Production of a composite hyaluronic acid/gelatin blood plasma gel for hydrogel-based adipose tissue engineering applications

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Abstract: Standard approaches to soft-tissue reconstruction include autologous adipose tissue transplantation, but most of the transferred adipose tissue is generally reabsorbed in a short time. To overcome this problem, long lasting implantable hydrogel materials that can support tissue regeneration must be produced. The purpose of this study was to evaluate the suitability of composite 3D natural origin scaffolds for reconstructive surgery applications through in vitro tests. The Young’s modulus of the glutaraldehyde crosslinked hyaluronic acid/gelatin (HA/G) plasma gels, composed of human platelet-poor plasma, gelatin and human umbilical cord hyaluronic acid, was determined as 3.5 kPa, close to that of soft tissues. The composite HA/G plasma gels had higher porosity than plain plasma gels (72.5% vs. 63.86%). Human adipose tissue derived stem cells (AD-MSCs) were isolated from human lipoaspirates and characterized with flow cytometry, and osteogenic and adipogenic differentiation. Cell proliferation assay of AD-MSCs on the HA/G plasma gels revealed the nontoxic nature of these constructs. Adipogenic differentiation was distinctly better on HA/G plasma gels than on plain plasma gels. The results showed that the HA/G plasma gel with its suitable pore size, mechanical properties and excellent cell growth and adipogenesis supporting properties can serve as a useful scaffold for adipose tissue engineering applications.


INTRODUCTION
Adipose tissue substitutes are required in reconstructive and plastic surgery. Adipose tissue is used for tissue reconstruction following a mastectomy and other tumor resections, posttraumatic defect reconstruction (especially burns), treatment of congenital abnormalities and augmentation of breast, cheek and chin.1–3 A source of excess adipose tissue is available from almost every individual. Therefore, autologous fat grafts would appear to be optimal for the restoration of soft tissue volume.3 However, autologous adipose tissue remains minimally effective due to insufficient neovascularization. In the long term, this leads to necrosis and apoptosis in free fat grafts and resultant tissue resorption over time.4 The attenuation of the fat graft requires repeated surgery.1

In tissue engineering applications, cell seeding with growth factors and proteins is accomplished on 3D porous scaffold and after that scaffolds are transplanted to recipient in order to repair functions of tissues or organs.5 Because lots of cells are needed for production of a tissue equivalent, stem cells have drawn attention due to their ability to proliferate easily in the laboratory and to differentiate into many cell types. The MSCs can decrease and prevent cell death and speed up the angiogenesis in the damaged tissues by secreting growth factors and cytokines, can differentiate into cells of the target tissue and can suppress immune rejection even in their allogeneic use.6,7 Adipose tissue-derived stem cells (AD-MSCs) are used in many soft tissue engineering applications. The most important advantage of AD-MSCs is that they can be obtained easily from standard lipoaspirate materials; they are found in high frequency in human adipose tissue.8

The niche (microenvironment) has a crucial effect on commitment of stem cells into the differentiation or self-renewal pathways. The scaffolds are mimicking the cell niche, so various biomaterial types are being tested in adipose tissue engineering. The natural polymers such as gelatin, collagen, silk, fibrin and agarose have been used as biomaterials for long time.9 These materials are biocompatible and available commercially for tissue engineering applications.10 The artificial extracellular matrix (ECM), in other words the scaffold, should be suitable for cell attachment, migration, proliferation and contribute to regeneration of the target tissue.11 The physical properties of the hydrogels...
are largely similar to living tissues. The similarities include high water content, softness, flexible structure and low interfacial tension with water or biological fluids.\textsuperscript{12} Hyaluronic acid-based hydrogels support cell attachment, proliferation, and expansion.\textsuperscript{13} Fibrin is widely used with other polymers to form composites because of its biocompatibility and easiness of manufacturing with desired properties.\textsuperscript{13} In comparison to other materials used for tissue engineering purposes, fibrin gel has advantages such as possibility of autologous production and controllable degradation rate.\textsuperscript{14} However, in use of allogeneic plasma to manufacture the fibrin gel, immunogenic reactions against foreign materials shall be reduced.\textsuperscript{14}

