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Biocompatibility of Hyaluronic Acid Hydrogels Prepared by Porous Hyaluronic Acid Microbeads

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Hyaluronic acid hydrogels (HAHs) were synthesized by immersing HA microbeads crosslinked with divinyl sulfone in a phosphate buffered saline solution to evaluate the biocompatibility of the gels by means of cytotoxicity, genotoxicity (*in vitro* chromosome aberration test, reverse mutation assay, and *in vivo* micronucleus test), skin sensitization, and intradermal reactivity. The HAHs induced no cytotoxicity or genotoxicity. In guinea pigs treated with grafts and prostheses, no animals died and there were no abnormal clinical signs. The sensitization scores were zero in all guinea pigs after 24 h and 48 h challenge, suggesting that the HAHs had no contact allergic sensitization in the guinea pig maximization test. No abnormal signs were found in New Zealand White rabbits during the 72 h observation period after the injection. There was no difference between the HAHs and negative control mean scores because skin reaction such as erythema or oedema was not observed after injection. Experimental results suggest that the HAHs would be suitable for soft tissue augmentation due to the absence of cytotoxicity, genotoxicity, skin sensitization, and intradermal reactivity.

Key words: biomaterials, porous materials, chemical synthesis, hyaluronic acid hydrogel

1. INTRODUCTION

Hydrogels have attracted attention as injectable fillers for repairing and regenerating a wide variety of tissues and organs due to their regeneration properties in various tissues, mechanical properties, softness, oxygen permeability, similarities with the body's highly hydrated composition, and excellent biocompatibilities [1-3]. The efficacy of injectable hydrogels has been demonstrated in minimal invasive surgery to correct aesthetic defects due to posttraumatic tissue defects, congenital malformation, and aging. They are known to be highly swollen, but the insoluble porous networks can be used to entrap biological body fluids, cells, and medicine. It is noted that higher equilibrium swelling promotes nutrient diffusion into the gel and cellular waste removal out of the gel, while their insolubility provides the structural integrity necessary for tissue growth [3-10].

Among biocompatible natural polymers, such as collagen, gelatin, fibrin, alginic acid, chitosan, and hyaluronic acid (HA), crosslink-stabilized HA is recognized as a naturally derived injectable filler due to its longevity of correction, reduced risk of immunogenicity and hypersensitivity, and controllable mechanical and degradation properties [7-14]. HA should be persistent in the body as a host cell without adverse effects such as immunological reaction or inflammatory response at approximate cell of the injected or implanted site. In addition, no carcinogenicity or mutagenicity should be detected during the degradation process. The tissue scaffold should possess certain mechanical properties to ensure appropriate space and structure during tissue regeneration. Cytotropism is also needed for cell proliferation and migration, tissue cell adhesion, and differentiation for regeneration. The scaffolds can be substituted by natural tissue cells after secretion of the extracellular matrix of cells during cell proliferation and growth.

HA is a linear polysaccharide formed from disaccharide units containing *N*-acetyl-*D*-glucosamine and glucuronic acid [8,15]. The HA molecules are stabilized to produce a crosslinked gel suitable for soft tissue implantation, providing improved resistance to enzymatic degradation within the dermis without compromising biocompatibility. HA hydrogels (HAH) were prepared as shown in Fig. 1 [8]. The as-dried microbeads were immersed in a phosphate buffered saline solution (PBS, NaH_2PO_4) to obtain HAHs (Fig. 2). HAHs are reported to be biologically inert and non-allergic and do not require allergy

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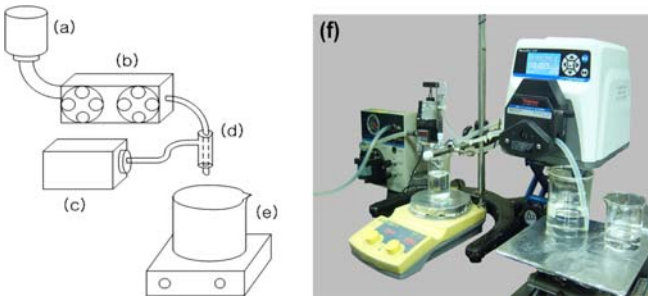

Fig. 1. A schematic diagram (a-e) and a photograph (f) of the HAH fabrication apparatus. (a)~(e) are the HA solution hopper, automatic dispenser pump, air pump, delivery line enclosed within a spray nozzle system, and a collector with a stirrer, respectively.

