Comparative study of hyaluronic acid fillers by *in vitro* and *in vivo* testing

K.Y. Park, H.K. Kim, B.J. Kim*

Departments of Dermatology, Chung-Ang University College of Medicine, Seoul, South Korea

*Correspondence: B.J. Kim. E-mail: beomjoon@unitel.co.kr

**Abstract**

**Background** Numerous hyaluronic acid (HA) fillers seem to have similar characteristics, although manufacturers insist that monophasic and biphasic HA fillers are different in many ways. Little information regarding this is available in the literature.

**Objectives** To determine characteristics of monophasic fillers vs. biphasic fillers.

**Material and methods** We tested three different (two biphasic and one monophasic) HA fillers both *in vitro* and *in vivo*. In the *in vitro* assay, cell toxicity, resistance to enzyme degradation, syringeability and morphology of particles were tested. *In vivo*, the efficacy and safety were investigated in the dorsal skin of hairless mice.

**Results** There was no cell toxicity in any of the three HA fillers. Resistance to enzymatic degradation and syringeability were better in the two biphasic HA fillers than in the monophasic filler. In particle morphology test, gel type monophasic HA filler was also found as a particle type, although there was a slight difference. Volume assessment in animal skin was superior with the monophasic than with the two biphasic HA fillers.

**Conclusion** Biphasic HA fillers have some advantages in hyaluronidase resistance, syringeability and lower risk for overcorrection, while monophasic HA fillers may be more suitable for volume augmentation due to swelling capacity.

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**Introduction**

Hyaluronic acid (HA) is an anionic, non-sulphated glycosaminoglycan distributed widely throughout connective, epithelial and neural tissues. HA is also a major component of skin, where it is involved in tissue repair. HA hydrates the skin because of its high water-binding properties and maintains proper tissue volume. Loss of HA leads to biological ageing and wrinkle development. During the past several years, a variety of HA-based fillers have been approved for the treatment of wrinkles, scars and facial contouring defects. HA dermal fillers typically fall under two categories, monophasic or biphasic, based on variations in cross-linking. Monophasic HA fillers are more cohesive, may last longer and may not migrate as much following its injection. However, biphasic HA fillers are more easily customized, to obtain the appropriate particle size to suit the indication and the anatomical area being treated. However, there is a paucity of information on the general difference between monophasic and biphasic HA fillers. Here, we have studied the different characteristics of monophasic vs. biphasic HA fillers.

**Materials and methods**

**Fillers** Two kinds of biphasic HA fillers (Perfectha Deep, ObvиеLine, France and Perlane, Q-Med, Uppsala, Sweden) and one monophasic filler (Juvederm Ultra-XC, Allergan Inc., Irvine, CA, USA) were tested. Juvederm and Perlane were approved by the U.S. Food and Drug Administration (FDA) as an option for the correction of facial wrinkles and folds in 2006 and 2007, respectively, whereas Perfectha Deep does not receive approval by FDA. Perfectha Deep is a biphasic product with 8000 particles/mL, which obtained CE marking in 2010. E-Brid technology, used in Perfectha Deep, is a unique cross-linking process, inducing formation of covalent bonds within and between HA particles. Juvederm Ultra-XC consists of stabilized, HA produced by Streptococcus equi bacteria, formulated to a concentration of 24 mg/mL in a physiological buffer, along with 0.3% lidocaine. Perlane particles are approximately 550 μm in size (10 000 particles/mL). All the fillers used in this study are...
produced by cross-linking with 1, 4-butanediol diglycidyl ether (BDDE).

**In vitro assay**

**Cell toxicity assay**  L929 immortalized mouse fibroblast cell line was tested for cellular toxicity. Cell viability was assessed neat and in 1/2 and 1/4 dilutions using 0.2 g/mL HA filler in Opti-MEM media (Gibco, Carlsbad, CA, USA) in a 37°C room for 72 h. Seeding of cells was performed on a 96-well plate, which was consistent with 1 x 104 cells per well, at 37°C overnight. Opti-MEM media was treated with 100 µL of releasing solution (100%, 75%, 50%, 25%) and incubated for 24 h. WST-1 (Takara Bio Inc., Shiga, Japan) was applied for 30 min for reaction and assessed at 450 nm (Spectramax 190; Molecular Devices, Sunnyvale, CA, USA).