In the present study we have explored the effect of scaffolds from naturally derived materials, namely, HA/G blood plasma gels and plain plasma gels on AD-MSC viability, proliferation, and adipogenic differentiation. Fibrinogen was precipitated from human platelet-poor plasma and hyaluronic acid isolated from human umbilical cords. Composite gels of fibrinogen with gelatin and hyaluronic acid (HA/G plasma gels) were produced afterwards. HA/G plasma gel characterization was made in comparison to plain plasma gels. Gels with higher porosity and pore size were obtained upon addition of gelatin and hyaluronic acid to the fibrinogen solution and young modulus of the gels was improved. The HA/G plasma gels supported AD-MSC attachment and proliferation and their porous structure was preserved at the end of 21-day culture. Adipogenic differentiation potential of AD-MSCs on HA/G plasma gels and plain plasma gels was investigated; lipid vesicle accumulation was more pronounced on the HA/G plasma gels, even in the absence of differentiation factors.

**MATERIALS AND METHODS**

**AD-MSC isolation and expansion**

Adipose tissue was obtained by lipoaspiration of subcutaneous fat of the abdominal region from female donors. The donors consented with the procedure and agreed with further research of aspirated fat in accordance with the ethical guidelines of the Kocaeli University Medical Ethics Committee. Fatty portions of liposaprate samples were collected with pipette and digested with 0.1% (w/v) collagenase (Gibco) at 37°C for 60 min in a shaking water bath. After the digestion process, cell suspension was centrifuged at 1500 rpm for 10 min. The pellet was resuspended in the phosphate-buffered saline (PBS, Gibco) for washing and centrifuged at 1800 rpm for 10 min. This washing process was repeated twice. The pellet was resuspended in 5 mL basal medium (DMEM/F12 (Gibco) containing 10% fetal bovine serum (FBS; Invitrogen/ Gibco), 1% ν-Glutamine, 1 ng mL\textsuperscript{-1} bFGF (Gibco) and 0.1% primocin (Invirogen) and filtered through 100-μm nylon mesh (BD Biosciences). The isolated adipose derived cells were plated in a 25 cm\textsuperscript{2} tissue culture flask (BD Biosciences) and were cultured in a humidified atmosphere of 5% CO\textsubscript{2} at 37°C. The cells were detached by using 0.25% trypsin-EDTA solution (Sigma) when 90% confluence was obtained. First-passage (P1) AD-MSCs were plated in tissue culture flask at 1 × 10\textsuperscript{5} cells cm\textsuperscript{-2} and cultured in the basal medium. When the cells were 80% confluent, they were passaged (P2). Medium was changed twice per week.

**Characterization of AD-MSCs**

The adipose tissue derived stem cells were characterized with flow cytometry at the third passage and also were differentiated into osteogenic and adipogenic lineages. Flow cytometry was performed using a FACS Calibur (BD Biosciences, San Diego, USA). The data were analyzed with Cell Quest software (BD Biosciences) and the forward and side scatter profiles gated out of debris and dead cells. The surface antigens (CD13, CD29, CD44, CD90, CD146, CD166, HLA ABC, CD3, CD8, CD11b, CD14, CD15, CD19, CD33, CD34, CD45, CD117, and HLA-DR) of the cells were characterized with flow cytometry, and osteogenic and adipogenic differentiation was performed as standard protocols.

**In vitro osteogenic differentiation**

Cells from passage three were seeded onto six-well plates (3000 cells cm\textsuperscript{-2}) in which type I collagen-coated coverslips were placed. The osteogenic medium was composed of MEM (Invitrogen/GIBCO) supplemented with 10 nM dexamethasone (Sigma-Aldrich), 50 μg mL\textsuperscript{-1} ascorbate-2-phosphate (Wako Chemicals), 10 mM β-glycerophosphate (Sigma-Aldrich), 0.1% primocin (Invivo gen) and 10% FBS (Invitrogen/GIBCO). The cells were cultured for 2 weeks in the osteogenic medium; the medium in the wells was replaced twice a week. At the end of the second week, osteogenic differentiation was assessed by staining with Alizarin Red (Sigma-Aldrich).

