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Effects of adipose-derived mesenchymal stem cell conditioned medium on human tenocytes exposed to high glucose

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Abstract

Introduction: Diabetic tendinopathy is a common invalidating and challenging disease that may be treated using stem cells. However, the effects of adipose-derived mesenchymal stem cell conditioned medium (ASC-CM) in diabetic tendinopathy have never been explored.

Objectives: The present study evaluated the effects of ASC-CM on morphology, cell viability, structure, and scratch wound closure of human tenocytes (HTNC) exposed to high glucose (HG).

Design: Experimental study.

Methods: HTNC were exposed to HG (25 mM) for 7, 14 and 21 days with or without ASC-CM for the last 24 h. CM was collected from 4×10^5 ASCs, centrifuged for 10 min at 200 g and sterilized with 0.22 μm syringe filter.

Results: At 7 days, HG-HTNC had decreased cell viability [72 ± 2%, $p < 0.01$ *versus* normal glucose (NG)] compared to NG-HTNC (90 ± 5%). A further decrement was detected after 14 and 21 days (60 ± 4% and 60 ± 5%, both, $p < 0.01$ *versus* NG and $p < 0.01$ *versus* HG7). While NG-HTNC evidenced a normal fibroblast cell-like elongated morphology, HG-HTNC showed increased cell roundness. In contrast, HG-HTNC exposed to ASC-CM showed a significant increase in cell viability, an improved cell morphology and higher scratch wound closure at all HG time points. Moreover, the exposure to ASC-CM significantly increased thrombospondin 1 and transforming growth factor beta 1 (TGF-β1) content in HG-HTNC. The TGF-β1 elevation was paralleled by higher Collagen I and Vascular Endothelial Growth Factor in HG-HTNC.

Conclusion: ASC-CM may restore the natural morphology, cell viability and structure of HTNC, promoting their scratch wound closure through TGF-β1 increase.

Keywords: adipose-derived mesenchymal stem cells, conditioned medium, diabetes, tendinopathy, thrombospondin-1, transforming growth factor beta 1

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Introduction

Tendinopathies are disabling musculoskeletal disorders mainly affecting athletes, but frequently observed also in the general population. These are characterized by both micro- and macro-structural tendon alterations, leading to prolonged mechanical pain.[1–4]

The management of chronic tendinopathies is still a challenge, since the numerous treatments available, including conservative options (non-steroidal anti-inflammatory drugs, steroids or hyaluronic acid injections, physiotherapy) and surgery often fail.[4–10] To this regard, the novel therapeutic options based on the regenerative medicine principles could be a useful tool for the treatment of tendinopathies.[11] Specifically, injections of adipose-derived mesenchymal stem cells (ASCs) in tendinopathic patients showed good safety and tolerability, assuring encouraging outcomes.[4] However, although the

adverse events related to ASCs injections are not serious, they may affect treatment compliance and satisfaction.[12] Therefore, a recent growing interest has been developed also for extracellular vesicles and/or for mesenchymal stem cells (MSCs) conditioned medium (CM) as novel tools for promoting tendon healing in preclinical studies, potentially able to avoid ASCs side effects.[13–19] Particularly, CM properties seem related to the many bioactive factors therein contained.[20] One of these factors is the transforming growth factor beta 1 (TGF-β1),[21–25] a cytokine that controls proliferation, differentiation, and other functions in most cells and that is extremely poor in the tendons of diabetic rats, thus contributing to the typical tendinous alterations induced by hyperglycemia.[26] Indeed, diabetes affects both the onset of tendon damage and its healing, due to specific factors such as the disorganization and the abnormal production of collagen fibers, the accumulation of advanced glycation end products and the vascular impairment.[2,22–24,27] Consequently, the tendon mechanical properties are weaker, the healing ability is lower, and the recurrence rate is higher due to the prolonged exposure to high glucose (HG) levels.[2,28–30]

However, no study has so far investigated the effects of ASC-CM on the morphology, cell viability, structure, and ability to recover from scratch damage of human tenocytes (HTNC) exposed to prolonged hyperglycemia, by reproducing the clinical conditions of diabetic tendinopathy. Moreover, no study has explored the possibility of reinforcing the low levels of endogenous TGF-β1 in diabetic tenocytes using ASC-CM.

Therefore, the present study aimed to assess the effect of human ASC-CM on the morphology, cell viability and TGF-β1 levels in HTNC exposed to HG concentration for several days, along with their capacity to recover from a scratch damage.

Materials and methods

Collection and processing of human lipoaspirated microfragmented adipose tissue

The reporting of this study conforms to the Minimum Information for Studies Evaluating Biologics in Orthopaedics (MIBO) stem cells checklist[31] (Supplemental Table 1).

The collection of the human microfragmented adipose tissue (μFAT), its processing for ASCs isolation and the subsequent *in vitro* procedures were approved by the Ethics Committee of the AOU University of Campania 'Luigi Vanvitelli' (protocol number 0035781/i, 15/12/2021). All the procedures adhered to the Declaration of Helsinki and Good Clinical Practice guidelines. Subcutaneous adipose tissue samples were obtained at the Unit of Orthopaedics, University of Campania 'Luigi Vanvitelli' (Naples, Italy), from subjects diagnosed with early osteoarthritis, undergoing abdominal lipoaspiration before knee or hip ASCs injections. All patients matched the following inclusion criteria: (I) diagnosis of unilateral/bilateral knee or hip osteoarthritis confirmed by radiography; (II) age $>$ 18 years; (III) joint pain refractory to conservative therapy; (IV) informed consent signed. The exclusion criteria were (I) presence of congenital joint anomalies; (II) recent joint trauma (within 3 months); (III) previous hip or knee prosthetic treatment; (IV) previous drug infiltration in the joint (within 12 months); (V) body mass index $<$ 18 kg/m^2; (VI) diagnosis of diabetes.

