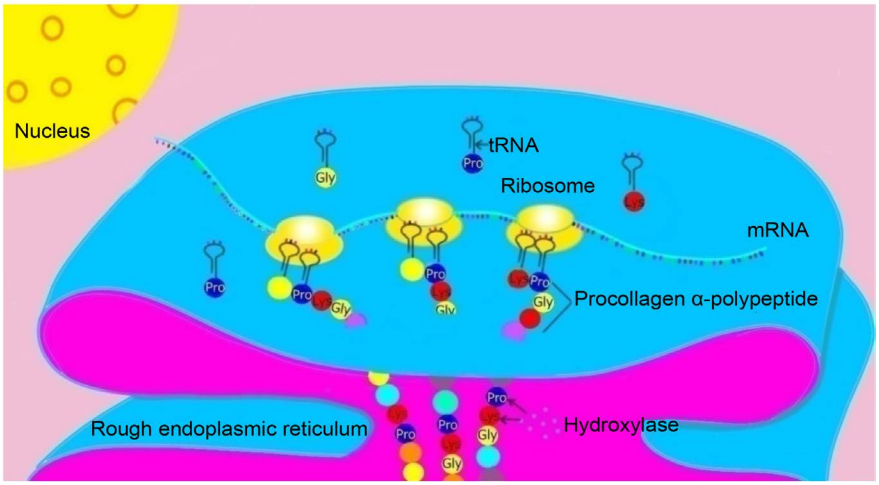


Figure 1. The Col I and Col III mRNA are transcribed into procollagen *α*-polypeptide on the ribosome. And then, procollagen *α* chains undergo a series of post-translational modifications in the rough endoplasmic reticulum.

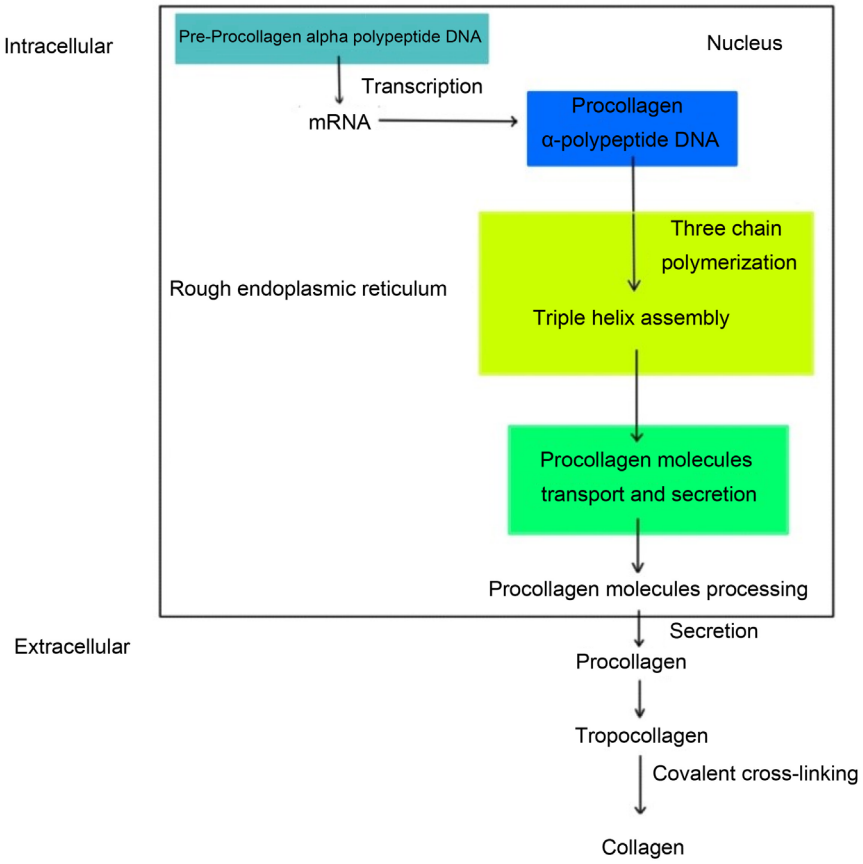


Figure 2. Assembly and secretion process of collagen.

procollagen molecules. Procollagen molecules in the dissolved state are spherical in conformation. Procollagen molecules processed by glycosylation in either the rough endoplasmic reticulum or the Golgi complex are then secreted to the outside of the cell. Procollagen molecules are then transformed into tropocollagen

molecules. Procollagen processing occurs during or shortly thereafter, followed by the assembly of fibrils. Finally, tropocollagen molecules are polymerized into collagen fibrils, and fibrils are stabilized by the formation of covalent cross-links [20] [23].

