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PLGA Microspheres in Hyaluronic Acid Gel as a Potential Bulking Agent for Urologic and Dermatologic Injection Therapies

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Abstract In this study, we investigated whether PLGA microspheres in combination with hyaluronic acid (HA) gel have appropriate properties as a bulking agent for urologic injection therapies and whether the implantation of PLGA microspheres and HA gel induces angiogenesis in the newly formed tissues. In order to investigate whether this bulking agent is injectable, this material was injected through 24-gauge needles into the subcutaneous dorsum of the mouse. The bulking agent was easily injected without needle obstruction. Histological analyses of the hybrid tissues at 2 weeks showed that host cells at the surrounding tissues migrated into the spaces between the implanted PLGA microspheres and formed tissue-like structures. An inflammatory response to the implants was mild at 2 weeks and diminished at 8 weeks. Importantly, extensive ingrowth of blood vessels was observed in the hybrid tissues formed by the injection of PLGA microspheres and HA, whereas blood vessels rarely formed in the hybrid tissues formed by the injection of PLGA microspheres only. The implant volume was conserved for almost the entire implantation period. Histological analyses of the distant organs of the bulking agent-implanted animals, such as the lungs, liver, heart, brain, kidney, and spleen, showed no evidence of the injected microsphere migration. These results show that PLGA microspheres in combination with HA possess the appropriate characteristics for a bulking agent for urologic injection therapies and induce extensive blood vessel formation in the hybrid tissues.

Key words: Angiogenesis, bulking agent, hyaluronic acid, injection therapy, poly(lactic-co-glycolic acid) microsphere

Injection therapies for urologic or esthetic treatments are gradually replacing traditional surgical therapies for the past decades. For cosmetic surgery, injectable collagen or polymethylmethacrylate (PMMA) microspheres have been used to restore facial wrinkles and concave cheeks [31, 32, 46]. The principle of these therapies is to repair a depressed tissue by filling with a bulking material, resulting in changes in the anatomy of the tissue. Similarly, vesicoureteral reflux or urinary incontinence can be corrected by injection therapies in this way. Since these urinary symptoms are caused by the weakness or malfunction of a muscle controlling urine flow in the ureter or urethra, injection of a bulking agent into the bladder neck or around the ureterovesical junction helps the muscles function appropriately. Comparing with conventional surgical therapies, the injection therapies are less invasive, less painful, and easier to perform [8, 20, 21, 23, 24, 26].

Several types of bulking agents for injection therapies have been developed and tested. The materials include polytetrafluoroethylene (PTFE) paste [41, 45], silicone [2, 4, 18, 43], PMMA microspheres [31, 32, 46], collagen [6, 14, 20, 34, 35], and autologous fat [29, 53]. Although these materials have been used to treat a large number of patients suffering from urinary diseases or facial wrinkles, these materials showed drawbacks: PTFE paste caused particle migration to distant organs and capillary obstruction [1, 5, 10, 36]; impurities of liquid silicone caused inflammation [3, 54]; PMMA, a non-degradable polymer, provoked chronic side effects such as granuloma formation [32]; and bovine collagen, the carrier gel for PMMA microsphere injection, exhibited allergic reactions [46]. Similarly, human-derived collagen products for injection therapies also contain a risk of a latent allergic reaction and viral infection. The biocompatibility of autologous fat transplantation would be the best among the materials mentioned above. However, it failed to preserve its original volume over time [23]. Furthermore, fat source is limited and an operation is required to obtain autologous fat [17].

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PLGA microspheres have been used for various applications, such as a tissue engineering scaffold [22], cell culture substrate [40], and drug delivery vehicle [11, 42]. In our previous study, poly(lactic-co-glycolic acid) (PLGA) microspheres were evaluated as a bulking agent to overcome the problems of the currently available bulking agents for injection therapies [9]. An ideal material for injection therapies must be easily injectable, non-migratory, non-inflammatory, volume stable, and biocompatible. PLGA microspheres were easily injectable. Upon PLGA microsphere implantation to the subcutaneous space of mice, host cells from the surrounding tissues migrated to the space between the implanted microspheres and formed hybrid tissues. The hybrid tissue volume was maintained unchanged, exhibiting a bulking effect. PLGA microspheres were non-migratory and biocompatible. However, blood vessel formation, which is critical to avoid cell necrosis in the hybrid tissues, rarely occurred in the hybrid tissues.

