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INTERVENTIONAL RADIOLOGY

ORIGINAL ARTICLE

Characteristics and efficacy of fish-derived gelatin microparticles as an embolic agent in a rabbit renal model: regulation of the degradation period by molecular weight

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PURPOSE
We aimed to evaluate the embolic effect of fish-derived gelatin microparticles (GMPs) and compare the degradation periods and biocompatibilities of different molecular weight (MW) GMPs in a rabbit model.

METHODS
GMPs were designed to degrade within 21 days (high MW GMP, 15-30 kDa) and 2 days (low MW GMP, 5-15 kDa) *in vivo*. Renal arteries of 24 rabbits were embolized using both high and low MW GMPs (155-350 μm). Rabbits were sacrificed either immediately after embolization, or after follow-up angiogram on days 2 and 21 of embolization, respectively (4 rabbits in each of the 6 subgroups). Pathological changes of recanalized vessels were evaluated using the Banff classification. For the *in vitro* study, each type of GMP was mixed with normal saline and morphological changes were compared for 14 days.

RESULTS
Fish-derived GMPs showed effective embolization. On 2-day follow-up angiography, occluded vessels were more recanalized to the peripheral branches in low MW group. On day 21, parenchymal perfusion defect recovered to a greater extent in low MW group than in high MW group. Mean Banff scores for intimal arteritis on 2-day follow-up and interstitial fibrosis on 21-day follow-up were higher in high MW group (1.75 ± 0.58 vs. 0.19 ± 0.4 and 2.56 ± 0.63 vs. 0.88 ± 0.89; $P < .001$). On *in vitro* assessment, low MW GMP lost the spherical shape and degraded, and was invisible on microscopy on day 6, whereas high MW GMP was only partially degraded after 2 weeks.

CONCLUSION
Fish-derived GMPs showed effective embolization in a rabbit model. Low MW GMPs degraded within 2 days with a low inflammatory response.

Gelatin is a hydrolyzed form of collagen that is usually derived from pig skins and cattle bones. It is commonly used in medical industries for manufacturing embolic materials, capsules, delivery systems for macromolecules, and scaffolds that support cell populations. [1-7] Among the embolic materials used clinically, gelatin sponge (GS) is the most widely used temporary embolic agent and it provides vessel occlusion for 3-6 weeks. It is available as a sponge, sheet, or powder, and can be easily customized according to the size of the target vessel.[5]

GS has been used in transarterial embolization for the treatment of hepatocellular carcinoma since the late 1970s.[8,9] After embolization with GS, the occluded vessels are recanalized within several weeks, and theoretically, the normal tissue in the embolized area is preserved. However, GS embolization may induce arterial stenosis or permanent occlusion because of organized thrombus and intimal hyperplasia.[10,11] To overcome these issues, quickly soluble gelatin particles were developed, and these gelatin particles caused significantly less damage to the recanalized artery on follow-up angiography.[12]

There has been an increasing interest in the use and development of biodegradable and bioabsorbable materials in the medical field. In the studies that compared the efficacy of different molecular weight (MW) gelatin particles, the low MW gelatin resulted in rapid

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hydration, complete dissolution, and was more biocompatible than the high MW gelatin.[13,14]

In the medical field, gelatin derived from fish skin or scale can serve as an alternative to that derived from mammals.[7,15] Fish gelatin has diverse properties different than those of porcine or bovine gelatin; for example, the melting and gelling temperatures of fish gelatin are relatively low compared with those of mammalian gelatin. The extraction yield of fish gelatin varies depending on the fish species and is lower than that of mammalian gelatin. However, fish gelatin has higher emulsion stability than porcine or bovine gelatin, and its lower gelling point is an advantage for systemic administration.[6,7,15,16] Furthermore, fish gelatin is available to anyone regardless of religion and culture, such as Islamic and Hindu societies, where consumption of porcine and bovine products is not allowed, respectively.[6,7]

To our knowledge, the present study is the first to use fish gelatin as an embolic material. Herein, we aimed to evaluate the embolic effect of fish-derived spherical gelatin microparticles (GMPs) and compare the degradation periods and biocompatibilities of different MW fish-derived GMPs for embolization via the transauricular arterial access in a rabbit renal model.