**In vitro adipogenic differentiation**

To induce adipogenic differentiation cells from passage three were seeded (3000 cells cm\textsuperscript{-2}) on fibronectin coated coverslips (BD Biosciences) in six-well plates. The adipogenic medium composed of MEM (Invitrogen/GIBCO) supplemented with 10% FBS (Invitrogen/GIBCO), 0.5 mM isobutyl-methyl xanthine (IBMX-Sigma-Aldrich), 10\textsuperscript{-6} M dexamethasone (Sigma-Aldrich), 10 μg mL\textsuperscript{-1} insulin (Invitrogen/GIBCO), 200 μM indomethacin (Sigma-Aldrich), and 0.1% primocin (Invivo gen) was added on the cells for a culture period of 2 weeks. The medium was replaced twice a week. Intracellular lipid vesicles indicating adipogenic differentiation were confirmed by Oil Red O (Sigma-Aldrich) staining.

**Hyaluronic acid isolation from umbilical cords**

Hyaluronic acid isolation from umbilical cords was performed according to Lago et al.\textsuperscript{14} Briefly, human umbilical cord residues (1 kg) were divided into small pieces (4 mm × 4 mm) and treated with PBS at 4°C overnight. The day after, the tissue pieces were treated with 4L 0.2% sodium chloride solution. The liquid part was collected and treated with 300–600 mL of 1% cetyltrimethylammonium bromide (CTAB) solution and hyaluronic acid aggregations were formed. The obtained precipitate was transferred into 0.9M calcium chloride solution to dissociate the HA-CTAB.
complex. The suspension was treated with 25% v/v aqueous ethanol; the nucleic acids were precipitated and separated by centrifugation (1000 g, 10 min). The resulting suspension was deproteinized four times through chloroform solvent extraction (10% of the total volume) performed every day. HA was precipitated by adding 75% v/v aqueous ethanol and acetone.

Characterization of umbilical cord derived hyaluronic acid
Nearly 1% umbilical cord derived HA solution was prepared and the percentage of nucleic acid and protein contamination was determined by measurement of absorbance at 260 and 280 nm with a pico-drop spectrophotometer. The hyaluronic acid sodium salt from rooster comb (>98% purity, Sigma) was used as a reference standard. The protein content of the hyaluronic acid solutions was determined with BCA test (Sigma–Aldrich) too, for confirmation. The umbilical cord derived HA and the commercial hyaluronic acid sodium salt from rooster comb were dried on glass slides and stained with alician blue. About 1% alician blue solution in 3% acetic acid (Riedel-de Haen) was prepared and the hyaluronic acid samples were stained in this solution for 30 min. After extensive washing the images were taken under a light microscope.

Fibrinogen isolation from human blood plasma
The human blood plasma of healthy donors that was out of date for transfusion was collected from Kocaeli University Blood Bank. The precipitation of fibrinogen was carried out using a combination of ethanol and cold treatment previously described by Park et al.15 Briefly, absolute ethanol was added to blood plasma and this mixture of ethanol-blood plasma was stored at −20 °C for 20 min. Later, samples were centrifuged at 3200 rpm at 4 °C for 8 min. The pellet was resuspended in 0.9% NaCl and samples were stored at 37 °C for 15 min to dissolve the fibrinogen. The concentrated fibrinogen solution was aliquoted and stored at −20 °C.