In this study, we designed a hybrid injectable bulking agent composed of PLGA microspheres and hyaluronic acid (HA). HA is a dominant glycosaminoglycan in connective tissues associated with various cellular processes involved in wound healing [19, 28, 37] and is known to promote angiogenesis [44, 49, 50]. Oligosaccharide HA fragments have been shown to induce angiogenesis in several animal models, including chick chorioallantois [55], in rat skin [56], and in skin graft preparations [30], as well as within collagen gels *in vitro* [39]. The angiogenic effect of HA may be associated with HA binding to endothelial cell surface receptors such as CD44 and the receptor for HA-mediated motility (RHAMM), leading to the promotion of cell adhesion, mobility, and proliferation [51, 55]. Due to the viscous property of HA solution, HA solution allows the microspheres to be homogeneously suspended in the solution and prevent the microspheres from obstructing the needles upon injection. We determined whether PLGA microspheres in combination with HA have appropriate properties for a bulking agent, and whether this bulking agent induces angiogenesis in the new hybrid tissues formed by the injection.

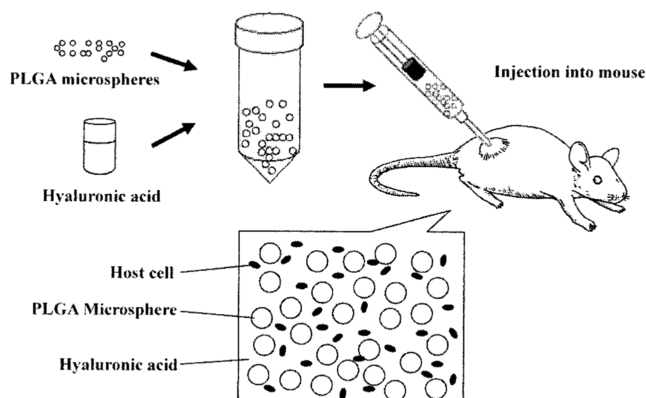

Fig. 1. Scheme of a bulking agent (PLGA microspheres and HA) injection into the subcutaneous space of a mouse.

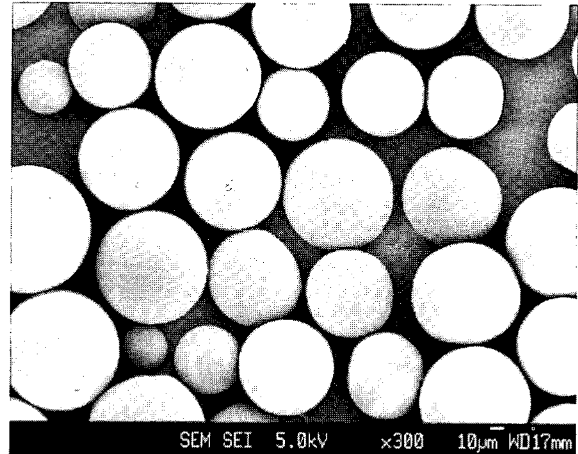

Fig. 2. Scanning electron microscopic image of PLGA microspheres.