A mean of 50 ml of lipoaspirate, collected and microfragmented by using Lipogems® device (Lipogems International S.p.A.; Milan, Italy) as previously described from the donor patient,[32–37] was sufficient to obtain a 10 ml volume of the final μFAT[33] used for a single autologous intra-articular injection. If any, the exceeding volume of μFAT after the intra-articular injection was used to suddenly isolate human ASCs.

Human ASCs isolation from μFAT

A mean of 3 ml of μFAT was digested in 7 ml of α-Minimum Essential Medium (αMEM; M4526, Merck; Milan, Italy), containing low glucose (5 mM), 1% of L-Glutamine (L-Glu; 25030081, Thermo Fisher Scientific; Milan, Italy), 1% of Penicillin-Streptomycin (P/S; AU-L0022, Aurogene; Rome, Italy) solution and collagenase type II (1 mg/ml, C2-BIOC, Merck; Milan, Italy). After an incubation at 37°C with agitation (200 rpm) for 30 min, the μFAT was filtered through cell strainers (70 μm mesh) and suspended in complete αMEM (5 mM glucose, 1% L-Glu, 1% P/S and 10% Fetal Bovine Serum – FBS; AU-S181H, Aurogene; Rome, Italy) before being centrifuged at 1500 rpm for 5 min at room temperature.[33] The final ASCs pellet was washed three times in Phosphate Buffer Saline (PBS; 14200, Thermo Fisher Scientific; Milan, Italy) before the cell counting. ASCs were grown in complete

αMEM at 37°C, 5% CO_2.[38] Particularly, 5×10^3 ASCs were seeded in 96-well plates to measure cell viability at different time points, while 16.6×10^3 ASCs/cm^2 were seeded on culture plates and daily observed with optical microscope until they reached an 80% confluence. Then, ASCs were trypsinized, counted and plated for ASCs characterization by immunofluorescence.

Assessment of human ASCs cell viability

5×10^3 ASCs were seeded into 96-well plates, to assess cell viability at different time points.[39,40] This was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, at each time point (day 4, 7 and 10), ASCs were incubated at 37°C for 4 h with medium MTT (1:10). Then 100 µL dimethyl sulfoxide (DMSO) were used to solubilize the formazan crystals. The absorbance at 570 nm was read by using a 96-well plate reader.

Human ASCs characterization

ASCs phenotype was determined by immunofluorescence, by verifying the presence of specific surface antigens (CD73, CD90, CD105) and the absence of hematopoietic markers such as CD34, CD45 and Human Leukocyte antigen-antigen D related (HLA-DR).[38,41,42] As previously described,[41] 1×10^3 cells/cm^2 were grown on cover slips at 37°C, 5% CO_2. After 18 h, cells were fixed with 3.7% paraformaldehyde (PFA; 1004968350, Merck; Milan, Italy) at room temperature for 15 min. The cover slips were then washed with PBS and blocked with PBS containing 3% bovine serum albumin (BSA; A7906, Merck; Milan, Italy) and 0.3% Triton X-100 (93443, Merck; Milan, Italy). These steps were followed by an overnight incubation at 4°C with the anti-CD73 (1:100; sc-398260, Santa Cruz; Santa Cruz, CA, USA), anti-CD90 (1:100; sc-53116, Santa Cruz; Santa Cruz, CA, USA), anti-CD105 (1:100; sc-18893, Santa Cruz; Santa Cruz, CA, USA), anti-CD34 (1:100; sc-74499, Santa Cruz; Santa Cruz, CA, USA), anti-CD45 CD34 (1:100; sc-1178, Santa Cruz; Santa Cruz, CA, USA) and anti-HLA-DR (1:100; sc-18875 Santa Cruz; Santa Cruz, CA, USA) primary human antibodies, diluted in PBS containing 1% BSA and 0.1% Triton X-100. Then, the cover slips were incubated at room temperature for 1 h with Alexa Fluor™ 594 rat (4 µg/ml; A-11007, Thermo Fisher Scientific; Milan, Italy), AlexaFluor™ 594 mouse (2 µg/ml; A-11032, Thermo Fisher Scientific; Milan, Italy) or AlexaFluor™ 488 mouse (2 µg/ml; A-32723, Thermo Fisher Scientific; Milan, Italy) secondary antibodies, diluted in PBS containing 1% BSA and 0.1% Triton X-100. ASCs were counterstained and mounted with VECTASHIELD Antifade Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (H-1200-NB, Novus Biologicals; Centennial, CO, USA). A fluorescence microscope (Leica, Wetzlar, Germany) was used to obtain the immunofluorescence images, analyzed with Leica FW4000 software (Leica, Wetzlar, Germany). The percentage (%) of positive cells was calculated as the number of positive stained cells on total cells counted for each field. A mean of 200 total cells was counted for each field ($N = 4$), by considering only DAPI counterstained cells.

Preparation of ASC-CM

4×10^5 ASCs (passage 3–5) were seeded in culture flasks and cultured in complete αMEM as described above.[43] Reaching an 80% confluence, cells were incubated for 24 h with a serum free αMEM.[42,44] The ASC-CM was then collected, centrifuged for 10 min at 200 g and sterilized with 0.22 µm syringe filter.[44]

Characterization of ASC-CM

TGF-β1 (total or active) and thrombospondin 1 (TSP-1) secreted by ASCs were assessed in ASC-CM by Enzyme-Linked Immunosorbent Assay (ELISA), using commercially available kits according to the manufacturer's protocols (EH0287, FineTest; Wuhan, China; 437707, Bio-legend; San Diego, CA, USA; MBS701627, MyBioSource; San Diego, CA, USA).