Methods

Preparation of GMPs

Fish-derived gelatin (5-30 kDa; Geltech) was prepared using subcritical water extraction (110-150°C, 20-30 atm). Subcritical water-processed gelatin solution (5%) was preheated to 37°C and mixed with formaldehyde to facilitate crosslinking. The mixture was stirred at 400-500 rpm for 12-36 hours and sprayed into 500 mL of paraffin oil using an encapsulation machine (Encapsulator B-390, BÜCHI Labortechnik AG). The mixture was stirred at 400 rpm at 37°C for 10 minutes to form spherical microparticles. Thereafter, stirring was continued for 30 minutes at 4°C, and the gelatin particles were washed thrice with isopropanol by centrifugation (1000-1500 rpm, 10 minutes, 4°C). After two cycles of freeze drying at -85°C for 24 and 12 hours, GMPs with a particle size of 155-350 μm were obtained. The GMPs used in this study were designed to degrade within 21 days (high MW GMP, 15-30 kDa) and 2 days (low MW GMP, 5-15 kDa) *in vivo*.

Main points

- Fish-derived spherical gelatin microparticles (GMPs) showed effective embolization and they can replace mammalian gelatin particles.
- The degradation period of GMPs can be controlled by molecular weight (MW) of the particles.
- Low MW GMPs degraded quickly and were more biocompatible with a low inflammatory response.

Animal models

The study protocol was approved by the Institutional Animal Care and Use Committee at our institution (2019-068). Twenty-four New Zealand white rabbits, weighing 2.7 to 3.8 kg, were used in this study. For general anesthesia, 6 mg/kg of xylazine hydrochloride (Rompun, Bayer) and 3 mg/kg of alfaxalone (Alfaxan, Careside) were administered intramuscularly to each rabbit. After anesthesia induction, the rabbits were restrained in the supine position on a fluoroscopic table (Integris H5000F, Philips Medical Systems); thereafter, a modified method for transauricular arterial access was performed. An auricular artery was punctured with an 18-gauge intravenous (IV) catheter (BD Angiocath Plus, Becton Dickinson) and the plastic sheath of the IV catheter was inserted into the arterial lumen. The plastic sheath was then plugged with the cap of a three-way stopcock, after removing the inner stylet needle.[17] Renal angiography was performed using a 1.9 F microcatheter (Tellus, Asahi Intecc) and a 0.016-inch guidewire (Meister 16, Asahi Intecc). Left renal angiography (n=19) was preferred, but the right renal arteries (n=5) were also used when a left renal artery could not be selected easily, or if vasospasm was detected. For embolization, 100 mg of each gelatin particle (high MW GMP: 15-30 kDa, 155-350 μm; Marine-Gel, PL micromed, and low MW GMP: 5-15 kDa, 155-350 μm; Smart-Gel, PL micromed) was mixed with 10 mL of normal saline and 10 mL of contrast media (Visipaque 320, GE Healthcare). A microcatheter was placed in the inferior segmental artery. The renal artery was embolized using 1 mL of the GMP mixture to achieve at least 1/3 of the parenchymal perfusion defect. Angiography was performed before and after embolization. The rabbits were sacrificed either immediately after the embolic procedure (groups A and B, n=4 each), or after the follow-up angiogram on day 2 (groups C and D, n=4 each) and day 21 (groups E and F, n=4 each) of embolization, respectively. High MW GMPs were used in groups A, C, and E, whereas low MW GMPs were injected in groups B, D, and F. Figure 1 demonstrates the experimental angiography process performed in each group. Angiographic findings were classified into 3 grades (Grade 1: parenchymal perfusion defect <1/3; Grade 2: 1/3< parenchymal perfusion defect <2/3; and Grade 3: parenchymal perfusion defect >2/3) and were interpreted by two board-certified interventional radiologists by consensus (Table 1).