Therefore, we propose a hypothesis of this study that the target of age-related change of vascular collagen is the increased translation of the Col I and Col III mRNAs.

2. Materials and Methods

2.1. Animals and Groups

12 newborn (young group) and 12 adult male Wistar rats aged 9 months (older group) were used for the study. All animal experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by Qingdao University and all experiments were approved by the Institutional Animal Care and Use Committee at Qingdao Municipal Hospital Medical Center.

2.2. Cell Culture

All cell culture reagents were obtained from Sigma unless otherwise noted. The Wistar rats were anesthetized with 15 mg/kg zolazepam (Zoletil100®; France), injected by heparin (1000 U/kg), and were euthanized 15 min later. Thoracic aortas of the rats were removed under sterile conditions and transferred to fresh plates. The adventitia and connective tissues were removed by micro dissection. The isolated aortas were then cut into 1 - 2 mm^2 flat segments and transferred into a fresh medium, which contained 20% fetal bovine serum (Gibco). The explants were incubated at 37˚C (5% CO_2) until VSMCs migrated from the aortas and reached confluence. Confluent cells were subsequently passed with a trypsin (0.25%) and EDTA (0.01%) solution. Early passage cells (2 - 5) were used for the studies, and VSMCs were confirmed with positive SM actin staining.

2.3. RNA Extraction and Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from thoracic aortic sections with Trizol (Invitrogen), following manufacture protocol. Purified RNA was reverse transcribed into cDNAs using iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed with iCycler iQ real-time PCR detection system (Bio-Rad). SYBR Green Real-time fluorescent quantitative PCR kit (Treasure biological engineering co. Dalian) Composition including:

SYBR® Premix Ex TaqTM II (Perfect Real Time) (2 × Conc.)	1.0 ml × 5
ROX Reference Dye (50 × Conc.)	200 μl × 1
ROX Reference Dye II (50 × Conc.)	200 μl × 1

The primers for Procollagen type I α-chain and Procollagen type III α-chain are listed in Table 1. The reaction mixture includes: SYBR® Premix Ex TaqTM II 5 μl, Forward Primer (2 μm) 0.5 μl, Reverse Primer (2 μm) 0.5 μl, RNase-free dH_2O 3.5 μl, synthesis of cDNA first template chain 0.5 μl. The conditions for real-time PCR were as follows: denaturation at 95˚C for 10 min, then 40 cycles at 95˚C for 20 s, annealing at 58˚C for 20 s and extension at 72˚C for 20 s. The mRNA levels were acquired from the expression ratio of target gene to β-actin.

2.4. Histological Analysis and Western Blot

Thoracic aortas were fixed in 10% formalin overnight, and then embedded in Tissue Tek® optimum cutting temperature compound. Frozen cross-sections (6 μm thick) were mounted onto slides. Slides were stained with Van Gieson solution.

Proteins were extracted as follows: about 50 mg of thoracic aortic sections was put into 1.5 mlEP tube, adding pyrolysis liquid and 500 μl tissue protein extraction agent, fully grind into homogenate and ice hatch. Then grinding liquid was centrifuged at 13,000 RPM for 15 min. 50 μl Liquid supernatant was collected, and protein concentration determination by the BCA method (Bio-Rad). Equal amounts of the protein samples (15 μg) were separated using SDS-PAGE and transferred to PVDF membranes. After blocking with 10% non-fat milk, the membranes were washed with PBST and incubated overnight using the 1:500 dilution beta-actin. After washing with PBST, the membranes were incubated with the 1:2000 dilution corresponding secondary horseradish peroxidase-labeled antibody. The bands were visualized with enhanced chemiluminescence (Millipore).