Human Tenocytes (HTNC) cell culture

Commercially available HTNC (P10968, Innoprot; Derio, Bizkaia, Spain), isolated from healthy human patellar tendon, were seeded in a T-75 flask precoated with poly-L-lysine (2 µg/cm^2; PLL, Innoprot; Derio, Bizkaia, Spain) and grown at 37°C, 5% CO_2 in Tenocyte Medium (TCM; P60177, Innoprot; Derio, Bizkaia, Spain). This is a basal medium supplemented with 5% FBS, 1% Tenocyte Growth supplement and 1% P/S (P60177, Innoprot; Derio, Bizkaia, Spain), according to the manufacturer's instructions. Reaching an 80% confluence, HTNC were trypsinized, seeded at a specific cell density for

each assay and then cultured in normal glucose TCM (5 mM; NG) or HG TCM (25 mM).[45,46] 20 mM mannitol was added to NG as positive osmotic control (M group) and did not affect HTNC morphology and viability (Supplemental Figure 1). Particularly, HTNC were exposed to NG or HG for 6, 13 and 20 days.[22,46,47] Then NG or HG medium was removed before adding ASC-CM for the last 24 h[44] (NG + ASC-CM, HG_7 + ASC-CM, HG_{14} + ASC-CM, HG_{21} + ASC-CM groups). Serum-free αMEM was used as control in NG (NG group) or HG groups (HG_7, HG_{14}, HG_{21} groups). HTNC were daily observed at the optical microscope. Three independent experiments were done, each performed in triplicates ($N = 9$).

HTNC cell viability

HTNC were seeded in PLL (2 µg/cm^2) pre-coated 96-well plates, at a density of 5×10^3/well[48] and treated as above described. Cell viability was measured by MTT assay[49,50] after 7, 14 and 21 days. Briefly, 10 µL of MTT reagent was added to each well. After an incubation at 37°C for 4 h, the MTT solution was substituted by 100 µL DMSO. A plate reader was used to read the absorbance at 570 nm (measured as optical density, OD) and the following equation was used to calculate HTNC cell viability, by using NG group as control: % viability = (mean OD treatment/mean OD control) × 100.

HTNC scratch assay

HTNC were seeded in PLL (2 µg/cm^2) pre-coated 6-well plates, at a density of 8×10^3 cells/well.[51] After 6, 13 and 20 days of NG or HG, cell monolayers were vertically scratched by using a 200 µL sterile pipette tip (T0). Then, HTNC were exposed for 24 h to serum free TCM or ASC-CM (T24). Cells were observed by using Leica DMi1 microscope at T0 and after 24 h.[51] Image J software 1.47 was used to measure the % of the initial wound covered by cells (wound closure) over the 24 h, normalized against the wound area at T0.[52,53]

ELISAs for HTNC

1×10^5 HTNC were seeded in PLL (2 µg/cm^2) pre-coated T-25 culture flasks[54] and treated as above described. After 7, 14 and 21 days, HTNC were trypsinized and centrifuged at 1000 rpm × 5 min, according to the manufacturer's protocol. After centrifugation, HTNC medium was separated by HTNC cell pellet. This was washed two times with PBS. The levels of total TGF-β1, TSP-1, Collagen I (Col I) and Vascular Endothelial Growth Factor (VEGF) were assessed in HTNC by ELISAs, using commercially available kits according to the manufacturer's protocols (EH0287, FineTest; Wuhan, China; MBS701627, MyBioSource; San Diego, CA, USA; ab285250, abcam; Milan, Italy; EH0327, FineTest; Wuhan, China).

Active TGF-β1 immunostaining in HTNC

HTNC were seeded in 24-well plates at a density of 2×10^4 cells/well[55], grown on a 1-cm diameter cover glasses[56] and treated as above described. After 7, 14 and 21 days, cells were fixed in 3.7% PFA for 15 min and the protocol for immunocytochemistry described above was followed. To target active TGF-β1 form, a specific anti-TGFβ1 primary antibody (1:250; sc-130348, Santa Cruz; Santa Cruz, CA, USA) was used, along with a secondary anti-mouse antibody (2 µg/ml; A-11032 Thermo Fisher Scientific; Milan, Italy). The % of active TGF-β1 positive HTNC was calculated as the number of positive stained HTNC on total HTNC counted for each field, by considering only DAPI counterstained HTNC. A mean of 250 total HTNC was counted for each field ($N = 4$).

Statistical analysis

Data were obtained from three independent experiments; each performed in triplicate ($N = 9$). Results were reported as mean ± standard deviation. GraphPad Prism 6.0 software (La Jolla, CA, United States) was used to perform the statistical analysis, by using Two-way repeated measures Analysis of Variance followed by post hoc Bonferroni for multiple comparisons. The strength of association between two parameters was evaluated by Pearson correlation analysis and reported as Pearson correlation coefficient (r). A p value < 0.05 was considered statistically significant.

Results

Characteristics of the µFAT donors

µFAT was collected from seven adult non-diabetic females (49–59 years). These were diagnosed with unilateral knee osteoarthritis (3), bilateral knee osteoarthritis (2) or unilateral hip osteoarthritis (2). All exhibited joint pain