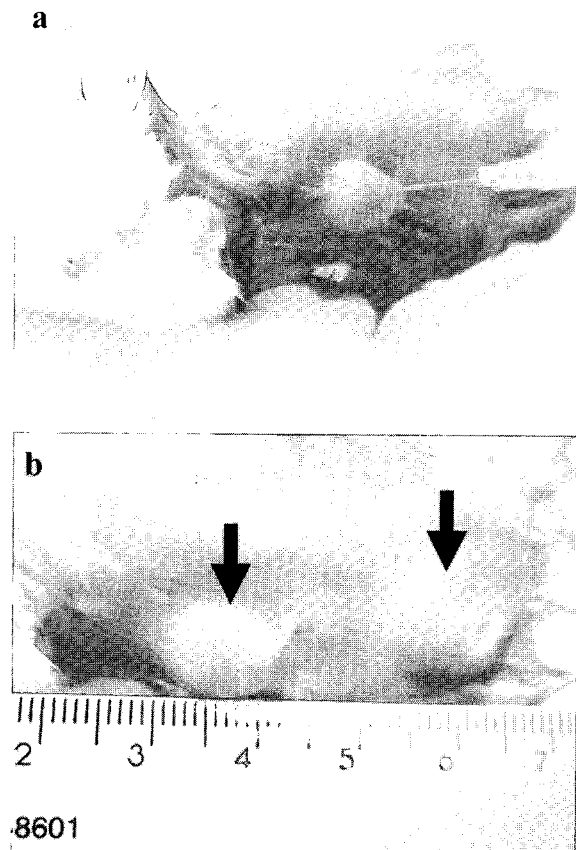

Fig. 3. Photographs of (a) injection of a bulking agent (PLGA microspheres and HA) through a 24-gauge needle into the subcutaneous dorsum of a mouse and (b) mounts (arrows) formed by the injection. The scale is in centimeters.

MATERIALS AND METHODS

PLGA Microsphere Fabrication

Microspheres were fabricated from 75:25 PLGA (molecular weight=100,000 Da, Birmingham Polymers, Birmingham, AL, U.S.A.) using an oil/water emulsion and solvent extraction/evaporation technique, as previously described [11]. Briefly, 600 mg of PLGA were dissolved in 12 ml of methylene chloride, and the solution was added to 400 ml of 0.5% aqueous polyvinyl alcohol (w/v) (molecular weight=30,000–70,000 Da, Sigma, St Louis, MO, U.S.A.), and the mixture was stirred vigorously (700 rpm) overnight at room temperature. The microspheres were collected by centrifugation, washed 3 times with deionized distilled water, lyophilized for 3 days, and filtered into size range of 70–100 μm through filters (70 and 100 μm, FALCON®, Becton Dickinson and Company, Franklin Lakes, NJ, U.S.A.). Approximately 50% of the microspheres fell within the 70–100 μm size range. Prior to implantation, microspheres were sterilized with ethylene oxide gas.

Implantation

ICR (Institute of Cancer Research) mice (two-month-old males, Jung-Ang Lab Animal Inc., Seoul, Korea) were anesthetized with ketamine hydrochloride (8 mg/kg body weight, Yuhan Corporation, Seoul, Korea) and xylazine hydrochloride (1.15 mg/kg body weight, Bayer, Seoul, Korea) and shaved. PLGA microspheres were mixed with 1% HA (w/v) (molecular weight=4,000,000 Da, LG Life Sciences Ltd., Seoul, Korea) in physiological saline with a volume ratio of 1:2 (PLGA:HA). After microspheres were homogeneously suspended in the HA gel, 0.2 ml of the microsphere (0.095 g) suspension was injected into the subcutaneous dorsum of the mice through 24-gauge needles (n=12). Injection of PLGA microspheres (0.19 g) in the 3% (w/v) carboxymethyl cellulose (CMC, Sigma) in physiological saline with a volume ratio of 1:1 (PLGA: CMC) served as controls (n=12). CMC is an important industrial polymer with various applications, such as a drag reduction agent, detergents, food additive, and drug additive [7]. One of the most important properties of CMC is viscosity building. Due to its viscous property, CMC allows the microspheres to be homogeneously suspended in the solution and prevents the microspheres from obstructing the needles upon injection. The scheme of implantation is described in Fig. 1.