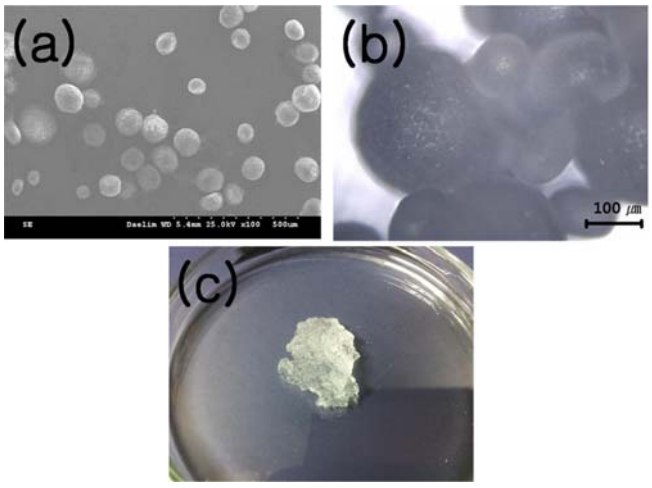

Fig. 2. Photographs of (a) HA microbeads, (b) hydrated beads, and (c) HAHs.

testing before implantation. Herein, we investigate the genotoxicity of a HAH because it is used within the dermis for several months after injection [5]. The cytotoxicity, genotoxicity, skin sensitization, and intradermal reactivity are investigated to assess the biocompatibility of the hydrogels.

2. EXPERIMENTAL PROCEDURES

2.1. Materials

HA solutions of 0.4 wt% concentration were prepared by dissolving sodium hyaluronate (Mw = 1×10^6 Da, Shiseido Co., Japan) in 0.05 mol/L NaOH at room temperature. pH in a range of 11 to 12 was achieved by adding 10 mol/L NaOH to the HA solution. The HA solution was then placed in the solution hopper attached to the syringe pump and fed into the delivery tube at a flow rate of 0.005 mL/min. Microbeads were collected from a solution mixture of divinyl sulfone (DVS, ≥98%, Sigma and Aldrich, Germany) and 2-methyl-1-propanol (99%, Aldrich), followed by a stirring process (200~400 rpm) for 24 h at room temperature. The crosslinked microbeads were then immersed in ethanol for 0.5 h to clean the beads by removing impurities such as DVS and 2-methyl-1-propanol. After 3 iterations of cleaning in ethanol, the microbeads were dried for 2 h at 60 °C under vacuum (20 torr). The as-dried microbeads were immersed in phosphate buffered saline solution (PBS, NaH_2PO_4) for 24 h to obtain HAHs (Fig. 2). The HAHs were then provided in a 1 mL sterilized syringe with a Luer-Lok cap, followed by autoclaving for 0.5 h at 130 °C. The presence of DVS after cleaning may cause adverse, allergic reactions and potential noxiousness of the HAHs because they are used within the dermis for several months. The presence of the unreacted residual crosslinker in HAHs is evaluated by using gas chromatography (GC, Agilent, HP6890N, USA). Four standard stock solutions of 10 mg/L, 250 mg/L, 500 mg/L, and 1,000 mg/L were prepared.

2.2. Biocompatibility

2.2.1. Cytotoxicity

The extract test method was conducted on the HAHs to evaluate the potential of cytotoxicity on the basis of the International Organization for Standardization (ISO 10993-5). The HAHs were extracted aseptically in single strength Minimum Essential Medium (1X MEM) with serum. The ratio of HAH to extraction vehicle was 4 g/20 mL. The test sample was used within 24 h after completion of the preparation. The test extract was placed onto three separate confluent monolayers of L-929 (NCTC Clone 929, ATCC, USA) mouse fibroblast cells propagated in 5% CO_2. For this test, confluent monolayer cells were trypsinized and seeded in 10 cm^2 wells (35 mm dishes). Simultaneously, triplicates of reagent control, negative control, and positive control were placed onto the confluent L-929 monolayers. All monolayers were incubated for 48 h at 37 °C in the presence of 5% CO_2. After incubation, the morphological change of the cell was examined to assess the biological reaction.

2.3. Genotoxicity

2.3.1. Chromosome aberration test (CAT)

CAT of the HAHs was carried out to evaluate their clastogenic potential in Chinese Hamster Lung fibroblast (CHL) cell line in the presence and absence of a metabolic activation system [16,17]. The cells were cultured in minimum essential medium (MEM, GIBCO BRL, Lot No. 1408867) supplemented with 1.5 mL of antibiotics (penicillin G sodium 10,000 units/mL, streptomycin sulface 10,000 mg/mL, amphotericin B 25 mg/mL in 0.85% saline, GIBCO BRL, Lot No. 1386464), and 55.5 mL of 10 vol% fetal bovine serum (FBS, GIBCO BRL, Lot No. 1276462) per 500 mL. During routine culture maintenance, cells were grown as a monolayer in T-75 culture flasks (TRP Co.) and were incubated for 2~3 days at 37 °C in a 5% CO_2 atmosphere. Each 0.9 mL of cell suspension was mixed with 0.1 mL of dimetyl sulfoxide and the mixture was kept in liquid nitrogen. Prior to the test, microbiological contamination of the cultured cells was checked.

The metabolic activation system (S-9 mix) consists of rat liver S-9 (Oriental Yeast Co., Lot No. 08011811) and cofactor-C (Oriental Yeast Co., Lot No. C08011611). Prior to CAT, a 3-(4,5 dimetylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay was performed [17]. Cell growth inhibition and lysis were examined, as given in the OECD test guidelines [16,17]. Cells were replaced by a new fresh medium and MTT (2 mg/mL) was added. After 4 h incubation, the absorbance was read at 540 nm. Cell viability determined by the MTT assay was over 82.6%. No cytotoxicity up to 1×extract in CHL cells was detected in the present study.