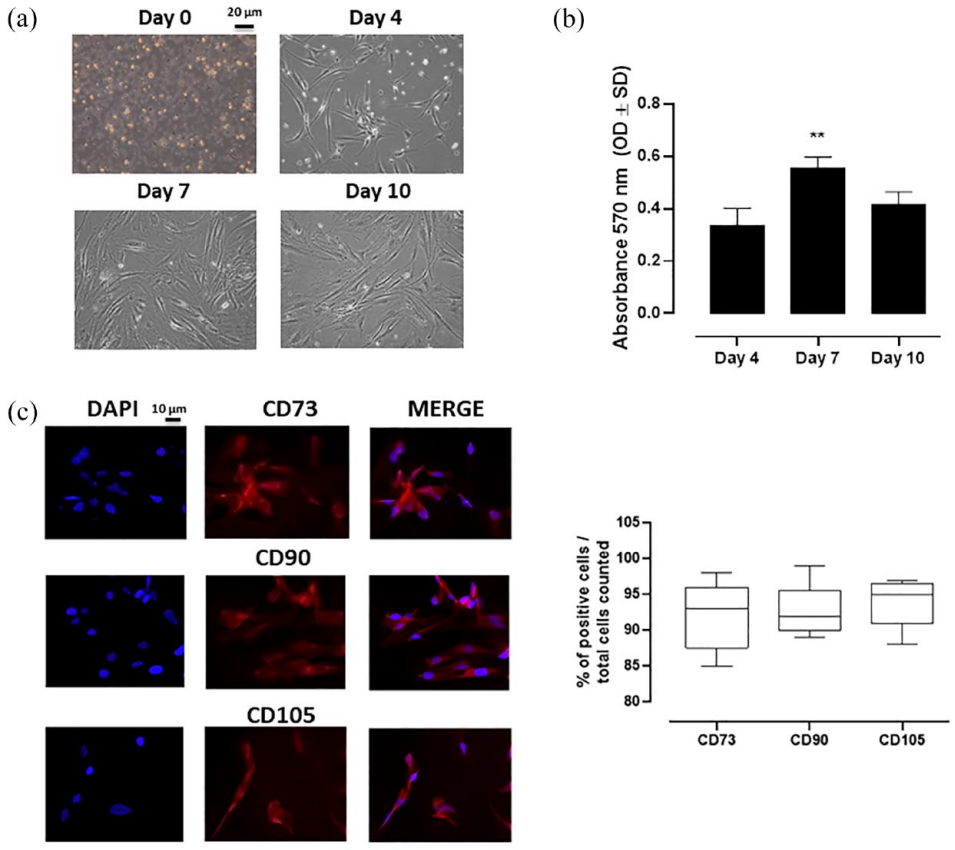


Figure 1. (a) Representative images of optical microscopy showing ASCs isolated from µFAT (Day 0), then after 4, 7 and 10 days (Day 4–7–10); scale bar: 20 µm; magnification 10×, (b) ASCs cell viability, expressed as OD values at 570 nm ± SD. ** $p < 0.01$ *versus* Day 4 and (c) representative immunofluorescence images of CD90/CD73/CD105 expression shown in red, with the nucleus stained in blue by DAPI (4′,6-diamidino-2-phenylindole). Scale bar: 10 µm; magnification 20×. The relative quantization was expressed by the percentage of positive cells (red) on total cells counted (blue) ± SD. Results are expressed as mean ± SD of nine observations.
ASC, adipose-derived mesenchymal stem cell; µFAT, microfragmented adipose tissue; OD, optical density.

refractory to conservative therapy and signed a written informed consent.

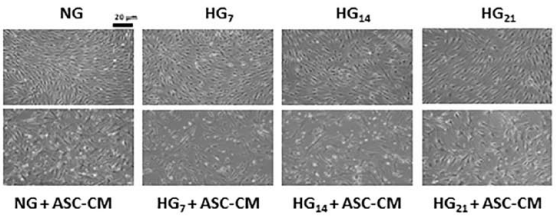
ASCs isolation and characterization

ASCs isolated from µFAT formed small colonies, which became larger starting from 4 days of culture [Figure 1(a)]. Within 7 days, ASCs appeared as adherent thin, long spindle-shaped fibroblast-like cells, gradually expanding in size [Figure 1(a)] and showing an increased cell viability [Figure 1(a) and (b)]. This was not evident between day 7 and day 10 [Figure 1(b)], probably due to the variability in ASCs cell viability evidenced from the different donors, possibly associated to sampling bias. ASCs characterization showed their immunoreactivity to the specific MSCs markers CD73, CD90 and CD105 up to 90–97% [Figure 1(c)], along with the lack of immunoreactivity (0.3–2%) to CD34, CD45 and HLA-DR (Supplemental Figure 2).

Effects of ASC-CM on HTNC

While HTNC in NG evidenced a fibroblast cell-like morphology, HTNC in HG showed increased cell roundness instead of the normal elongated shape [Figure 2(a)]. Moreover, HG condition significantly decreased HTNC cell viability compared to NG, starting from 7 days of HG [Figure 2(b)]. A further decrement of HTNC cell viability was detected starting from day 14 of HG, still present after 21 days [Figure 2(b)]. After the exposure to ASC-CM, HTNC in HG were spindle-shaped or enlarged triangles, recovering an appearance of fibroblasts [Figure 2(c)]. Also,

(a)



(b)

	Tenocytes viability (% ± SD)	P-value
NG	90 ± 5	
NG+ASC-CM	115 ± 13	P < 0.01 vs NG
HG_7	72 ± 2	P < 0.01 vs NG
HG_7 + ASC-CM	83 ± 5	P < 0.01 vs HG_7
HG_{14}	60 ± 4	P < 0.01 vs NG, HG_7
HG_{14} + ASC-CM	70 ± 4	P < 0.01 vs HG_{14}, HG_7 + ASC-CM
HG_{21}	60 ± 5	P < 0.01 vs NG, HG_7
HG_{21} + ASC-CM	73 ± 4	P < 0.01 vs HG_{21}, HG_7 + ASC-CM

(c)

		Wound closure at T24 (% ± SD)	P-value
NG	αMEM	30 ± 4	
	ASC-CM	68 ± 6	P < 0.01 vs NG
HG_7	αMEM	23 ± 6	P < 0.01 vs NG
	ASC-CM	55 ± 4	P < 0.01 vs HG_7
HG_{14}	αMEM	20 ± 3	P < 0.01 vs NG,
	ASC-CM	37 ± 5	P < 0.01 vs HG_{14}, HG_7 + ASC-CM
HG_{21}	αMEM	17 ± 3	P < 0.01 vs NG, HG_7
	ASC-CM	36 ± 6	P < 0.01 vs HG_{21}, HG_7 + ASC-CM