**Resistance to enzymatic degradation**  Four milligrams of each HA filler reacted with 10 U/mg of hyaluronidase (BMI KOREA, Seongnam, Korea) for 8 h at 37°C. The remaining HA quantity was evaluated in the supernatant using a carbazole assay.6 The optical density was measured at 530 nm.

**Syringeability**  Syringeability test was performed to identify how much ‘injection force (F)’ is required to pull out filler from the syringe. Injection force was mainly affected by three parameters: the solution viscosity, the injection flow rate and the syringe or needle diameters. Perfectha Deep filler syringe was inserted in texture analyzer (XT2i; Stable micro system, Surrey, UK) with a 27 1/2G needle at 25°C and a moving speed fixed at 1 mm/s by recording its delivered injection force. Increase in the viscosity, density, particle size and concentration of solids in suspension hinders the syringeability of suspension.

**Particle morphology analysis**  Particle sizes were evaluated by scanning electron microscopy (SEM). HA gels were immersed in corn oil, and particle morphology was investigated by optical imaging with a folliscope (LeadM Corp., Seoul, Korea).

**In vivo animal test**

Three different kinds of 0.2 mL of HA fillers were injected into the dorsal skin of the anterior limbs of hairless mice (76-week-old female mice per group, total 21 mice). Morphological pattern of injected filler was assessed by digital photography (D9, Nikon, Tokyo, Japan). After 6 weeks, the total remaining amount of HA filler was measured in five mice, and histological evaluation was performed in two mice. These procedures were repeated in each of the three groups.

**Results**

Cell toxicity was not seen with any of the three HA fillers using an L929 mouse fibroblast immortalized cell line (Fig. 1). Resistance to enzyme degradation by carbazol assay was higher in the two biphasic HA fillers compared with that in the monophasic filler (Fig. 2). Syringeability was better in the two biphasic HA fillers than in the monophasic filler (Fig. 3). In optical imaging with the folliscope, the two biphasic fillers were particulated, but the monophasic filler was agglomerated with no particulation. However, gel type monophasic HA filler was also found as a particle type in the SEM images, though there were slight differences as follows. The mean size of the particles was uniformly 500 µm for Perfectha Deep and Perlane, while variably 250~500 µm particles existed mixing with solution for Juvederm Ultra-XC (Fig. 4). In vivo testing showed about twice as much oedema in the monophasic filler than in the two biphasic HA fillers immediately after the injection. Volume assessment in animal skin was superior with the monophasic than with the two biphasic HA fillers (Fig. 5).

**Discussion**

In recent years, use of HA-based dermal fillers has expanded in minimally invasive aesthetic dermatology. HA filler is widely

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**Figure 1**  *In vitro* cytotoxicity assay. There was no significant cytotoxicity in any of the tested fillers. All tested fillers showed greater than 90% cell viability.

**Figure 2**  Resistance to enzymatic degradation. The two biphasic fillers were more resistant to degradation by hyaluronidase than the monophasic filler.
used in the cosmetic treatments of dermal atrophy, nasolabial folds, lip augmentation and restoration of facial contour. HA is a natural, biocompatible polymer with a high molecular weight and excellent safety record. However, HA quickly degrades in its natural state and has a rapid turnover through enzymatic and free radical metabolism. If the naturally occurring form of HA were to be injected into the skin, it would be lost rapidly. To produce a more resilient form of HA, the naturally occurring form is processed into a cross-linked gel. Stabilized HA is produced predominantly using streptococcus species bio-fermentation. The acid polymer is formed from two monosaccharides, D-glucuronic acid and N-acetyl-D glycosamine. Once purified, the polymer chains are cross-linked for stability. Cross-linking stabilization of HA is required to increase resistance to biodegradation by enzymes and free radicals. The preferred cross-linking agents are BDDE and 1, 2, 7, 8-diepoxyoctane, in which both ether and ester linkages are created, further enhancing duration. Manufacturers tend to use as little cross-linking agent as possible so as to limit the irritation sometimes associated with some of these chemicals. By varying the cross-linking technique, the monophasic or biphasic nature of the HA fillers is determined.