Analyses

To obtain scanning electron microscopic images, PLGA microspheres were mounted on an aluminum support

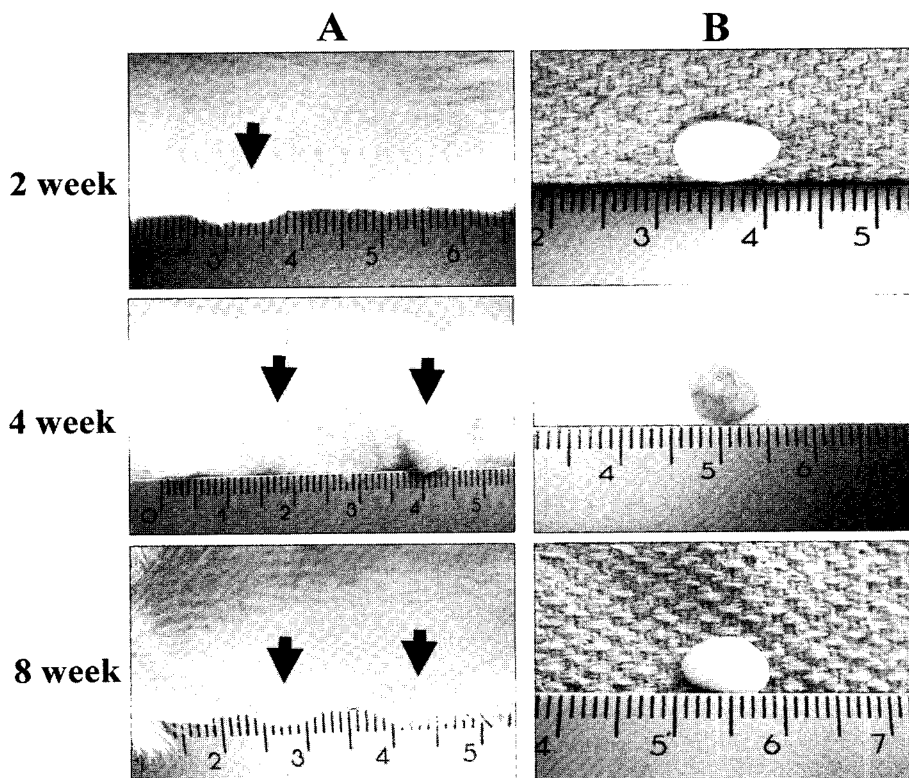

Fig. 4. Column A; Gross views of mounds formed by the bulking agent (PLGA microspheres and HA) injection at various time points. Column B; Gross views of hybrid tissues formed by the bulking agent injection at various time points.
The arrows indicate the injection sites. In columns A and B, the scales are in centimeters.

and coated with platinum under a vacuum. A scanning electron microscope (JSM-6330F, JEOL, Tokyo, Japan) was operated to image the samples at 5 kV. After microsphere implantation, the volume of retrieved implants was measured by immersing the implants in physiological saline in a graded pipette. To minimize possible saline absorption into the implants, the volume was measured instantly. For histological analyses, implants were fixed in 10% formaldehyde (v/v) in phosphate buffered saline, dehydrated with a graded ethanol series, embedded in paraffin, and cut at a thickness of 4 μm. The sections were stained with hematoxylin and eosin (H&E) for morphological analysis and with Masson's trichrome for collagen detection. The organs of the microsphere-implanted mice, including the lungs, liver, heart, brain, kidneys, and spleen, were harvested and examined histologically to determine microsphere migration to the distant organs.

RESULTS AND DISCUSSION

SEM observations showed that the fabricated PLGA microspheres had an absolutely spherical morphology with a smooth surface (Fig. 2). This morphology of the