The 25 cm^2 culture flasks were seeded with 1×10^5 cells, each in 5 mL medium, and incubated for 1 day. The specimens were added to each flask. The composition of the treatment mixtures was as follows: the cells were treated with the 5 mL specimen without metabolic activation (groups 1 and 2 in Table 1) and were treated with the 4.5 mL specimen and 0.5 mL S-9 mix with metabolic activation (group 3). MMC and cyclophosphamide (CAP) were added to each culture at final concentrations of 0.1 and 5.0 mg/mL. The group 1 and 3 cultures were treated with the HAHs for 6 h and washed once with 5 mL of MEM and re-incubated in 5 mL of fresh medium for 18 h. Group 2 cultures were treated with the HAHs for 24 h without washing. Pure extracts and 0, 1, ½, and 1/4 dilutions were added to cells. Approximately 22 h after treatment, colcemid (GIBCO BRL, Lot No. 1354870) was added to each culture at a final concentration of 0.2 μg/mL and the cultures were incubated for an additional 2 h.

The number of cells with chromosomal aberrations was counted on 200 well-spread metaphases per concentration and control equally divided amongst the duplicates. The recorded structural aberrations recorded were as follows: chromatid and chromosome gaps, chromatid type deletions and exchanges, and chromosome type deletion and exchanges. Each slide was scanned systematically, and each set of metaphase was examined with 1000× magnification. The chromosome number of each metaphase was counted and recorded. A hundred metaphases that were well spread and had a chromosome count between 23~27 were evaluated for aberrations. The microscope state coordinates were recorded for each aberrant metaphase. Each type of aberration was recorded and the number of aberrant metaphases (showing one or more aberrations, including/excluding gaps) and total aberrations (including/excluding gaps) were calculated. The results were expressed as number of findings per 100 metaphases. Regardless of the presence of aberration, and an additional 100 metaphases were examined to determine the frequency of diploid (DP, 23~26 chromosome), polyploidy (PP, 37≤chromosome), and endoreduplication (ER). Any metaphase with one or more aberrant, regardless of its type, was classified as an aberrant metaphase.

2.3.2. Reverse mutation assay (RMA)

Mutagenic potential of the extract in the bacteria reverse mutation assay system using a pre-incubation assay was evaluated. Details of the experiment have been described elsewhere [18-20]. Four strains of *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and one strain of *Escherichia coli*, WP2*uvr*A, were obtained from MOLTOX™ *Salomonella* Mutagenicity Assay Kits (STDiscs™, ECDiscs™) [19,20]. Different positive control substances were used for each strain, as summarized in Table 2.

A culture medium was prepared with 0.8 g of Nutrient Broth (NB, Becton Dickinson Co., Lot No. 4092047) and 0.5 g of NaCl (Sigma Co.) per 100 mL distilled water and autoclaved. Each 25 mL of NB was inoculated with STDiscs™ and ECDiscs™ tester strains and incubated in a 37 °C gyratory incubator with a speed of 180 rpm for 10~13 h. Ampicillin was added to a final concentration of 25 mg/mL (TA98, TA 100).

Table 1. The composition of treatment mixtures

G[1]	S-9 mix	Test article	Treat time (h)	Concentration (μg/mL in media)	No[2]
		MEM[a]	6	-	2
		HAH	6	1/1×diluents	2
1	w/o[3]	HAH	6	1/2×diluents	2
		HAH	6	1/4×diluents	2
		MMC[b]	6	0.1	2
		MEM	24	-	2
		HAH	24	1/1×diluents	2
2	w/o	HAH	24	1/2×diluents	2
		HAH	24	1/4×diluents	2
		MMC	24	0.1	2
		MEM	6	-	2
		HAH	6	1/1×diluents	2
3	with	HAH	6	1/2×diluents	2
		HAH	6	1/4×diluents	2
		CPA[c]	6	5.0	2

[1]test group, [2]No. of flasks, [3]without
[a]minimum essential medium (MEM), [b]mitomycin C (MMC), [c]cyclophosphamide (CPA)

Table 2. Types and concentrations of positive control substances for each test strain

Strain	Positive control substance (μg/100 μL/plate)	
	Without activation	with activation
TA98	2-nitrofluorene (10.0)	2-aminoanthracene (2.0)
TA100	sodium azide (5.0)	2-aminoanthracene (2.0)
WP2*uvr*A	mitomycin C (0.5)	2-aminoanthracene (5.0)
TA1535	sodium azide (0.5)	2-aminoanthracene (5.0)
TA1537	9-aminoacridine (80.0)	2-aminoanthracene (5.0)