Biphasic products consist of gel particles of stabilized HA suspended in a weakly or even non-stabilized HA fluid, and in contrast, monophasic products are known as solely stabilized and non-particulate HA gels. However, we observed that monophasic HA filler was also found as a particulate gel type mixing with solution in SEM. As the result, it seems better to understand that the monophasic HA filler has weak strength of gel-particle and can easily transform by external force. The body’s breakdown of biphasic gels may also differ from that of monophasic gels. Biphasic gels are thought to have a rapid initial degradation of HA fluid and a slower degradation of the stabilized gel particles, whereas monophasic gels are thought to degrade more uniformly. This property seems to be relevant to maintain more considerable volume in monophasic filler vs. biphasic fillers in mice models.

The different physical characteristics of filler make us to expect differences in suitability for any given application. However, whether these differences have an effect on clinical outcome remains controversial. Nevertheless, most providers feel that there are subtle differences among fillers that allow a greater degree of precision and variation in the effects of a given treatment. Flynn et al. performed a prospective, controlled, investigator-blind, single-centre study of 12 patients receiving 0.2 mL injections of commercially available HA fillers in the forearm or buttock skin. Fillers used were one biphasic (Restylane, Q-Med, Uppsala, Sweden) and three monophasic forms (Surgiderm 24XP; monodensified, Juvederm Ultra; monodensified, and Esthélis Basic; polydensified). The histopathological evaluations indicated that HA fillers diffused differently based on the production technology. Biphasic fillers produced large pools of HA distributed as beads of material in the lower portion of the dermis, compressing collagen fibres. Monophasic monodensified fillers showed pools of HA throughout the reticular dermis, breaking up the collagen fibres. Monophasic polydensified fillers were spread throughout the reticular dermis in a diffuse, evenly distributed manner. The investigators concluded that the different types of cross-linked HA behave differently, yet consistently, in the dermis among patients. Relating to our results, biphasic HA fillers might shown lower risk for overcorrection through localising ability, while monophasic fillers have volumizing efficacy through spreading capacity. Other clinical pilot study compared a monophasic HA dermal filler with a biphasic filler for the correction of nasolabial folds. The authors concluded that the effect after injection of monophasic HA and biphasic HA
was generally comparable, although there was a trend in favour of mono-HA.\textsuperscript{10}

Many different filler materials have been tested and the differences in tissue responses and injection techniques make it very difficult to correlate the clinical persistence of these different materials to physical properties. Attempts to compare the available monophasic vs. biphasic dermal fillers have been limited. It is the first report that investigated \textit{in vitro} and \textit{in vivo} characteristics of both monophasic and biphasic HA fillers. Our study suggests that biphasic HA fillers have some merits in hyaluronidase resistance, better application for syringe injection, and lower risk for overcorrection. Therefore, biphasic HA fillers may be better for wrinkle restoration. In contrast to biphasic HA fillers, monophasic HA filler may be more suitable for volume augmentation possibly due to its swelling capacity. However, it is the limitation of this study that we did not assess the values that affect the performance of HA filler, including elastic modulus ($G'$), viscous modulus ($G''$), degree of cross-linking and percentage of free HA. Further related human study is needed to determine the different characteristics of monophasic and biphasic HA fillers.

\section*{References}
\begin{enumerate}
  \item Prager W, Steinkraus V. A prospective, rater-blind, randomized comparison of the effectiveness and tolerability of Belotero (R) Basic versus Restylane (R) for correction of nasolabial folds. \textit{Eur J Dermatol} 2010; 20: 748–752.
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