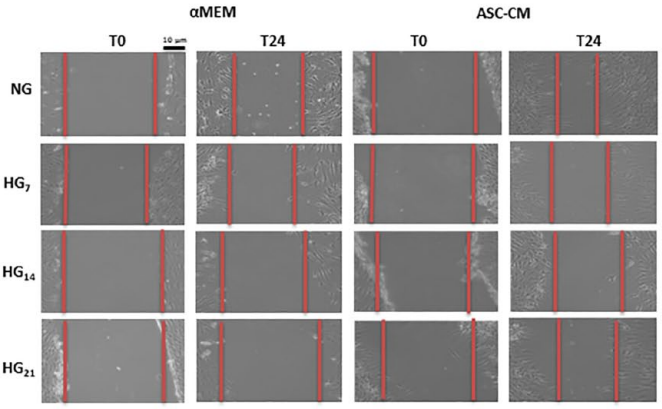


Figure 2. (a) Representative optical microscope images of HTNC morphology in NG or HG (6, 13 and 20 days) followed by 24 h of αMEM as control (NG, HG_7, HG_{14}, HG_{21}) or ASC-CM. Scale bar: 20 μm; magnification 10×, (b) HTNC cell viability in NG or HG (6, 13 and 20 days) followed by 24 h of αMEM as control (NG, HG_7, HG_{14}, HG_{21}) or ASC-CM. HTNC metabolic activity was measured as OD at 570 nm and calculated as (mean OD treatment/mean OD control) × 100 and (c) representative images of wound healing in HTNC at T0 (day 6, 13, 20) and after 24 h in serum free αMEM as control or serum free ASC-CM. The percentage of scratch wound closure at T24 was measured by ImageJ and normalized against the wound area at T0; scale bar: 10 μm; magnification 20×. Results are expressed as mean ± SD of nine observations.
ASC-CM, adipose-derived mesenchymal stem cell conditioned medium; HG, high glucose; HTNC, human tenocytes; αMEM, α-minimum essential medium; NG, normal glucose; OD, optical density.

HTNC viability was recovered by ASC-CM after the HG exposure, with a significant increment of HTNC cell viability at all HG time points [Figure 2(c)]. The positive effects of ASC-CM on HTNC cell damage induced by HG levels was confirmed by the scratch assay, reporting a significant increase of the scratch wound closure both in NG and at all the HG time points, with the highest recovery in HTNC exposed to ASC-CM after 7 days of HG [Figure 2(c)].

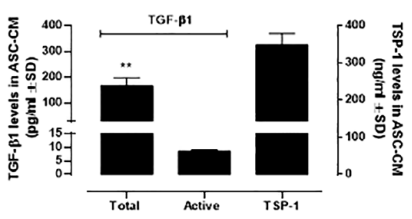
Determination of TGF-β1 and TSP-1 in ASC-CM

The levels of total TGF-β1 in ASC-CM were 168 ± 30 pg/ml, significantly higher ($p < 0.01$) compared to the minimum levels of active TGF-β1 detected (9 ± 0.4 pg/ml), by indicating a marked presence of latent TGF-β1 in ASC-CM [Figure 3(a)]. TSP-1 levels in ASC-CM were 326 ± 44 ng/ml [Figure 3(a)].

ASC-CM increases total and active TGF-β1 in HTNC

HG exposure starting from 7 days induced a significant decrease of total and active TGF-β1 in HTNC [Figure 3(b) and 4). This was present also at HG_{14} and at HG_{14}. As expected, the stimulation of HTNC with ASC-CM lead to a significant increase in total TGF-β1 levels in NG and at all the HG time points [Figure 3(b)]. The internalization of latent TGF-β1 present in ASC-CM by HTNC was confirmed by the assessment of total TGF-β1 content in ASC-CM after the 24 h of HTNC stimulation (Supplemental Figure 3). The total TGF-β1 elevation in HTGN cultured

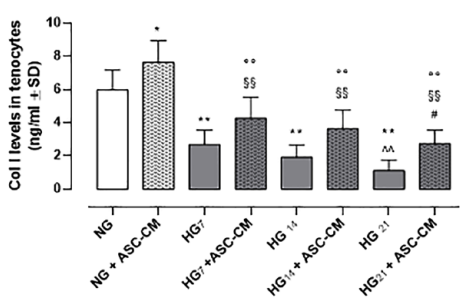
(a)



(b)

HTCN	Total TGF-β1 levels (pg/ml ± SD)	P-value	Col I levels (ng/ml ± SD)	P-value	VEGF levels (pg/ml ± SD)	P-value
NG	800 ± 224		5.9 ± 1.1		92 ± 12	
NG+ASC-CM	1211 ± 163	P < 0.01 vs NG	7.6 ± 1.3	P < 0.05 vs NG	136 ± 32	P < 0.01 vs NG
HG_7	400 ± 173	P < 0.01 vs NG	2.7 ± 0.8	P < 0.01 vs NG	43 ± 7	P < 0.01 vs NG
HG_7 + ASC-CM	844 ± 213	P < 0.01 vs HG_7	4.3 ± 1.3	P < 0.05 vs HG_7	78 ± 11	P < 0.01 vs HG_7
HG_{14}	162 ± 91	P < 0.01 vs NG and HG_7	1.9 ± 0.7	P < 0.01 vs NG	33 ± 5	P < 0.01 vs NG
HG_{14} + ASC-CM	589 ± 136	P < 0.01 vs HG_{14} and HG_7 + ASC-CM	3.6 ± 1.1	P < 0.01 vs HG_{14}	63 ± 14	P < 0.01 vs HG_{14}
HG_{21}	173 ± 113	P < 0.01 vs NG and HG_7	1.1 ± 0.6	P < 0.01 vs NG and HG_{21}	33 ± 8	P < 0.01 vs NG
HG_{21} + ASC-CM	606 ± 191	P < 0.01 vs HG_{21} and HG_7 + ASC-CM	2.7 ± 0.8	P < 0.05 vs HG_{21} and HG_7 + ASC-CM	68 ± 14	P < 0.01 vs HG_{21}