The S-9 and cofactor-A were thawed in a water bath and mixed to a ratio of one to nine just before use and kept in an ice bath. RMA was performed with a maximum dosage of 1× extract of the HAH. The extract was diluted to 5 dosage levels with normal saline. Each tester strain was exposed to extracts with and without the metabolic activation system. Mutagenicity was then assayed by the method described by Maron and Ames [20]. The 12×75 mm sterile tubes were maintained at room temperature and the following were added to the tubes: 0.1 mL of HAH, 0.5 mL of S-9 mix or PB (sodium-phosphate buffer, pH 7.4), and 0.1 mL of bacterial culture. This mixture was pre-incubated in a 37 °C gyratory incubator (180 rpm) for 30 min. Two mL of molten top agar maintained at 43 °C or 45 °C was added to each tube. The contents of test tubes were then mixed and poured onto a minimal glucose agar plate. All assays were carried out in triplicate. After the top agar was hardened, the plates were inverted and placed in a 37 °C incubator for 48 h. After incubation, the revertant colonies were counted and the results were expressed as the number of revertant colonies per plate. The formation of a background lawn and other abnormalities was examined by using a microscope. Vehicle (0.1 mL) and S-9 mix (0.1 mL) without bacterial culture were poured with top agar onto minimal glucose agar and cultured at 37 °C for 48 h to check the sterility.

2.3.3. Micronucleus test in ICR mice

An *in vivo* micronucleus test in ICR mice was performed to evaluate the genotoxic potency according to the OECD test guideline No. 474 [21]. Five mice (8 week old, male) of each group were intraperitoneally administered at volumes of 50, 25, and 12.5 mL/kg body weight (BW) for 1 day. The negative control group was supplied with saline (50 mL/kg) and the positive control group with MMC at a dosage level of 2 mg/kg BW. All animals were sacrificed at 16, 24, 48, and 72 h after the final treatment and slides were prepared. Two-thousand polychromatic erythrocytes (PCE) per animal were evaluated for the presence of micronucleated polychromatic erythrocytes (MNPCE).

Animals were weighed and sacrificed by cervical dislocation and bone marrow preparations were made. Bone marrow cells were collected in 3 mL of fetal bovine serum (Gibco, BRL), centrifuged, and smeared on a slide. Preparations were dried and fixed by submerging in absolute methanol. Fixed slides were stained with Giemsa solution. Four slides per mouse were made, randomly coded, and examined under 1000× magnification. Results were expressed as the number of MNPCEs in 2000 PCEs and the mean values ± standard deviation for each group of samples. In addition, the PCE to total erythrocytes (PCE+normochromatic erythrocytes (NCE)) ratio, which is an indicator of cytotoxicity of bone marrow cells, was calculated by counting 1000 cells. Round or oval shaped bodies, the size of which ranged from approximately 1/5 to 1/20 of the diameter of PCE, were counted as micronuclei. Tests for differences of PCE/(PCE+NCE) ratio and the changes of BW in mice were carried out using a one-way analysis of variance (ANOVA). Statistical significance was considered at a probability $p < 0.05$.

2.4. Skin sensitization test

The maximization test for delayed hypersensitivity was conducted on the HAHs to evaluate the skin sensitizing potency on the basis of ISO 10993-10 [22]. The HAHs and a sterile physiological saline (Table 3) were used as a treated group and a negative control group, respectively. They were injected intradermally on the back of guinea pigs (Dunkin Hartley, Samtako Bio, Korea) to examine the skin sensitization. One day before the test, 0.1 mL of HAHs was injected intradermally on the back of the animal (Table 4, Fig. 3). Six days after the intradermal injection (second induction phase (7 day)), the same area used during the first induction phase was clipped free of fur and treated with 10% sodium lauryl sulfate/vaseline without covering. Then, 0.5 mL of HAHs, absorbed in gauze was attached to cover the intradermal induction site of each animal, and held using an elastic adhesive bandage for 48 h. The dressing and gauze were removed 48 hours after the occlusive contact and the HAHs were not wiped off because the application sites of the HAHs were clean. On the day prior to the challenge patch (challenge phase (21 day)), the fur was removed from the sides and flank areas. At 14 days after completion of the topical induction phase, all animals were wrapped with an elastic bandage to maintain well occluded sites for 24 h exposure. The sites were wiped gently with gauze after the patch removal.

Clinical signs, survival, and body weight of all animals were monitored during the experimental period. Skin reaction was performed by observing the appearance of the challenge skin sites 24 and 48 h after removal of the dressings. The skin reactions were described and graded for erythema and oedema

Table 3. Experimental design

Group	Animal No.	Test solutions	
		Induction phase	Challenge phase
G1 treatment	1-10	HAH	HAH
G2 negative control	11-15	Physiological saline	HAH

Table 4. Test article for the intradermal induction

Group	Animal No.	Intradermal injection solution	Volume (mL)	Injection site
Treatment (Saline)	1~10	physiological saline: FCA(1:1)	0.1	①
		physiological saline: FCA(1:1)	0.1	①
		HA hydrogel	0.1	②
		HA hydrogel	0.1	②
		HA hydrogel: FCA(1:1)	0.1	③
		HA hydrogel: FCA(1:1)	0.1	③
Negative control (Saline)	11~15	physiological saline: FCA(1:1)	0.1	①
		physiological saline: FCA(1:1)	0.1	①
		Blank(physiological saline)	0.1	②
		Blank(physiological saline)	0.1	②
		Blank(physiological saline): FCA(1:1)	0.1	③
		Blank(physiological saline): FCA(1:1)	0.1	③