Preparation of hyaluronic acid/gelatin plasma gels and plain plasma gels
Hyaluronic acid and gelatin were cross-linked using ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride/N-hydroxysuccinimide (EDC/NHS). 1% EDC and 0.2 M NHS were prepared in MES buffer. Nearly 500 μL of EDC/NHS solution was added to the 500 μL 1% hyaluronic acid solution and incubated for 30 min at room temperature. The 2-mercaptoethanol (Merck) was added to the sample solution to a final concentration of 0.14% to stop EDC binding. The 6% gelatin solution prepared in PBS with 150 mM NaCl was added to sample solution and incubated overnight (Final hyaluronic acid/gelatin ratio: 2/98 w/w). HA/G plasma gel was formed by mixing 120 μL concentrated human fibrinogen (480 mg mL−1), 30 μL crosslinked hyaluronic acid/gelatin solution and 30 μL 40 mM CaCl2 solution in each well of a 48-well plate. Later, 50 μL thrombin (40 U mL−1) was added to each well and mixed quickly by moving the pipette tip in the well. The mixture was stored at 37 °C for 2 h for polymerization. The plain plasma gel was prepared with concentrated human fibrinogen, water, 40 mM CaCl2 and thrombin (40 U mL−1). HA/G plasma gels were maintained in 1% (v/v) solution of gluteraldehyde (GA) at room temperature for 30 min for crosslinking. The scaffolds were washed in HBSS three times a day for 4 days. The crosslinked plain plasma gels were prepared the same way but water was used instead of the hyaluronic acid/gelatin solution.

Scanning electron microscopy
The porous and fibrous morphologies of HA/G plasma gels and plain plasma gels were observed under a Scanning Electron Microscope (SEM) model QUANTA 400F Field Emission. AD-MSC seeded HA/G plasma gels were fixed with 4% paraformaldehyde and washed with PBS. Later, the gels were frozen in −80 °C and lyophilized using Cool safe 110-4 (Labogen), and then grossly cut with a blade to observe with SEM. The gels were coated with 10 nm thick Au-Pd and were observed under low-vacuum conditions.

Mechanical test
Compression test of the gels (9.2 mm in diameter) was conducted with LR 30 K mechanical tester (Lloyd instruments) which can apply a force up to 2N in the compression range of 10% and 1 mm min−1 speed. Load was applied to gels until destruction. Three samples were tested from each gel group.

Measurement of gel porosity and pore size
A 10-mm-thick cryosection was obtained from HA/G plasma and plain plasma gels under the same freezing conditions. The cryosections were incubated with 4% paraformaldehyde in PBS at room temperature for 15 min to stabilize gels’ morphological features. The gels were stained with coomassie brilliant blue (Merck) to determine their porosity and pore size through microscopy. For preparation of the dye, 1:1 (v/v) methanol and distilled water were mixed. Glacial acetic acid (Riedel-de Haen) up to 10% of the solution and coomassie brilliant blue were mixed to obtain 0.25% dye solution and the final solution was filtered through a 0.45-μm membrane filter. The dye solution was applied on the crosslinked cryosections and incubated for 30 min. The cryosections were stored in mixture of water, methanol and acetic acid without dye for 30 min for destaining. Images were taken under a light microscope, and gel porosity and pore size were determined from these images by using the Scion Image program.

Cell viability and proliferation assay
The viability, adhesion and proliferation rate of AD-MSCs on HA/G plasma gels was determined by tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-1) test (Roche). AD-MSC suspensions were seeded at 7.5 × 104 cells cm−2 on HA/G plasma gels and coverslips in 48-well plates and were incubated in 5% CO2 atmosphere at 37 °C for 1, 5, 7, 14, and
21 days. The 1st day, culture medium was removed, the samples were washed twice with PBS and transferred into 10% WST-1 reagent containing plate. The plate was incubated for 2 h at 37°C in dark. The gels and coverslips without cells were used as blanks. Absorbance of the solution from the wells was measured at 480 nm with a UV-visible spectrophotometer (VersaMax, Molecular Device, USA). For each group, experiments were repeated three times and measurements were completed in triplicate. In the other days (5th, 7th, 14th, and 21st day) the above mentioned protocol was repeated.