In vitro study

One vial (100 mg) of each GMP was mixed with 20 mL of normal saline and maintained at 37°C for 2 weeks. The size distribution of the particles was evaluated with an optical microscope (BX53T, Olympus) by measuring the diameter of 30 randomly selected

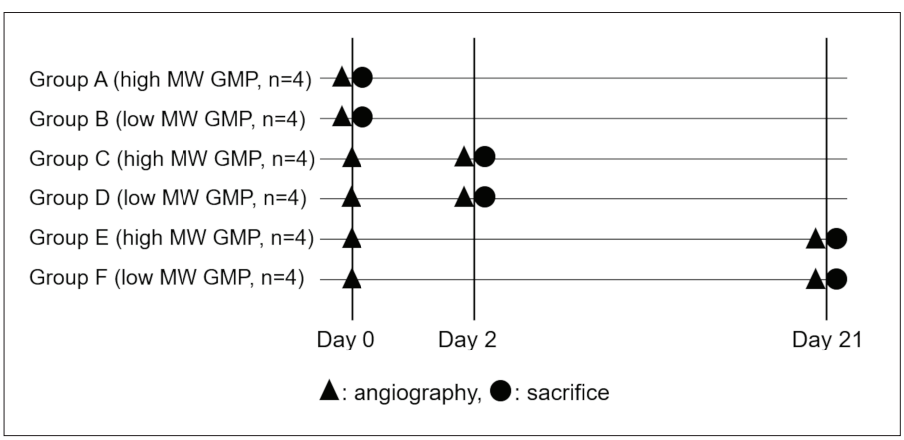

Figure 1. The experimental angiography and sacrifice procedures. MW, molecular weight; GMP, gelatin microparticle.

Table 1. Grading of the angiographic findings

	A (n=4)		B (n=4)	
Groups	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)
Immediate	2.25 ± 0.50	2 (2-2.5)	2.25 ± 0.50	2 (2-2.5)
2-day F/U	-		-	
21-day F/U	-		-	
	C (n=4)		D (n=4)	
Groups	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)
Immediate	2.00 ± 0.00	2 (2-2)	2.75 ± 0.50	3 (2.5-3)
2-day F/U	1.25 ± 0.50	1 (1-1.5)	1.75 ± 0.50	2 (1.5-2)
21-day F/U	-		-	
	E (n=4)		F (n=4)	
Groups	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)
Immediate	2.50 ± 0.58	2.5 (2-3)	2.50 ± 0.58	2.5 (2-3)
2-day F/U	-		-	
21-day F/U	1.25 ± 0.50	1 (1-1.5)	1.00 ± 0.00	1 (1-1)

Grade 1, parenchymal perfusion defect <1/3; Grade 2, 1/3 < parenchymal perfusion defect < 2/3; Grade 3, parenchymal perfusion defect > 2/3.
SD, standard deviation; IQR, interquartile range; F/U, follow-up.

Table 2. The Banff classification of renal allograft pathology[18,19]

Quantitative criteria for intimal arteritis ("v") scores
v0 - No arteritis
v1 - Mild-to-moderate intimal arteritis in at least one arterial cross section
v2 - Severe intimal arteritis with at least 25% luminal area lost in at least one arterial cross section
v3 - Transmural arteritis and/or arterial fibrinoid change and medial smooth muscle necrosis with lymphocytic infiltrate in vessel
Quantitative criteria for mononuclear cell interstitial inflammation ("i") scores
i0 - No or trivial interstitial inflammation (<10% of unscarred parenchyma)
i1 – 10% to 25% of parenchyma inflamed
i2 – 26% to 50% of parenchyma inflamed
i3 - More than 50% of parenchyma inflamed
Quantitative criteria for interstitial fibrosis ("ci") scores
ci0 - Interstitial fibrosis in up to 5% of cortical area
ci1 - Interstitial fibrosis in 6% to 25% of cortical area (mild)
ci2 - Interstitial fibrosis in 26% to 50% of cortical area (moderate)
ci3 - Interstitial fibrosis in >50% of cortical area (severe)

particles in a microscopic field. The mean diameters of GMPs were compared before and 5 minutes after mixing with normal saline. The microscopic morphological changes and soluble time of GMPs were also compared.