FCA: Freund's Complete Adjuvant

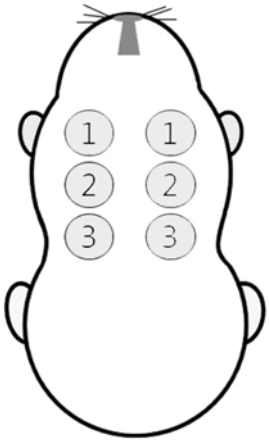

Fig. 3. Schematic diagram of the injection sites.

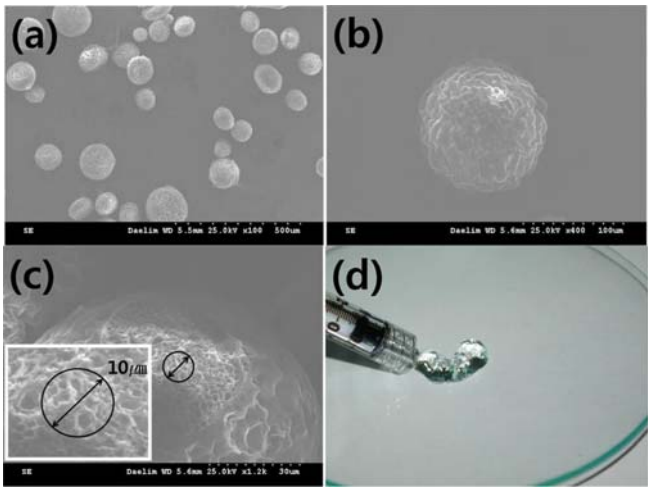

Fig. 4. SEM images of (a-c) porous microbeads and (d) hydrogel.

according to the grading given in the classification system for each challenge site and at each time interval.

2.5. Intradermal reactivity test

The test was performed by screening the HAHs for potential irritation effects as a result of intracutaneous injection in two male New Zealand White rabbits (Samtako Bio, Korea), as given by ISO 10993-10 [23]. A 0.2 mL dose of the HAHs was injected by an intracutaneous route into five separate sites on the back of each rabbit. Similarly, the negative controls were injected. Observation for erythema and oedema was conducted at 24, 48, and 72 h after injection.

3. RESULTS AND DISCUSSION

Morphologically the microbeads were white colored spheres having a smooth surface, as depicted in Fig. 4. Although no pores on the surface of the microbeads were visible, pores inside the microbeads were easily seen. The microbeads were a 3-dimensional (3-D) porous network structure channeled with 2~3 μm pores, as shown in Fig. 4(c). The HAHs (Fig. 4(d)) crosslinked with DVS were prepared by immersing the microbeads in a PBS solution [15]. Covalent linkages between polymer chains were obtained by the reaction of functional groups of a crosslinking agent (vinyl group, CH_2:CH-) and HA (hydroxyl group, -OH). It is known that the porous network can be adjusted by the concentration of the dissolved polymer and the amount of crosslinking agent [14].

Microbeads were crosslinked with DVS, followed by cleaning in distilled water and ethanol. The microbeads were then immersed in PBS solution for 2 h to obtain HAHs. The presence of DVS after cleaning may have adverse effects and causes allergic reactions to the HAHs because they are used within the dermis for several months [15]. The presence of the crosslinker's residue in the HAHs after cleaning was evaluated by using a GC, as shown in Fig. 5. No peak of DVS was detected, suggesting that the crosslinker was successfully removed.

A cytotoxicity test determines whether a product or compound will have a toxic effect on living cells. The confluence of the monolayer was recorded as (+) if present and (–) if