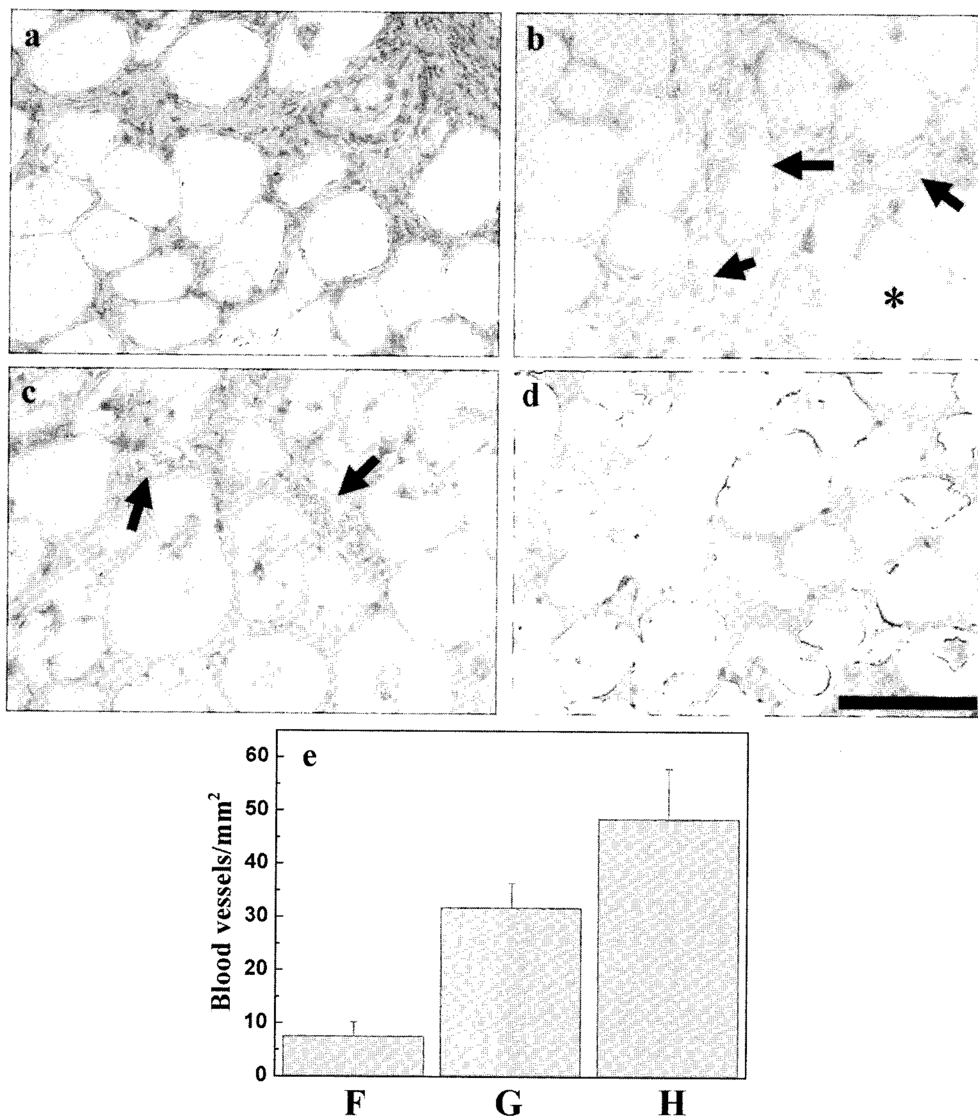

Fig. 5. H&E stained sections of hybrid tissues formed by the injection of PLGA microspheres suspended in 0.1% (v/v) HA gel at (a) 2, (b) 4, and (c) 8 weeks and (d) by the injection of PLGA microspheres suspended in 3% (v/v) CMC solution at 8 weeks.
Blood vessels (arrows) formed extensively in the tissues by the injection of PLGA microspheres and HA at 4 and 8 weeks, but were hardly seen in the tissues formed by PLGA/CMC injection at 8 weeks. (e) Blood vessel density in the hybrid tissues formed by the injection of (F) PLGA/CMC at 8 weeks or PLGA/HA at (G) 4 and (H) 8 weeks. $P<0.05$ between any two groups. The asterisk in (b) indicates PLGA microsphere. The scale bar in (d) indicates 100 μm. All photographs were taken at the same magnification.

microspheres may not provoke host cells and may prevent inflammation [31]. Because too large microspheres make injection through fine needles difficult or too small microspheres may migrate to distant organs, microspheres sized 70–100 μm were used for implantation through injection. PLGA microspheres mixed with HA at 1:2 volume ratio were injected easily through 24-gauge needles without morphological deformation and obstruction of the needles.