Histopathology

The extracted renal specimens were fixed in 10% formaldehyde solution and embedded in paraffin. Coronal histological sections of the tissue were obtained, and 5 µm thick sections cut from each paraffin block were stained with hematoxylin and eosin. The pathological changes in the vessel wall and renal parenchyma, and degradation of the GMPs were evaluated. The Banff classification of renal allograft pathology, including the quantitative criteria for intimal arteritis ("v"), mononuclear cell interstitial inflammation ("i"), and interstitial fibrosis ("ci") were used to quantitatively analyze inflammatory response of renal arteries and renal parenchyma (Table 2).[18-21] The histopathological analysis was performed by a pathologist with 9 years of experience.

Statistical analysis

Statistical analysis was performed using the SPSS software package (version 26.0, IBM Corp.). Mann-Whitney U test was performed to compare the mean diameters of GMPs in the *in vitro* study and Banff scores in pathologic evaluation. $P < .05$ was considered statistically significant.

Results

All embolization procedures were successfully performed without any procedure-related complication. Both high and low MW GMPs caused effective embolization, at least 1/3 of the parenchymal perfusion defect, on angiograms obtained immediately after the embolization procedure in 24 rabbits. In each group, some areas of the perfusion recovery were observed on the follow-up image, but the degree was slightly different between high MW and low MW groups (Table 1). On 2-day follow-up angiography, the occluded vessels were recanalized to more peripheral branches, including arcuate arteries, in the low MW group, whereas diffuse luminal narrowing and irregularity in partially recanalized vessels were observed in the high MW GMP group (Figure 2). Compared to the follow-up angiograms on day 21 after embolization, the embolized arteries were reperfused to a greater extent in the low MW GMP group, and large areas of parenchymal perfusion defect had also recovered. However, the high MW group showed multifocal stenosis of partially recanalized arteries with persistent poorly enhanced areas (Figure 3).

On histopathological evaluation, remnant GMPs were detected in the embolized artery in most of the renal specimens (15/16) in the high MW GMP group on 2-day follow-up; however, the GMPs were only partially degraded and still blocked the lumen of the vessels. Most of the high MW GMPs were degraded within 21 days, and GMP remained in only one of the 16 samples. In contrast, the low MW GMPs were almost degraded within 2 days, and gelatin fragments were found in only 4 of the 16 samples on 2-day follow-up. The mean Banff score of the quantitative criteria for intimal arteritis ("v") was significantly higher in the high MW GMP group

Table 3. Quantitative analysis according to Banff scores

	High MW GMP		Low MW GMP		
	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)	*P*
Intimal arteritis ("v")					
2-day F/U	1.75 ± 0.58	2 (2-2)	0.19 ± 0.40	0 (0-0)	<0.001
21-day F/U	0.50 ± 0.89	0 (0-1)	0.00 ± 0.00	0 (0-0)	0.14
Interstitial inflammation ("i")					
2-day F/U	1.81 ± 0.40	2 (2-2)	1.38 ± 0.50	1 (1-2)	0.035
21-day F/U	1.81 ± 0.54	2 (1.5-2)	0.44 ± 0.51	0 (0-1)	<0.001
Interstitial fibrosis ("ci")					
2-day F/U	0.69 ± 0.48	1 (0-1)	0.81 ± 0.40	1 (1-1)	0.56
21-day F/U	2.56 ± 0.63	3 (2-3)	0.88 ± 0.89	1 (0-2)	<0.001

MW, molecular weight; GMP, gelatin microparticle; SD, standard deviation; IQR, interquartile range; F/U, follow-up.