2.5. ELISA Assay

Procollagen I and procollagen III were determined by ELISA according to the manufacturer's instructions. The cell culture supernatant was centrifuged to remove particulates, and either analyzed immediately or aliquoted and stored at −20˚C. The user should determine the sample dilution fold by a crude estimation of rat [ProcollagenIPropeptide (PCIP) and Procollagen III Propeptide (PCIIIP) ELISA Kit, USCNK] amount in samples. The detection wavelength was 450 nm, and concentration of the samples was interpolated from the standard curve.

Table 1. ProCol I and ProCol III primers for real-time PCR.

NAME	Primer sequence	Position	Length	Tm	GC%
PreproCol-1α1F	AGGCGAACAAGGTGACAGAG	3363	20	56.9	55
PreproCol-1α1R	ACCGTTGAGTCCGTCTTTGC	3537	20	57.9	55
PreproCol-3α1F	AAAGGGTGAAGTCGGTGCTC	2190	20	57.2	55
PreproCol-3α1R	CAGACCAGGAGAACCAGAAC	2379	20	57.6	55

2.6. Statistical Analysis

Data are expressed as $\bar{X} \pm SD$ of three independent experiments and analyzed with SPSS 13.0. A two-tailed Student's *t*-test was used to analyze the differences between the two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. No Changes in mRNA Expression of Collagen Type I α-Chain and Collagen Type III α-Chain between Two Groups

Real-time PCR was used to compare the mRNA expression of the target genes between the young group and the older group. Real-time PCR semi-quantitative analysis displayed that there was no significant difference between the two groups of type I and type III collagen *a* polypeptide chain mRNA (Figure 3). The Real-time quantitative result showed that the mRNA expression level of collagen type I *a*-chain in the older group was slightly higher than that of the young group (p = 0.05), but the difference was not statistically significant (Figure 4). Furthermore, there was no difference in mRNA expression level of collagen type III *a*-chain between two groups (Table 2).

3.2. Age-Related Increase in Procollagen I and III Protein Levels

The results of VG staining showed that the expression of procollagen I and procollagen III was significantly higher in the older group compared with the young

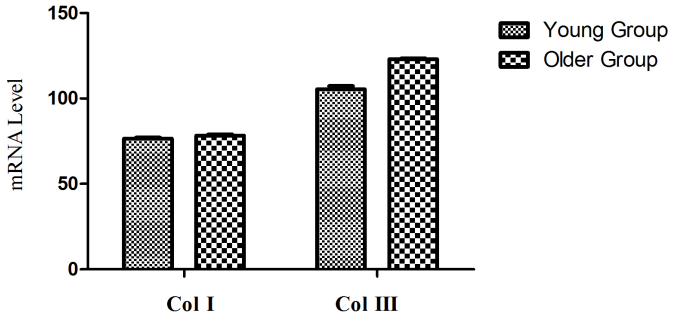


Figure 3. The results of real-time semi-quantitative PCR between the young group and the older group ($P > 0.05$).

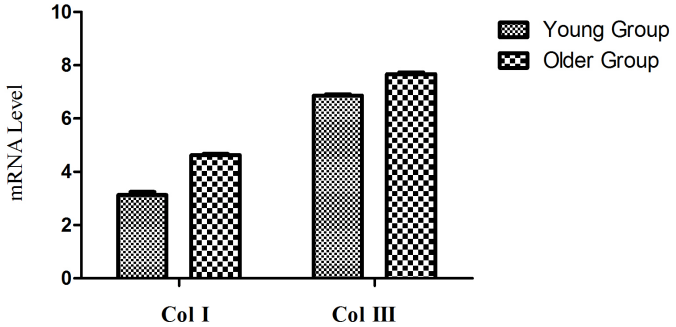


Figure 4. The results of real-time quantitative PCR between the young group and the older group ($P > 0.05$).

Table 2. Real-time quantitative and semi-quantitative PCR showed the comparison of the Col I and Col III mRNA.