To determine whether the bulking agent injection can induce new tissue formation and angiogenesis *in vivo*, PLGA microspheres in HA solution were injected beneath the dorsal skin of mice. Subcutaneous mounds were created at the injection sites in all animals (Fig. 3 and Fig. 4A). There was no evidence of complications, including swelling or erythema, at the surface of any of the injection sites despite the fact that none of the animals were administrated antibiotics. Hair grew back over the subcutaneous mass of the injected materials. Gross views of the implants retrieved at 2, 4, and 8 weeks indicated the formation of tissue-like structures by the bulking agent injection (Fig. 4B). There was no evidence of erythema around any of the implants. The size and shape of the implants were maintained approximately for the entire implantation period (Fig. 4B). Histological analyses of the implants indicated that host cells from the surrounding tissues migrated to the space between the injected microspheres and formed new hybrid tissue structures (Figs. 5a, 5b, 5c). Migrating cells seemed to be fibroblasts or muscle cells, as the injected microspheres were located between skin and muscle layers. The cell ingrowth helped embed the microspheres in the extracellular matrix and form the hybrid tissue structures. Lymphocytic infiltration was noted at 2 weeks, but the inflammatory reaction diminished remarkably at 8 weeks (Figs. 5a, 5c). Masson's trichrome staining revealed the presence of collagen, which might have been produced by the fibroblasts or muscle cells in the newly formed tissue (Fig. 6).

Importantly, numerous blood vessels formed in the hybrid tissues generated by the injection of PLGA microspheres and HA at 4 and 8 weeks (Figs. 5b, 5c). In contrast, blood vessels were rarely observed in the new tissues generated by the injection of PLGA microspheres suspended in 3% (v/v) CMC solution (Fig. 5d). The blood vessel density was 6 times ($P<0.05$) higher in the tissues formed by the injection of PLGA microspheres suspended in HA gel than in the tissues formed by the injection of PLGA microspheres suspended in CMC solution at the same time point (Fig. 5e). Vascularization of the newly formed tissues is critical, since cells more than approximately 200 μm from a blood supply are either metabolically inactive or necrotic, owing to the limitation of nutrient (e.g., oxygen) diffusion [12, 25, 27].

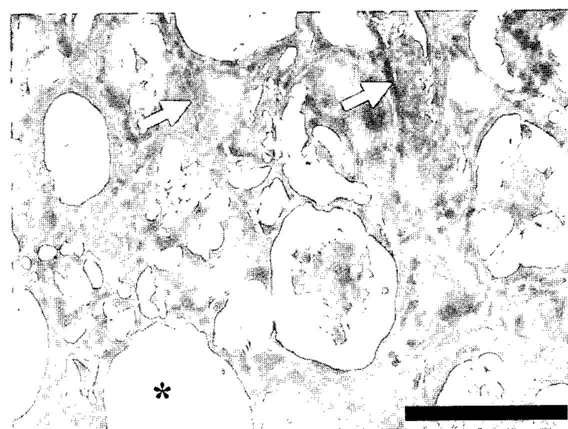

Fig. 6. Masson's trichrome stained section of hybrid tissues formed by the injection of PLGA microsphere and HA at 8 weeks, indicating the presence of a significant amount of collagen in the hybrid tissues.

Collagen was stained blue. The arrows indicate newly formed blood vessels. The asterisk indicates PLGA microsphere. The scale bar indicates 100 μm.