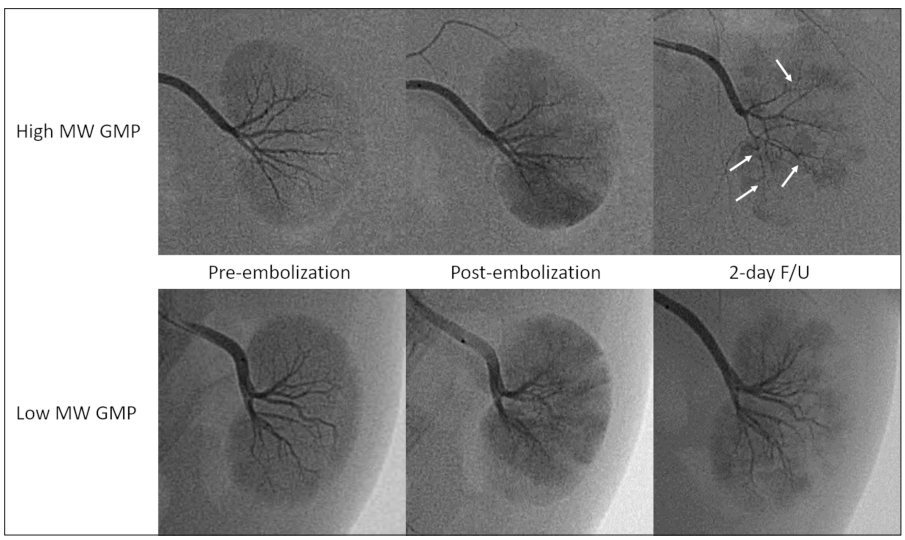

Figure 2. Two-day follow-up (F/U) angiographies of high MW GMP and low MW GMP groups. Branches of interlobar arteries are not visible in either the high and low MW GMP groups on post-embolization images. On 2-day F/U angiograms, diffuse luminal narrowing and irregularity of partially recanalized vessels *(white arrows)* are found in the high MW GMP group. However, the embolized arteries are recanalized to the more peripheral branches in the low MW group, and the arterial lumens are relatively preserved.

on 2-day follow-up (1.75 ± 0.58 vs. 0.19 ± 0.4, $P < .001$). When comparing interstitial inflammation ("i"), the difference of Banff scores was significant between the high and low MW GMP groups both on post-embolization day 2 (1.81 ± 0.4 vs. 1.38 ± 0.5, $P = .035$) and day 21 follow-up (1.81 ± 0.54 vs. 0.44 ± 0.51, $P < .001$). Interstitial fibrosis ("ci") was also more severe in the high MW GMP group than in the low MW GMP group on 21-day follow-up (2.56 ± 0.63 vs. 0.88 ± 0.89, $P < .001$) (Table 3 and Figure 3).

In *in vitro* studies, both high MW and low MW GMPs had an almost spherical shape when they were dried, without a significant difference in their mean diameter (289 ± 59.21 μm vs. 284 ± 54.24 μm, $P = .57$). Their spherical shapes were maintained even after mixing with normal saline, and clumping was not observed. At 5 minutes after mixing with saline, the mean diameter of the low MW GMP became larger than that of the high MW GMP (309.3 ± 62.8 μm vs. 441 ± 77.48 μm, $P < .001$) (Figure 4). Moreover, the peripheral portion of the low MW GMP became transparent on microscopy as it absorbed fluid faster than the high MW GMP. When stored at 37°C, the low MW GMPs gradually lost the spherical shape and degraded over time. On day 6, the low MW GMPs were totally dissolved macroscopically, and only tiny debris were observed on microscopy. In contrast, the high MW GMPs maintained a relatively round appearance and were only partially degraded on microscopic evaluation at 2 weeks (Figure 5).

Discussion

Transcatheter arterial embolization using GS particles has been widely performed since several decades in patients with hepatocellular carcinoma, uterine fibroids, gastrointestinal bleeding, postpartum hemorrhage, or multiple traumas.[8,22-25] As gelatin is a temporary embolic agent, GS embolization typically provides vessel occlusion that lasts for 3-6 weeks, and recanalization of the occluded vessels is expected. However, arterial impairment after embolization with GS particles because of thrombus and neointimal hyperplasia has been reported previously; these conditions may induce arterial stenosis or occlusion and increases the difficulty of the next treatment.[10-12,26]

In the present study, high MW GMP showed a temporary embolic effect for approximately 21 days. On follow-up day 21 post-angiography, occluded arteries were partially recanalized and multifocal stenosis of the vessels was detected, which was similar to the result obtained when porcine gelatin was used for embolization.[10,11] As a result, fish gelatin can be used as an embolic agent with similar effects to general GS particles. In addition, fish gelatin can be useful in areas where porcine or bovine gelatin is not available for religious reasons.[6,7]