Adipogenic differentiation of AD-MSCs on the gels
AD-MSCs were seeded at 2.5 × 10⁴ cell cm⁻² density on the HA/G plasma and plain plasma gels and when the cells reached 70% confluence, adipogenic differentiation was initiated with the adipogenic medium: MEM (Invitrogen/GIBCO) supplemented with 10% FBS (Invitrogen/GIBCO), 0.5 mM isobutyl-methylxanthine (IBMX-Sigma-Aldrich), 10⁻⁶ M dexamethasone (Sigma-Aldrich, Fluka Chemie AG, Buchs, Switzerland), 10 μg mL⁻¹ insulin (Invitrogen/GIBCO), 200 μM indomethacin (Sigma-Aldrich) and 0.1% primocin (Invivogen). The medium was replaced twice a week. At the end of 3-week culture, the presence of intracellular lipid vesicles, which indicate adipogenic differentiation was confirmed by Oil Red O (Sigma–Aldrich) staining with 0.5% Oil red 0 in methanol.

Statistical analysis
All experiments were repeated a minimum of three times. Data are reported as mean ± standard deviation (SD) and all statistical analyses were performed using SPSS 14.0. In addition, data were analyzed using the paired t test. Differences between the experimental and control groups were regarded as statistically significant when p < 0.05.

RESULTS
Isolation and characterization of human AD-MSCs
The mesenchymal stem cells isolated from human adipose tissue were used in this study. After 5 days following enzymatic isolation, the attached cells at the flask surface became fibroblast-like in morphology. The cells were cultured until 70% confluence and subcultured and cryopreserved for further characterization and use in the study. Defined markers exist that specifically identify MSCs. The flow cytometry data indicated that the adipose tissue derived third passage cells expressed CD13, CD29, CD44, CD90, CD146, and CD166, but not CD3, CD8, CD11b, CD14, CD15, CD19, CD33, CD34, CD45, CD117, or HLA-DR. These findings are consistent with the undifferentiated mesenchymal stem cell surface antigen profile, i.e., the cells possessed immunophenotypic characteristics of MSCs (Fig. 1).

Characterization of umbilical cord derived hyaluronic acid
About 640 mg hyaluronic acid was obtained from about 1 kg of human umbilical cord residues. A 1% umbilical cord derived HA solution was analyzed for nucleic acid and protein contamination through measurement of absorbance at 260 and 280 nm, respectively, with a pico-drop spectrophotometer. The hyaluronic acid sodium salt from rooster comb (> 98% purity, protein content of <2%, Sigma) was used as a reference standard. The rooster comb derived hyaluronic acid contained 16.7 ng μL⁻¹ double-stranded DNA and 0.2 mg mL⁻¹ direct protein according to the data obtained from the pico-drop spectrophotometer. On the other hand, the umbilical cord derived HA solution contained 39.4 ng μL⁻¹ double-stranded DNA and 0.6 mg mL⁻¹ direct protein.

Considering the amount of direct protein, protein content of umbilical cord derived HA was 6% and the percentage of purity was determined as 94%, accordingly. In addition, DNA content of umbilical cord derived HA was determined as 2.4 times of the DNA content of the rooster comb derived HA. Umbilical cord derived HA, hyaluronic acid sodium salt from rooster comb, and gelatin solution (as a negative control) were stained with alcian blue (Fig. 2). The blue coloration of the umbilical cord derived material proves its glycosaminoglycan (GAG) content; hyaluronic acid is the most abundant GAG of the umbilical cord matrix.

Characterization studies of HA/G plasma gels and plain plasma gels
The scaffolds were 9.2 mm in diameter and acquired their final form at about an hour after mixing the components. HA/G plasma gels and plain plasma gels were strong, compact and easy to handle.

Mechanical test
The compression test was applied to investigate the elastic moduli of HA/G plasma gels and plain plasma gels. The compressive strengths of the scaffolds were determined from stress-strain curve by applying the load until the scaffolds were destroyed. Elastic modules of plain plasma gels and HA/G plasma gels were calculated as 2.14 ± 0.045 kPa and 3.53 ± 0.23 kPa, respectively, from the slope of the linear portion of the stress-strain curve generated from the Displacement (mm) vs. Force (N) graphs [Fig. 3(A,B)]. Addition of hyaluronic acid and gelatin to the fibrin gel caused an increase in elastic modulus.