Group	n	Col I	Col I mRNA	Col III	Col III mRNA
		Semi-quantitative	quantitative	Semi-quantitative	quantitative
Young	12	76.62 ± 1.05	3.13 ± 0.54	105.40 ± 2.66	6.86 ± 0.41
older	12	78.37 ± 2.42	4.63 ± 1.03	123.10 ± 3.81	7.68 ± 0.63
P		>0.05	0.05	>0.05	>0.05

group (**Figure 5**). Western blot results showed that the protein levels of procollagen I and III were significantly increased in the older group compared with the young group (**Figure 6**).

3.3. ELISA Assay

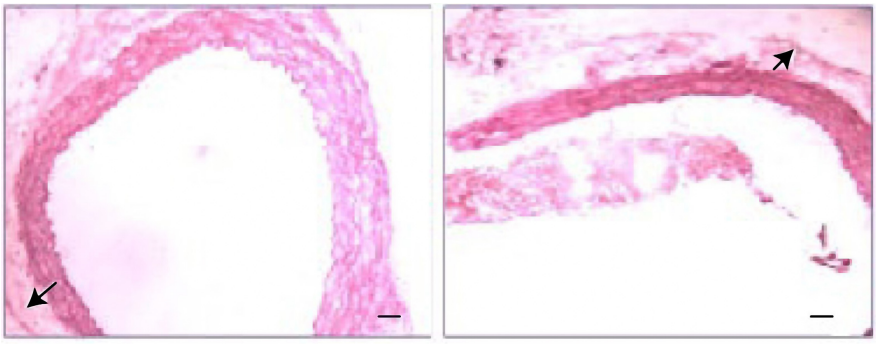
Compared with the young group, the ELISA assay suggested that the OD value increased in the older group, and it also showed PCI (76.0 ± 5.2 ng/ml *VS* 64.0 ± 4.2 ng/ml) and PCIII (78.0 ± 5.3 ng/ml *VS* 66.0 ± 4.3 ng/ml) ($P < 0.05$). The results were consistent with the Western blot results.

4. Discussion

The present study revealed that the expression levels of procollagen I and III alpha-polypeptide were significantly increased in VSMCs of aged rats, while the levels of Col I and Col III mRNA remained unchanged in adult group. This implies that the target of the age-related changes in the vascular Col I and Col III is the regulatory mechanism of translation.

Although the study of vascular remodeling molecular mechanism has not yet been fully revealed, this study may prove that regulatory pathways on age-related changes in procollagen alpha polypeptides are one of the molecular mechanisms in vascular remodeling. Our previous clinical studies innovatively showed that aging is an independent factor affecting vascular remodeling, in which, the aging mechanism of vascular remodeling was firstly proposed [8]. It also suggested that the VSMCs and age-related regulatory mechanisms are related to the increase in collagen secretion, influencing vascular remodeling and aging molecularly.

Composed of a distinct collagen subtype, the aorta is mainly made up of collagen I and collagen III [24]. Though the molecular biology mechanism of this phenomenon has not yet been clarified, a hypothesis that that aging reduces the content of miRNAs in vascular smooth muscle cells, thus inhibiting the effect of the protein translation, is proposed to explain this phenomenon. Thus, the vascular smooth cell enhances translation of type I and type III procollagen mRNAs. MicroRNAs regulate gene expression by inhibiting translation or reducing mRNAs [25] [26]. Clinical studies showed the decrease of miR-18 and miR-19 in age-induced cardiac fibrosis and dysfunction mouse strains, which



Young Group Older Group

Figure 5. Expression of procollagen I and III between two groups by VG staining (original magnification, ×100). Vessel wall of thoracic aortas in young group rats compared to that of older group rats (n = 12, $P < 0.05$).