The volume of the hybrid tissues formed by the injection of PLGA microspheres and HA was measured to determine whether the hybrid tissues conserved their original volume over time. The volume of the hybrid tissues was approximately maintained for the entire implantation period (Fig. 7). The slight volume decrease from 0.20 ml to 0.19 ml at 8 weeks was likely caused by the absorption and degradation of the HA solution, a carrier solution utilized to inject microspheres. HA solution is known to resorb within 2 weeks after injection [15, 33]. The volume was likely conserved by the ingrowth

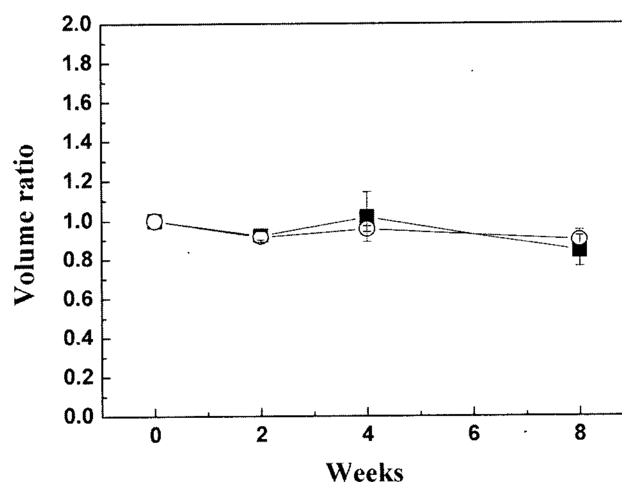

Fig. 7. The profile of volume of the hybrid tissue formed by microsphere injection over time.

For each injection, 0.2 ml of microspheres suspended in 0.1% (v/v) HA gel (square) or 3% (v/v) CMC solution (open circle) were injected. Volume ratio=V_t/V_0, where V_0=initial implant volume (0.2 ml) and V_t=implant volume at each time point.

of host cells that filled the void space between the microspheres. As the PLGA microspheres degrade, more cells may migrate to or proliferate within the new tissues, resulting in the conservation of the hybrid tissue volume.

To determine whether the injected microspheres migrated to distant organs, several distant organs of the mice implanted with microspheres and HA were examined histologically. Serial sections of the lungs, liver, heart, brain, kidneys, and spleen were thoroughly examined in search of migrant microspheres or any evidence of side effects anticipated by the migration such as embolization and inflammation. The examinations showed no sign of microsphere migration or granuloma formation (Fig. 8). This result is promising when compared with PTFE paste implantation, which resulted in PTFE particle migration to the distant organs [24]. The migration was likely caused by the small size (less than 40 μm in diameter) of the PTFE particles. PTFE particles, measuring mostly between 4–40 μm in size, may be exposed to the blood stream and conveyed to distant organs [24]. In addition, there is a possibility of particle migration by phagocytes that can uptake particles up to 20 μm *in vitro* [39]. The PLGA microspheres used in the present study ranged from 70 to 100 μm in diameter, which would have no or extremely low possibility of microsphere migration after injection and needle obstruction upon injection.

Besides urinary applications, PLGA microspheres and HA could be applied to esthetic treatments, such as lip augmentation, wrinkle correction, and other treatments