Recently, several studies have reported the advantage of rapidly degradable embolic materials in animal models and clinical studies. Kawai et al.[12] compared the solubility times of gelatin particles by adjusting the cross-linkage temperature. They suggested that the 2-day soluble GS particles resulted in no significant difference regarding adverse events or tumor response, while causing significantly less damage to the recanalized artery.[12,27] Verret et al.[28] reported on uterine artery embolization using resorbable microspheres, which were adjusted to degrade in 24 hours when mixed in phosphate-buffered saline; these microspheres showed complete recanalization of the uterine arteries without remnant inflammatory response in sheep models.

Physical properties such as compressibility and viscosity between two different GMPs were not evaluated in this study. But, theoretically, low MW GMP contains more particles in 1 vial (100 mg) than high MW GMP, even if there is not much difference in numbers, because the weight of one particle of

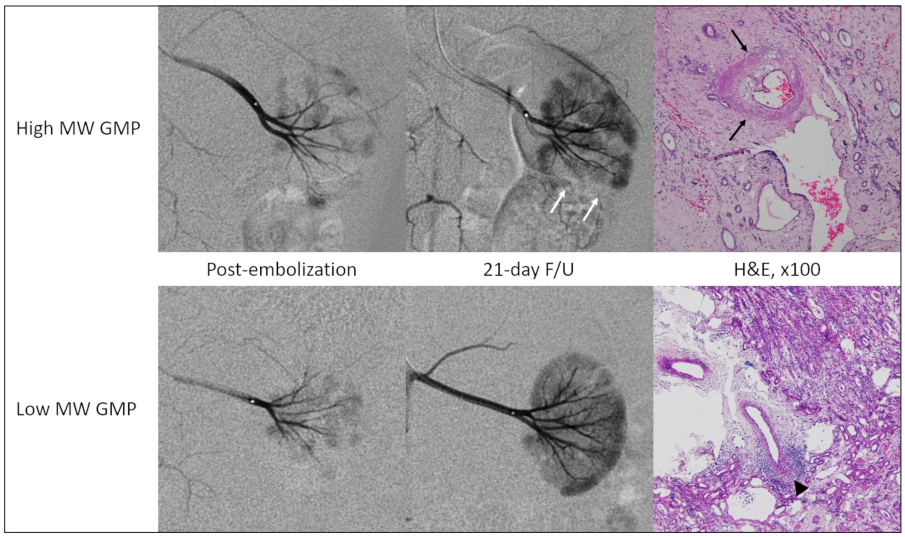

Figure 3. Day 21 F/U digital subtraction angiographies and microscopic images of the high MW GMP and low MW GMP groups. Multifocal stenosis of vessels with persistent poorly enhanced areas *(white arrows)* are detected in the high MW group. However, the occluded vessels are reperfused to a greater extent in the low MW GMP group, and large areas of parenchymal perfusion defect show recovery. On histopathologic analysis, the arterial wall shows thickening with fibrosis *(black arrows)*, and a large area of interstitial fibrosis is found in the high MW group. In the low MW group, only mild focal perivascular inflammation *(arrowhead)* is detected. Interstitial inflammation is also found, which is considered as a reversible change. H&E: hematoxylin and eosin stain.