(c)



(d)

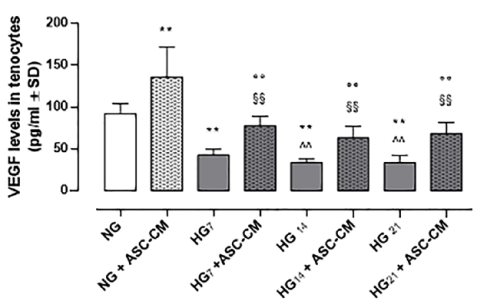


Figure 3. (a) Total TGF-β1 (pg/ml ± SD), active TGF-β1 (pg/ml ± SD) and TSP-1 levels (ng/ml ± SD) in ASC-CM; $^{**}p < 0.01$ *versus* active TGF-β1, (b) total TGF-β1 (pg/ml ± SD), (b and c) Col I (ng/ml) and (b and d) VEGF (pg/ml) levels in HTNC after exposure to NG or HG (6, 13 and 20 days) followed by 24 h of αMEM as control (NG, HG_7, HG_{14}, HG_{21}) or ASC-CM. Results are expressed as mean ± SD of nine observations. $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* NG; $^{\circ\circ}p < 0.01$ *versus* HG at the same time point; $^{\wedge\wedge}p < 0.01$ *versus* HG_7; $^{\S\S}p < 0.01$ *versus* HG_7 + ASC-CM, $^{\#}p < 0.05$ *versus* HG_{14} + ASC-CM.

ASC-CM, adipose-derived mesenchymal stem cell conditioned medium; Col I, Collagen I; HG, high glucose; NG, normal glucose; TGF-β1, transforming growth factor beta 1; TSP-1, thrombospondin 1; αMEM, α-minimum essential medium; VEGF, Vascular Endothelial Growth factor.

in HG and exposed to ASC-CM was paralleled by a significant increase of Col I and VEGF levels, markedly decreased by hyperglycemic conditions [Figure 3(c) and (d)].

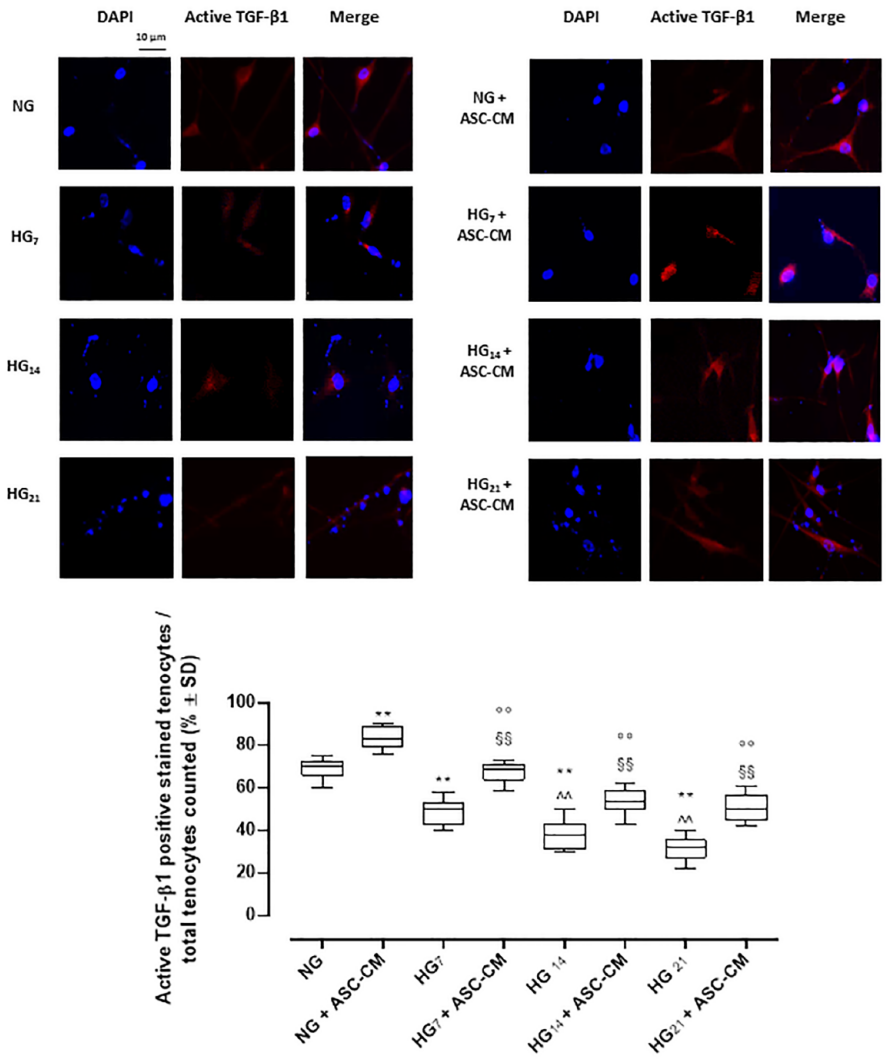
Interestingly, HTNC in NG or HG evidenced also increased levels of active TGF-β1 staining after ASC-CM exposure (Figure 4).

TSP-1 levels in HTNC cultured in HG and exposed to ASC-CM

Since low levels of active TGF-β1 detected in ASC-CM, the increment of TGF-β1 active form in HTNC could be due to the higher TSP-1 levels evidenced by these cells after ASC-CM stimulation [Figure 5(a)]. The Pearson correlation analysis confirmed a significant positive association between active TGF-β1 and TSP-1 levels in HTNC ($r = 0.76$, $p < 0.01$) [Figure 5(b)]. Active TGF-β1 and TSP-1 levels in ASC-CM after the 24 h of HTNC stimulation are reported in Supplemental Figure 3.