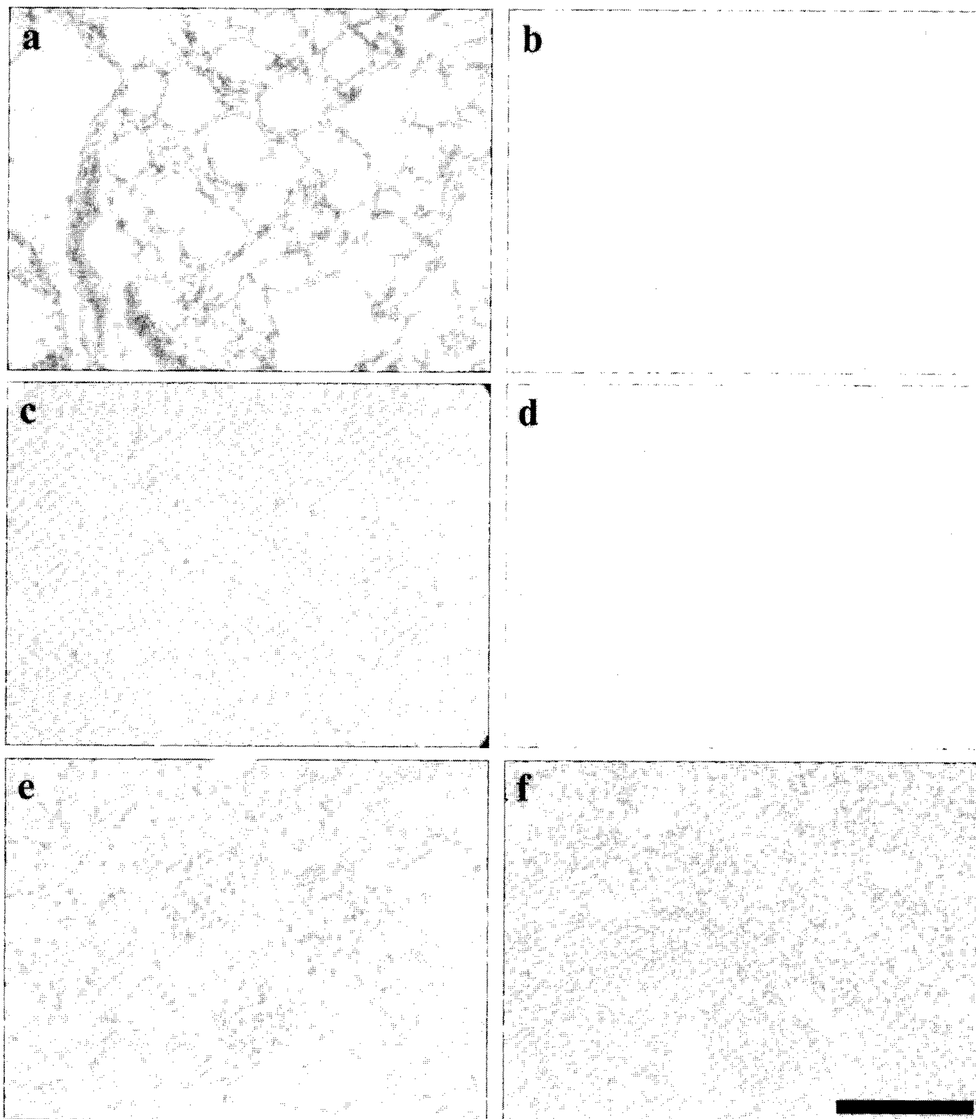

Fig. 8. Histological analyses of the organs of mice implanted with PLGA microspheres and HA at 4 weeks (H&E staining).
(a) The lungs, (b) liver, (c) heart, (d) brain, (e) kidneys, and (f) spleen. These data showed no evidence of microsphere migration. The scale bar indicates 100 μm. All photographs were taken at the same magnification.

requiring tissue filling. Injection materials for facial corrections include collagen [48], PMMA [31], silicone [57], and PTFE [13]. Adverse reactions reported on these materials in esthetic treatments [47] are approximately similar to those in urinary treatments, but allergic or hypersensitive problems matter much more in esthetic treatments. As previously mentioned, the collagen treatment may instigate an immune response [14, 34]. PLGA microspheres have several advantages over these materials. PLGA is a biocompatible synthetic polymer, approved by the Food and Drug Administration [16, 52]. PLGA does not contain biological byproducts or pathogens and is not recognized as an antigen or allergen.

In summary, these results show that PLGA microspheres in combination with hyaluronic acid could be a potential bulking agent for urologic or dermatologic applications. The bulking agent was easily injected through a 24-gauge needle into the subcutaneous space of mice and successfully generated a hybrid tissue structure. The volume of the tissue was conserved for approximately 8 weeks. There was no sign of microsphere migration to distant organs. Importantly, new blood vessels formed extensively throughout the implants, whereas the injection of PLGA microspheres only without HA resulted in the formation of only a few number of blood vessels. Extensive blood vessel formation may retain the viability of the newly formed tissues. Additional studies are necessary to examine implant fate and long-term volume change to verify the feasibility of this bulking agent for urologic and dermatologic applications.

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