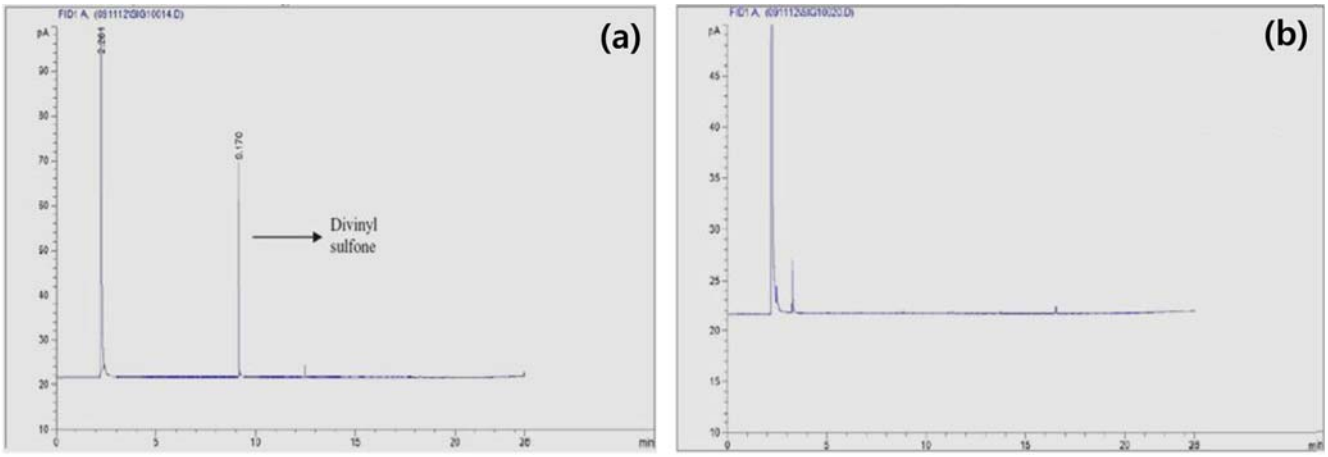

Fig. 5. GC graphs of (a) standard solution of the divinyl sulfone and (b) the HAHs. Note that the HAHs were crosslinked by divinyl sulfone for 24 h.

Table 5. Results of L-929 cytotoxicity test

Well	Confluent Monolayer	% Cells Detached	% Cells Without Intracellular Granulation	% Rounding	% Lysis	Reactivity	Cytotoxicity Scale
Test	(+)	0	0	0	0	None	0
Reagent	(+)	0	0	0	0	None	0
Negative	(+)	0	0	0	0	None	0
Positive	(-)	90	5	N/A	N/A	Severe	3

(+) : present, (-) : absent, N/A : Not applicable

absent. Under the conditions of this study, the 1X MEM test extract showed no evidence of causing cell lysis or toxicity, as listed in Table 5. For the test to be valid (ISO 10993-5), the reagent control and the negative control must have no reactivity (cytotoxicity scale 0) and the positive control must be severely cytotoxic (cytotoxicity scale 3). The reagent control and negative control showed no cytotoxicity and the positivity control showed cytotoxicity in more than 75% cells, as expected. It was determined that the test sample had no reactivity. Therefore, the HAHs are likely to be suitable for soft tissue augmentation due to the lack of cytotoxicity.

CAT of HAHs was performed to evaluate its potential to induce clastogenicity in the CHL cell line in the presence and absence of the metabolic activation system [16,17]. The extracts were performed at a ratio of 4g per 20 mL of sterilized MEM at 37±2 ˚C for 24 h. From the MTT assay results no cytotoxicity up to 1× extract in CHL cells was detected and the treated cell viability was more than 82.6%. Cells were exposed to extracts with and without the metabolic activation system and appropriate negative and positive controls were included. The cells with chromosomal aberrations were counted on 200 well-spread metaphases per concentration. 100 metaphases that had a chromosome count between 23 and 27 were evaluated for aberrations. There was no statistically significant increase in the number of structural or numerical aberrant metaphases at any dose both in the presence and absence of S-9 mix. The HAHs were determined to be negative in CAT, suggesting that CA does not induce clastogenicity.

RMA of the extracts of HAHs was performed to evaluate its potential to induce reverse mutation in the auxotroph strains of TA98, TA100, TA1535, TA1537, and WP*2uvr*A by using a pre-incubation method [18-20]. Cytotoxicity of the HAH extracts was not observed in the TA100 strain. RMA was performed with a maximum dosage of 1× extract of HAHs. The extracts were diluted to 5 dosage levels (1, 1/2, 1/4, 1/8, 1/16) with normal saline. Each tester strain was exposed to extracts with and without the metabolic activation system. Appropriate negative and positive controls were included. No contamination of the extracts and S-9 mix was detected. They showed no thinning of complete absence of the background lawn compared to the negative control (saline). No significant change in the number of revertant colonies at all tested strains compared with the negative control (saline) was observed, as shown in Fig. 6. In addition, the number of revertant colonies per plate at all tested strains compared with the positive control (Table 2) was significantly higher than those of strains compared with saline. This observation revealed that the HAHs were negative in bacterial RMA, suggesting that the HAHs had no mutagenicity.

A micronucleus test in bone marrow cells of male ICR mice was performed to evaluate the *in vivo* genotoxic potential of the HAH extracts [21]. Two-thousand PCEs per ani-