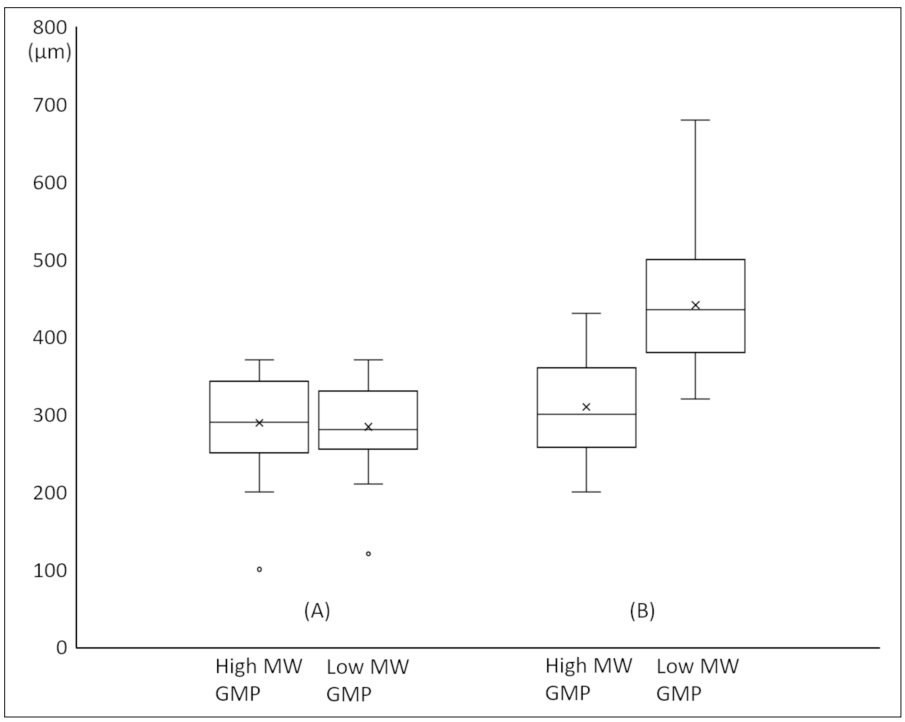

Figure 4. Comparison of diameters of the high MW and low MW GMPs before (A, box plots on the left) and 5 minutes (B, box plots on the right) after mixing with normal saline. At 5 minutes after mixing with saline, the mean diameter of the low MW GMPs appear significantly larger than that of the high MW GMP ($P < .001$).

the same diameter is lighter in low MW GMP. According to the study by Lai,[14] the viscosity of gelatin increased with increasing MW. Therefore, low MW GMP has more particles with lower viscosity than high MW GMP in 1 vial, and it has the advantage of being easily delivered to the distal portion. These may also help with a strong embolic effect of low MW GMP, in our opinion.

The degradation property of gelatin particles is determined by controlling the cross-linking time and temperature, cross-linked materials, morphology, size, and MW of the particles.[3,11,27,29] It has been previously reported that the mechanism of hydration of gelatin is a capillary phenomenon of water molecules penetrating the tiny interstices of a collagen-like four-dimensional structure in the gelatin.[30] Gelatin is easily dissolved in water by heating, as hydrolysis of peptide bonds and crosslinks occur, known as gel formation [3]. Therefore, gelatin fragment was invisible after gel formation when we mixed the GMP with normal saline and placed it at 37°C; this occurred within 6 days in low MW GMP, while it took more than 14 days in high MW GMP, in our study. Gelatin hydrolysis is accelerated by enzymes in the body fluid, especially macrophage-associated gelatinase in the blood.[3,27,31] That is the reason why we can use the gelatin sponge particle as a temporary embolic agent. The present study aimed to verify whether low MW GMP degrades quickly and whether embolization using low MW GMPs shows rapid degradation of the gelatin particles and early recanalization of the occluded vessels on follow-up angiographies. Low MW GMPs absorbed fluid quickly in our *in vitro* experiment, which coincides well with previous reports.[13,14] Therefore, we speculate that low MW GMPs absorb blood rapidly in the vessels and react with gelatinases to promote GMP degradation.

On histopathologic analysis of renal specimens, most low MW GMPs degraded within 2 days, and remnant gelatin particles were rarely found in the recanalized arteries, which is consistent with angiographic findings. In the follow-up groups, perivascular and interstitial inflammation was mild in the low MW GMP group; consequently, the low MW GMPs were demonstrated to be more biocompatible. As the blood vessels were reopened earlier in low MW GMP group, interstitial fibrosis was also significantly decreased, and large areas of renal parenchyma were well preserved. In contrast, renal infarction was prominent in the high MW GMP group because of the prolonged embolic effect and impairment of arteries. Because the renal parenchyma is sensitive to ischemia, irreversible damage to the renal glomeruli and tubules can occur.[32] The results of the present study correspond well with those reported earlier, which reported that low MW gelatin resolved rapidly and was more cytocompatible than high MW gelatin.[13,14]