Gel porosity and pore size
The gels were stained with coomassie brilliant blue to determine their porosity and pore size. The porosity of HA/G plasma and plain plasma gels were calculated as 72.05% ± 5.85% and 63.86% ± 4.27%, respectively, by using the Scion Image program. The transverse length of the pores was measured to get an idea about the pore size of the scaffolds (Fig. 3). The average pore diameter of the HA/G plasma gels and plain plasma gels were calculated as 133 ± 20 μm and 58 ± 9 μm, respectively. Therefore, addition of hyaluronic acid and gelatin to the fibrin gel caused an improvement in both pore size and porosity. The pore size of a scaffold has an effect on various aspects of the tissue culture such as intercellular communication, cell expansion on and within the scaffold, transportation of nutrients and waste material.16,17 Having a larger pore size and

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FIGURE 1. Morphological and phenotypic characteristics of AD-MSCs. The cells isolated from human adipose tissue exhibited large, flattened, or fibroblast-like morphology during the onset of culture: A—Passage zero (P0)—1st day, B—P1–4th day, C—P4–6th day (original magnification: A—×40, B—×200, and C—×200). D—A representative flow cytometric analysis of cell-surface markers of AD-MSCs at P3; cells were labeled with antibodies against hematopoietic antigens (CD106, CD146, CD11b, CD14, CD15, CD34, CD45, CD117 HLA-DR, and STRO-1) and MSC markers (CD13, CD29, CD44, CD90, CD146, CD166, CD73, CD71, CD105, and HLA A,B,C) or immunoglobulin isotype antibodies (Green line: histogram of isotype control immunoglobulin). E—Fluorescent microscopic images of undifferentiated AD-MSCs (control) and AD-MSCs cultured under adipogenic conditions for 14 days (Oil Red O staining—red: lipid vesicles; blue: nuclei, original magnification: ×40). F—Light microscopic images of undifferentiated AD-MSCs (control) and AD-MSCs cultured under osteogenic conditions for 14 days (Alizarin Red staining, original magnification: ×40). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
higher porosity, the HA/G plasma gels are more suitable for tissue engineering applications than the plain plasma gels. Moreover, HA/G plasma gels may better resist deformation in vivo due to their thicker pore walls.

**Scanning electron microscopy (SEM)**

Scanning electron microscopy was used to visualize the porous and fibrous microstructure, pore size distribution and surface topography of the scaffolds (Fig. 4). SEM micrographs of the gels revealed an intact skin layer at the top of the plain plasma gels, while this structure was more porous in the HA/G plasma gels. In the cross-section of the gels, a fibrous and porous structure was observed. According to SEM micrographs, upper, bottom and cross-sectional structure of HA/G plasma gels is more porous than their counterparts. Fibrin polymerizes to form a fibrous gel, while hyaluronic acid and gelatin provide a much more porous structure. The pore size distribution at the plain plasma gels’ bottom layer is very uneven with some parts being nonporous in contrary to that of HA/G plasma gels (Fig. 4).

AD-MSCs seeded on the HA/G plasma gels were cultured for 21 days and prepared for SEM analysis. The scanning electron micrographs revealed enlarged pore diameters at the surface of gels seeded with the cells [Fig. 5(D)] probably due to degradative enzymes released by the cells to their microenvironment within 21 days. The gels incubated in the same growth media but in the absence of cells had a smaller pore size at their surface [Fig. 5(B)]. The pores in the cross-sectional area appeared to be a little bit compressed in the presence of the cells, but the interconnected structure and architecture was preserved.

**Cell viability and proliferation on the gels**

Gelatin, hyaluronic acid and fibrin were used as main components of the scaffolds. Being all biocompatible materials, they are not expected to have any cytotoxic effects, but since glutaraldehyde was used in crosslinking and stabilizing the gels the scaffolds were tested for their biocompatibility. AD-MSC proliferation was determined as a function of time on coverslips (as a control) and HA/G plasma gels. Figure 6 shows the...
FIGURE 4. Scanning electron micrographs of the plain plasma gels and the HA/G plasma gels. Top and cross-section scale bars: 1 mm; Top scale bars: 200 μm, cross-section scale bars: 100 μm, bottom scale bars: 200 μm.

proliferation of cells with time on these substrates; the amount of cells on coverslips was much more than on the gels on the first day. However, cell number on the gels increased later in culture. Cell number on the gels was significantly higher than on the coverslips on day 21 (\( p < 0.05 \)).