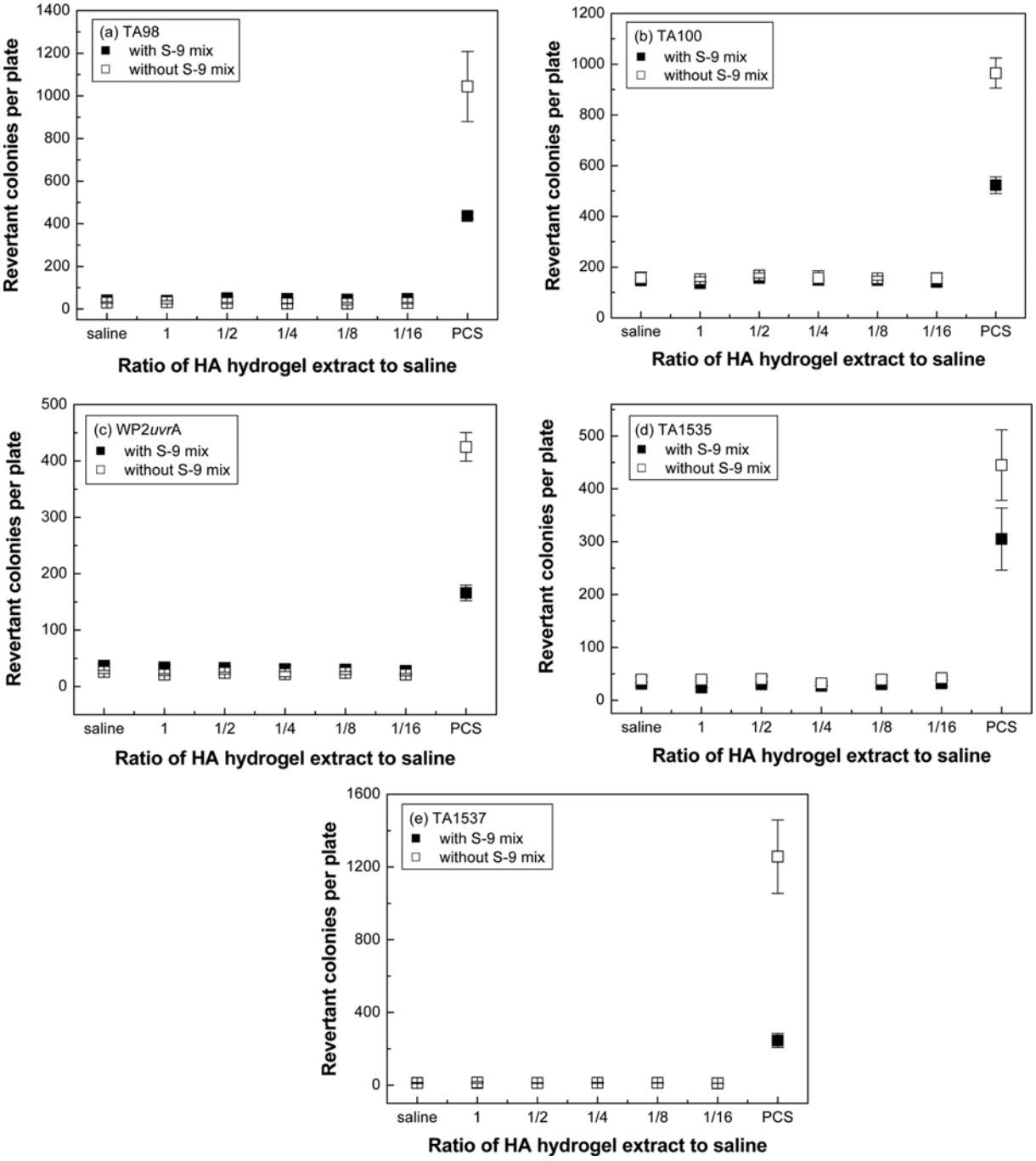

Fig. 6. Reverse mutation assays with HAH extracts diluted with saline. Note that PCS denotes the positive control substance. Each test strain exposed to extracts with and without S-9 mix ((a) TA98, (b) TA100, (c) WP2*uvr*A, (d) TA1535, and (e) TA1537) used different PCSs, as listed in Table 2.

mal were evaluated for the presence of MNPCE. It was clear that there were no effects on mortality, clinical signs, and BW changes. No statistically significant increase was found in the frequency of MNPCEs at any treated groups compared with the negative control group, as shown in Fig. 7. In addition, there was no significant difference in the ratio of PCE/(PCE+NCE), which is an indicator of cytotoxicity, in any of the treated groups.

The ratio of PCE/(PCE+NCE) and MNPCE/2000PCE was examined and the results are shown in Fig. 8. Test items were intraperitoneally administered at volumes of 50, 25, and 12.5 mL/kg BW. The maximum dosage volume of the test item was 50 mL/kg BW. The positive control was administered with MMC at a dosage level of 2 mg/kg BW. No test item-related

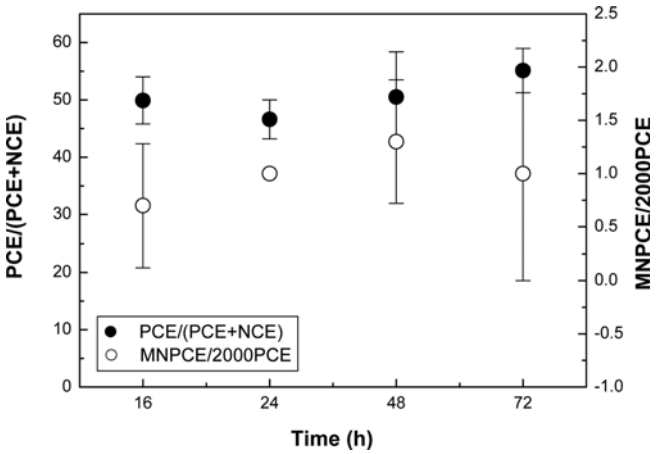

Fig. 7. PCE/(PCE+NCE) and MNPCE/2000PCE of micronucleus test in mice intraperitoneally treated with HA gels as a function of sampling time.