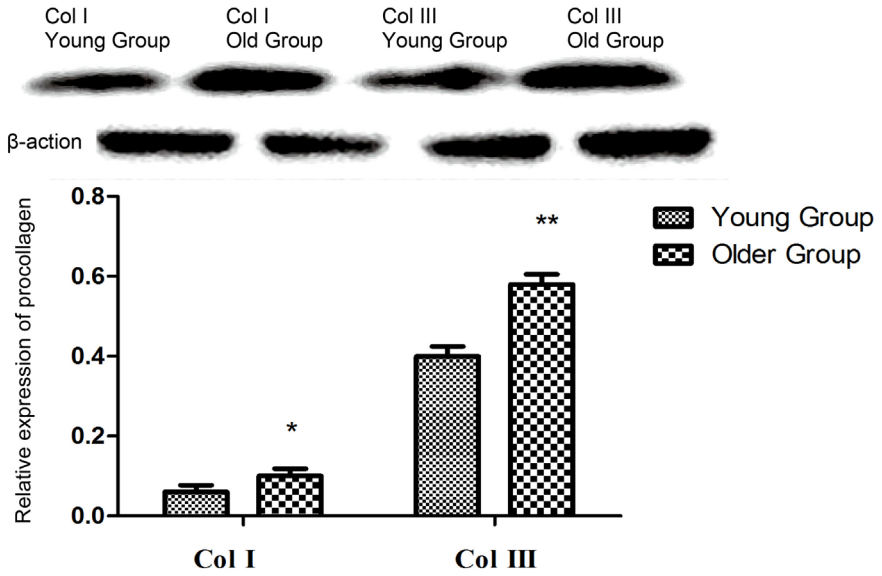


Figure 6. The results of Western blot for levels of procollagen I and III between two age-groups (original magnification, ×500). Protein levels of procollagen I and III in older group compared with that in young group (n = 12, $P < 0.05$).

can suppress collagen expression in cardiac myocytes [27] [28]. Furthermore, upregulated in old mice between young mice, the miR-29 family has been well known to repress matrix proteins in the heart and liver, and the increasing expression of miR-29 family members in the aorta of aged mice was associated with a decline of matrix protein expression, such as collagens and elastin [29] [30]. Therefore, we can conclude that aging can change the content of microRNA that can inhibit translation. This supports the hypothesis we have proposed.

What's more, this phenomenon can mainly be explained by the fact that DNA can be damaged by aging and can be repaired again [31]. Several recent observations support the hypothesis that cell senescence can be a highly dynamic as well as multi-step process. Senescence may be either chronic or acute, which deter-

mines the speed of aging [32]. Thus, age groups in this experiment may not be completely represented in the process of aging, and the gene level may not change according to aging at a specific age.

Regulatory mechanisms in age-related changes of procollagen alpha polypeptides can be mainly derived from the ribosome. The role of the ribosome in aging is an academic research hotspot currently. The rRNA modifications related to aging, together with ribosomal stress responses, played an influential role in tracking the process of immature procollagen alpha polypeptide translation. The specific collagen mRNA nucleotide sequence on the ribosome may alter organismal physiological behavior, linking RNA-mediated translational regulation to the modulation of lifespan and differential stress responses [33]. Studies suggest that ribosomal subunits themselves are considered as regulatory elements or filters that mediate the interactions between particular mRNAs and components of the translation machinery, and differential binding of particular mRNAs to eukaryotic 40S ribosomal subunits before translation may also affect the rate of polypeptide chain production selectively [34].

However, there are considerable debates about the mechanisms of procollagen alpha polypeptide transcription and protein expression during aging, with mitochondrial energy metabolism and oxidative stress always the core of these studies [35]. Recent animal experiments show that the peroxisome proliferator-activated receptor-γ coactivators PGC-1α/β-independent signal pathway play an important role in the aging process; aging regulates PGC-1α/β-independent nuclear-mitochondrial communication, leading to a specific loss of mitochondrial, encoded OXPHOS subunits, however, without nuclear gene transcription [36].

5. Conclusion

In conclusion, the age-related changes of procollagen alpha polypeptide gene mRNA and protein expression in VSMC and the possible underlying mechanisms were discussed in this research, laying a solid foundation for the further exploration of the theory of aging and the future study of anti-angiogenesis and atherosclerosis. The limitation of this study is that we will add a more aging group in the future.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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