Furthermore, in the *in vitro* study, both high and low MW GMPs remained spher-

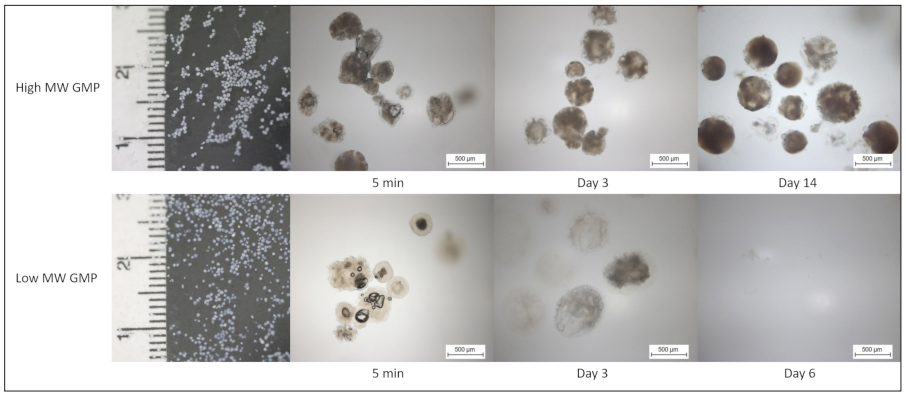

Figure 5. Sequential morphological change in the high MW and low MW GMPs (stored at 37°C). The spherical shape of both high MW and low MW GMPs is maintained after mixing with normal saline. Peripheral portion of low MW GMP appears transparent 5 minutes after mixing with normal saline, as it absorbs fluid faster. On day 6, the low MW GMPs are totally dissolved, and only tiny debris is observed. In contrast, the high MW GMP appears partially degraded until 14 days.

ical, without clumping, when mixed with normal saline. This characteristic of the fish-derived spherical GMPs reduces aggregation on the vascular wall, prevents proximal embolization, and allows them to be accurately delivered to the target vessel. Therefore, using low MW GMPs for embolization can reduce damage to the proximal portion of the target artery and prevent the formation of collateral blood vessels. In addition, as it provides a short-term embolic effect, low MW spherical GMPs might be useful to avoid ischemia in large areas in patients with gastrointestinal bleeding whose hemorrhagic focus is difficult to be superselected. A smaller size of low MW GMP (50-100 μm) can also be considered as a replacement for imipenem/cilastatin sodium, which is used as an embolic agent for transcatheter arterial microembolization in musculoskeletal pain management, without worrying about antibiotic resistance. However, further studies are required to determine the efficacy and safety of low MW GMPs for clinical use.

This experimental study had several limitations. First, the renal artery model was used because it is easy to cannulate and has a diameter suited to this experiment. However, the renal artery is an end-artery without collateral circulation, and renal infarction induced by renal artery occlusion might affect the degradation time of the GMPs. Ideally, evaluation of degradation period and inflammatory reaction of the vessels should be performed using internal iliac or hepatic arteries, both of which are not end arteries. However, it is difficult to access and perform an angiography for these arteries in the rabbit model. Therefore, additional studies using larger animal models would be required. Second, even though the same volume of GMP mixture was injected into the renal arteries of all rabbits, the extent of renal artery occlusion and parenchymal perfusion defect varied. It is believed that there were differences in the kidney size and blood vessel diameter according to the body weight, and that it was impossible to contain the exact same amount of gelatin particles in the GMP mixtures, both of which could have influenced the embolic effect. Therefore, angiographies and pathological findings of kidneys embolized to a similar degree in each group were compared to analyze the results.

In conclusion, fish-derived spherical GMPs showed effective embolization in a rabbit model. Embolization using low MW fish GMPs had a short-term embolic effect lasting 2 days and provided early recanalization of the occluded arteries. By lowering the MW of the GMP, biocompatibility with low inflammatory response was also achieved.

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Conflict of interest disclosure

The authors declared no conflicts of interest.

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