Discussion

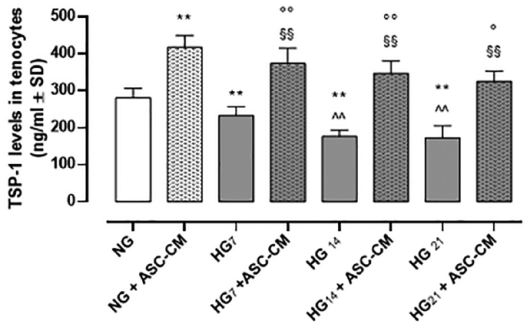
To date, integrative and regenerative medicine have gained a lot of interest in the field of musculoskeletal degenerative disorders and related pain.[11,34,57] Particularly, PRP (platelet-rich



HTCN	Active TGF-β1 positive cells/total cells (% ± SD)	P-value
NG	69 ± 5	
NG+ASC-CM	84 ± 5	P < 0.01 vs NG
HG_7	48 ± 6	P < 0.01 vs NG
HG_7 + ASC-CM	68 ± 4	P < 0.01 vs HG_7
HG_{14}	38 ± 7	P < 0.01 vs NG and HG_7
HG_{14} + ASC-CM	54 ± 6	P < 0.01 vs HG_{14} and HG_7 + ASC-CM
HG_{21}	32 ± 5	P < 0.01 vs NG and HG_7
HG_{21} + ASC-CM	51 ± 6	P < 0.01 vs HG_{21} and HG_7 + ASC-CM

Figure 4. Representative images of immunocytochemistry showing active TGF-β1 in HTNC after exposure to NG or HG (6, 13 and 20 days) followed by 24 h of αMEM as control (NG, HG_7, HG_{14}, HG_{21}) or ASC-CM, with relative quantization reported as % of positive cells (red for TGF-β1, blue for the nucleus) on total cells counted. Results are expressed as mean ± SD of nine observations. Scale bar: 10 µm; magnification 20×. $^{**}p < 0.01$ *versus* NG; $^{\circ\circ}p < 0.01$ *versus* HG at the same time point; $^{\wedge\wedge}p < 0.01$ *versus* HG_7; $^{\S\S}p < 0.01$ *versus* HG_7 + ASC-CM.
ASC-CM, adipose-derived mesenchymal stem cell conditioned medium; HG, high glucose; HTNC, human tenocytes; NG, normal glucose; TGF-β1, transforming growth factor beta 1; αMEM, α-minimum essential medium.

(a)



HTCN	TSP-1 levels (ng/ml ± SD)	P-value
NG	281 ± 25	
NG+ASC-CM	418 ± 32	P < 0.01 vs NG
HG_7	233 ± 24	P < 0.01 vs NG
HG_7 + ASC-CM	374 ± 41	P < 0.01 vs HG_7
HG_{14}	177 ± 17	P < 0.01 vs NG and HG_7
HG_{14} + ASC-CM	347 ± 34	P < 0.01 vs HG_{14} and HG_7 + ASC-CM
HG_{21}	173 ± 33	P < 0.01 vs NG and HG_7
HG_{21} + ASC-CM	326 ± 28	P < 0.05 vs HG_{21}, P < 0.01 vs HG_7 + ASC-CM

(b)

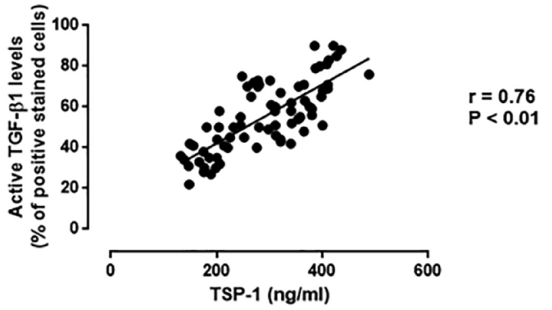


Figure 5. (a) TSP-1 content (ng/ml ± SD) in HTNC after exposure to NG or HG (6, 13 and 20 days) followed by 24 h of αMEM as control (NG, HG_7, HG_{14}, HG_{21}) or ASC-CM; (b) Pearson correlation analysis between active TGF-β1 levels in HTNC (% of positive cells/total cells counted) and TSP-1 levels (ng/ml) in HTNC. Results are expressed as mean ± SD of nine observations. $^{**}p < 0.01$ *versus* NG; $^{\circ}p < 0.05$ and $^{\circ\circ}p < 0.01$ *versus* HG at the same time point; $^{\wedge\wedge}p < 0.01$ *versus* HG_7; $^{\S\S}p < 0.01$ *versus* HG_7 + ASC-CM.
ASC-CM, adipose-derived mesenchymal stem cell conditioned medium; HG, high glucose; NG, normal glucose; TGF-β1, transforming growth factor beta 1; TSP-1, thrombospondin 1; αMEM, α-minimum essential medium.

plasma) and MSCs seem to be promising in reducing pain and improving function in osteoarthritic joints.[58,59] Recently their use had been suggested also in tendinopathies with debated results.[4,60,61] Interestingly, in case of shoulder calcific tendinopathy ultrasound-guided percutaneous lavage seems to be one of the most efficacious treatment.[62]

Among the regenerative medicine options, ASCs have well documented good clinical outcomes in various diseases, including degenerative osteoarthritis,[34,36,37,63] diabetic ulcer[64–66] and non-diabetic tendinopathies.[4] ASCs injections in non-diabetic tendinopathic patients were associated to rapid and long tendon pain recovery.[4] However, some studies to test the effects of ASC-CM in non-diabetic tendinopathies are also emerging. CM from ASC has been injected in several animal models of tendinopathies, with satisfactory results for tendon and ligament healing processes, due to the increased proliferation and viability of tenocytes evidenced after CM injections.[16,67–71] Beside this, however, no study has so far tested the possibility of using ASC-CM against the damage caused by exposing HTNC to prolonged HG levels, reproducing *in vitro* the clinical setting of diabetic tendinopathy.