Adipogenic differentiation of AD-MSCs on the gels

Representative images of the Oil Red O-stained cells on HA/G and plain plasma gels are shown in Figure 7. Lipid vesicle accumulation in the cells was followed by examining the 2D cultures through light microscopy; lipid droplet formation started after 7 days and on the day 21, when the culture was stopped, the cells had a more differentiated phenotype, with larger intracellular lipid vesicles. More lipid accumulation was observed in AD-MSCs on HA/G plasma gels under adipogenic differentiation conditions [Fig. 7(D)]. Lipid accumulation was observed in AD-MSCs on HA/G plasma gels even when cultured in control medium, i.e. in the absence of adipogenic factors.

**FIGURE 6.** Comparative cell proliferation results of AD-MSCs on HA/G plasma gels and coverslips. **C.S:** coverslip. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**FIGURE 7.** Micrographs of oil red O stained AD-MSCs cultured on the gels in adipogenic induction media and in control media for 21 days. Cryosections of the gels were obtained and stained with Oil Red O. A—AD-MSCs grown on a plain plasma gel in control medium, B—AD-MSCs grown on a plain plasma gel in adipogenic differentiation medium, C—AD-MSCs grown on a HA/G plasma gel in control medium, D—AD-MSCs grown on a HA/G plasma gel in adipogenic differentiation medium. Scale bars: 50 \( \mu \text{m} \). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
of differentiation factors [Fig. 7(C)]. In the plain plasma gels, lipid accumulation could be visualized only in a small number of cells [Fig. 7(B)]. AD-MSCs did not show any adipogenic differentiation on the plain plasma gels when cultured in the control medium [Fig. 7(A)]. Therefore, the composite plasma gel with hyaluronic acid and gelatin is able alone to induce adipogenic differentiation of AD-MSCs.

**DISCUSSION**

In tissue engineering applications potential cell sources include autologous cells from the patient, allogeneic cells from a donor, and xenogeneic cells. Autologous cells seem to be most promising due to the exclusion of immune rejection and ethical issues. AD-MSCs are easily isolated for autologous use in plastic or aesthetic surgeries nowadays and were used in this study. AD-MSCs can be isolated by collagenase digestion following liposuction. MSCs have been reported to be negative for the hematopoietic markers CD34 and CD45, whereas they are positive for Stro-1, CD29, CD44, CD90, and CD105. Most of the surface proteins expressed by MSCs have been demonstrated to also be expressed by AD-MSCs, with the exception of Stro-1. Stro-1 is supposed to represent the nearest approximation to identify the “pure” MSCs, although a few hematopoietic cells weakly express Stro-1.

It is very advantageous to use natural biomaterials such as fibrinogen, hyaluronic acid, and gelatin as a scaffold material in tissue engineering; they are bioactive, biocompatible and they possess mechanical properties similar to that of soft tissues. On the other hand, it is difficult to control their physicochemical properties and their rate of dissolution. Many biological materials have been used in a controlled manner in recent studies. Films composed of alginate and gelatin mixed in different ratios were shown to induce cell proliferation and hydrogels composed of hyaluronic acid and gelatin were successfully used in the field of cartilage tissue engineering. In a study by Lei et al., expansion of mouse MSCs on hyaluronic acid gels was investigated; here the number of cross-links and concentra- tion of RGD (arginine-glycine-aspartic acid) directed cellular behavior. It was revealed by this study that cells on more rigid hydrogels spread more, migrate less, and as a result their growth rate falls down. All these studies point out the importance of scaffold properties and provision of the target tissue microenvironment in the culture in obtaining a desired cellular behavior. Accordingly, natural ECM materials were used in this study to produce scaffolds in the form of gels with elastic moduli very close to that of fat tissue. Addition of hyaluronic acid and gelatin to the fibrin increased the elastic modulus of the gels, but it was still in an acceptable range.