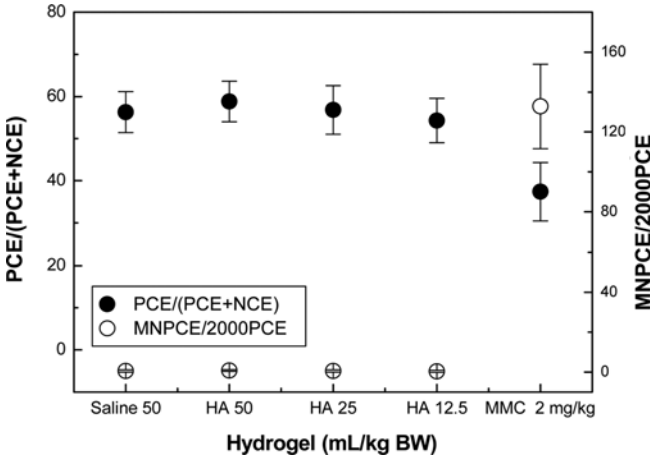

Fig. 8. PCE/(PCE+NCE) and MNPCE/2000PCE of micronucleus test in mice intraperitoneally treated with test item. Note that saline 50 and HA 50 indicate saline 50 mL/kg BW and hyaluronic acid gel 50 mL/kg BW, respectively.

change on BW was monitored as compared with the negative control. The ratio of PCE/(PCE+NCE) and MNPCE/2000PCE against the negative control remained constant, as depicted in Fig. 7. However, a significant difference of the PCE/(PCE+NCE) and MNPCE/2000PCE ratio against the negative and positive controls was observed. Therefore, it is conceivable that HAHs do not induce any genotoxicity in the micronucleus test using bone marrow cells of male ICR mice.

3.1. Skin sensitization test

The appearance of the challenge skin sites 24 and 48 h after removal of the dressings was observed [22]. The skin reactions were described and graded for erythema and oedema according to the grading given in classification system for each challenge site. Grades of 1 or greater in the test group generally indicate sensitization, provided less than 1 is seen on control animals. If grades of 1 or greater are noted on control animals, then the reactions of test animals that exceed the most severe control reaction are presumed to be due to sensitization. Numerical grading scales for the evaluation of skin reaction, 0, 1, 2, and 3, indicate no visible change, discrete or patchy erythema, moderate and confluent erythema, and intense erythema and swelling, respectively. In guinea pigs treated with grafts and prostheses, no animals died and no abnormal clinical signs were detected. The extracts did not induce any allergic reactions. The sensitization scores were zero in all guinea pigs and the sensitization rates were also observed 0% at 24 h after challenge, as listed in Table 6. The results observed 48 h after challenge were the same, suggesting that the HAHs had no contact allergic sensitization in the guinea pig maximization test.

3.2. Intracutaneous(intradermal) reactivity test

No abnormal signs were observed in any animals during the 72 h observation period after the injection [23]. There were no differences between the body weight of individual animals on the day of injection and those on the last day of injection and those on the last day of the observation period. The difference between the HAH and negative control mean scores was 0 because skin reactions such as erythema and oedema were not observed after injection in this test, indicating that the HAHs were not reactive materials.

4. CONCLUSIONS

HAHs were assessed to evaluate their biocompatibility by means of cytotoxicity, genotoxicity, skin sensitization, and intradermal reactivity. They were covalently bonded by the reaction of functional groups of DVS and HA. HA microbeads formed a 3-D porous network structure channeled with 2~3 μm pores. The extract test method was conducted on the HAHs to evaluate the potential of cytotoxicity. The reagent control and negative control showed no cytotoxicity. Genotoxicity (*in vitro* CAT, RMA, and *in vivo* micronucleus test)

Table 6. Evaluation of skin response

Group	Observation	Mean score	Sensitization rate (%)	Number of animal									
				1	2	3	4	5	6	7	8	9	10
Treatment	24 h	0	0	0	0	0	0	0	0	0	0	0	0
	48 h	0	0	0	0	0	0	0	0	0	0	0	0
Negative Control	24 h	0	0	0	0	0	0	0	-	-	-	-	-
	48 h	0	0	0	0	0	0	0	-	-	-	-	-

was examined to evaluate the long-term toxicity. The HAHs induced no genotoxicity. In addition, no skin reactions were observed in guinea pigs and no intracutaneous reaction was detected in New Zealand White rabbits after injection. Therefore, the HAHs possessing a 3-D porous network structure and excellent biocompatibility could be applicable to scaffolds and drug (cell) delivery carriers for tissue engineering.

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