Here, in fact, it is shown for the first time that an ASC-CM improves the impaired morphology and structure of HTNC from patellar tendon exposed to a prolonged period of HG levels. This implies an amelioration in the maintenance and restoration of tendon tissue, as well as a reduction of extracellular matrix (ECM) structural and biochemical changes, during diabetic tendinopathy.[72,73]

From the molecular point of view, the current *in vitro* setting showed that tenocytes exhibited alterations in cell morphology along with low TGF-β1 levels, recovered after ASC-CM exposure. This was translated as a higher wound closure of a mechanical scratch caused to the cells,

thus resembling the *in vivo* recovering from a tendon rupture. To this regard, it is to note that a significant down-regulation of both latent and active TGF-β1 was evident in HTNC exposed to HG. These were paralleled by reduced cell viability and decreased wound healing, as expected from a reduced TGF-β1 content and activation.[74] Notably, TGF-β isoforms and their receptors are strongly associated with tendon formation, differentiation, and regeneration, as well as to stimulation of collagen transcription in tendon fibroblasts and tissue repair.[75–78] However, contrasting evidence is reported about the influence of diabetes on TGF-β1 expression in tendons. Indeed, while HG conditions were associated to increased TGF-β1 levels in tenocytes from porcine patellar tendons,[79] with consequent scar formation and tenocytes death,[80] TGF-β1 was found to be downregulated in tenocytes from the Achilles tendon of diabetic Sprague Dawley rats, characterized by fiber disorganization and increased interfibrillar spaces.[22] Moreover, injured tendons in diabetic rats showed a weaker immunoreactivity for TGF-β1 compared to injured tendons of non-diabetic rats.[27]

After ASC-CM, TGF-β1 elevation was paralleled by higher levels of Col I and VEGF in tenocytes exposed to HG. Although the evidence about the expression of Col I and VEGF in hyperglycemia-induced tendon damage is controversial,[23,71,81,82] our results are in line with the reduction of Col I and neoangiogenesis reported in preclinical and clinical settings of diabetic tendinopathy.[21,81,83–85] The TGF-β1 related effects in diabetes[51,74,81,86–89] on the levels of Col I, the main component of tendon dry mass[90] and of VEGF, the most important angiogenic factor involved in tendon healing,[91] support the potential role of TGF-β released by ASC-CM in improving tenocytes mediated tendon healing process under HG stimulus.

Tendon repair is also associated with alterations of TGF-β activation.[92] The activation of the latent precursor of TGF-β is obtained after its binding to TSPs,[93] extracellular matrix proteins involved in tendon pathophysiology, tendon healing, vascularization and able to activate the latent TGF-β1 isoform.[94–97] TSP-1 deficiency has been associated with impaired wound healing,[94] supported by impaired activation of the TGF-β1 precursor. Furthermore, TSP-1 is present in the CM of distinct types of mesenchymal stem cells,[98,99] and, interestingly, in ASC-CM.[100] Thus TSP, like TGF, may be pivotal for proper tendon development and maintenance. Increased TSP-1 levels in tenocytes could have pleiotropic effects in addition to TGF-β1 activation, as it appears to co-localize with Col I,[101] the most abundant isoform in healthy tendons[102] and it seems to reduce the inflammatory process in osteoarthritis when released in ASC-CM.[100] Obviously, as with TGF-β1, no evidence of TSP-1 levels achieved in diabetic tenocytes after ASC-CM stimulation has been reported. On the latter aspect, the present study is the first to demonstrate that TSP-1 was reduced in tenocytes exposed to HG levels, while it was increased after their exposure to ASC-CM and positively associated with active TGF-β1 levels.

In conclusion, from a translational point of view, the present study paves the way for further investigations regarding the use of CM as a promising strategy for the management of diabetic tendinopathy. Of course, the present study did not explore other cytokines and factors present in ASC-CM, as well as further appropriate inhibition studies are needed to strengthen the proposed mechanisms related to the effects of ASC-CM in the several cell lines involved in the musculoskeletal system. However, even if the present study cannot give clinical evidence for the application of a CM to patients, it is in line with beneficial effects of human MSC-CM on wound healing in diabetic rats[103] and suggests novel perspectives in the field of new pharmaceuticals for regenerative medicine in tendon healing. Indeed, the use of CM for tendinopathies could have several advantages compared to ASCs, such as the easier manufacturing, freezing, packaging and transport. Moreover, CM could also avoid the rejection problems for the recipient patient when using allogeneic ASCs.[104]

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the AOU University of Campania 'Luigi Vanvitelli', Naples - Italy (protocol number 0035781/i, 15/12/2021). A written informed consent to participate was obtained from all the participants to the study.

Consent for publication

A written informed consent for publication was obtained from all the participants to the study.

Author contributions

Maria Consiglia Trotta: Conceptualization; Formal analysis; Writing – original draft.

Annalisa Itro: Data curation; Formal analysis; Writing – original draft.

Caterina Claudia Lepre: Investigation; Writing – original draft.

Marina Russo: Investigation; Writing – original draft.

Francesca Guida: Methodology; Writing – original draft.

Antimo Moretti: Methodology; Writing – review & editing.

Adriano Braile: Methodology; Writing – review & editing.

Umberto Tarantino: Methodology; Writing – review & editing.

Michele D'Amico: Conceptualization; Writing – review & editing.

Giuseppe Toro: Conceptualization; Validation; Writing – review & editing.

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Competing interests

The authors declare that there is no conflict of interest.

Availability of data and materials

All data are included within the article and its Supplemental Materials.

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Supplemental material

Supplemental material for this article is available online.

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