Long term stability of scaffolds is necessary to provide enough time for cell proliferation and matrix production without causing any implant collapse. Fibrin gels are potentially attractive scaffolds for use in tissue engineering; however their use is often impaired by fast dissolution and lack of shape stability. Commercially available fibrin sealants tend to shrink and disintegrate in vitro and in vivo due to fibrinolysis after a few days and almost completely dissolve within 3–4 weeks. Therefore, in this study after the production of HA/G plasma gels, they were crosslinked with glutaraldehyde to obtain a long-term scaffold shape stability for adipose tissue engineering applications.

Fiber based scaffolds induce tissue regeneration by providing structural communication. In a study by Moël et al., less loss of soluble collagen to the medium was observed when the cells were loaded to PCL coated fibrous PLGA scaffolds in a fibrin gel carrier and a more mature ECM was deposited in shorter period of time. The scaffolds with fiber structure have high similarity to ECM. However, high porosity scaffolds are preferred since they provide better cell communication, nutrient and gas transportation. In this study, as a result of hyaluronic acid and gelatin addition to the fibrin gel, both porous and fibrous scaffolds were produced. HA/G plasma gels had larger pore size and higher porosity in comparison to plain plasma gels. This also makes them more suitable for use in soft tissue engineering. HA/G plasma gel macro and microstructure was preserved when incubated in medium for 21 days; in presence of AD-MSCs the gel material was metabolized which led to increase in surface pore size and a little bit downfall in inner pores, but still the structure was preserved. After cell seeding the gels were cultured under static conditions and this may explain the accumulation and growth of the cells being mainly on the top portion where they can have an access to nutrients and oxygen. The cell penetration would be better in a perfused culture. On the other hand, according to the WST-1 assay results, cell proliferation rate on the gels increased after day 14 (Fig. 6) when compared to cell proliferation rate on the glass slides. The AD-MSCs on the coverslips had a higher proliferation rate between day 1 and 7. Although the gels contain natural extracellular matrix material that is expected to be very much suitable for attachment and growth of AD-MSCs, the glutaraldehyde used to crosslink the gels in order to have a stable structure may have a growth retarding effect. We presume that AD-MSCs need a week to adapt to the crosslinked material and can proliferate further after synthesis of their own ECM. Cell proliferation on the coverslips slowed down very much after day 14 due to contact inhibition while the cells on the gels continued to proliferate probably by migrating towards and occupying the inner parts of the 3D structure. Gel surface and glass slide surface area are similar, so increase in cell number on the gels can only be explained with AD-MSC penetration and growth within the pores. Therefore HA/G plasma gels did not have any negative effects on cell proliferation in a long run.

The cell shape, cytoskeletal components and ECM structure and composition have been found to strongly influence adipocyte differentiation and function. Furthermore, adipocyte precursor cell adhesion, proliferation, and differentiation can strongly be influenced by components of the ECM which play a pivotal role in the adipose tissue development. Both the biological material and the mechanical properties of a scaffold may affect MSC
differentiation. Young modulus of breast adipose tissue was determined as close to 1.9 kPa by Samani et al.\textsuperscript{38} AD-MSCs on the HA/G plasma gels differentiated into adipocytes, as evidenced by intracellular lipid vesicle deposition, in presence and even absence of soluble differentiation factors. Adipogenic differentiation observed in control medium may be either due to mechanical properties of the gels being close to that of adipose tissue, or due to presence of hyaluronic acid or gelatin in the structure of the gels. Adipogenic differentiation of AD-MSCs was not observed on the plain plasma gels, which had mechanical properties closer to that of native adipose tissue, when cultured in control medium. This phenomenon emphasizes more the importance of biological composition of the scaffolds.

As a conclusion, the results of this study showed that a composite HA/G plasma gel with suitable pore size and mechanical properties can support both AD-MSC growth and adipogenic differentiation. Therefore the HA/G plasma gel developed in this study can serve as a useful scaffold for adipose tissue engineering applications.

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