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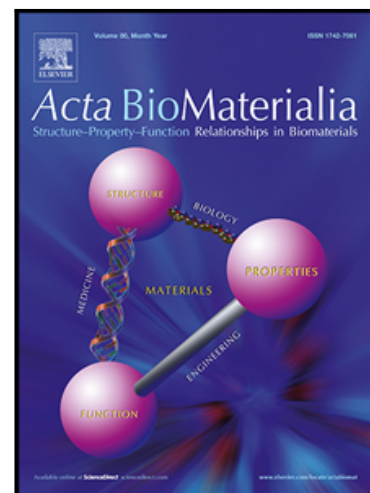
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**Pre-culture of Mesenchymal Stem Cells within RGD-modified Hyaluronic Acid Hydrogel
Improves their Resilience to Ischaemic Conditions**

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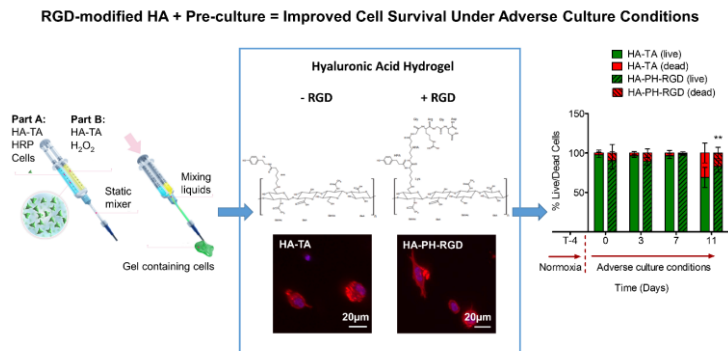
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Graphical Abstract



Abstract

The incorporation of the RGD peptide (arginine-glycine-aspartate) into biomaterials has been proposed to promote cell adhesion to the matrix, which can influence and control cell behaviour and function. While many studies have utilised RGD modified biomaterials for cell delivery, few have examined its effect under the condition of reduced oxygen and nutrients, as found at ischaemic injury sites. Here, we systematically examine the effect of RGD on hMSCs in hyaluronic acid (HA) hydrogel under standard and ischaemic culture conditions, to elucidate under what conditions RGD has beneficial effects over unmodified HA and its effectiveness in improving cell viability. Results demonstrate that under standard culture conditions, RGD significantly increased hMSC spreading and the release of vascular endothelial factor-1 (VEGF) and monocyte chemoattractant factor-1 (MCP-1), compared to unmodified HA hydrogel. As adhesion is known to influence cell survival, we hypothesized that cells in RGD hydrogels would exhibit increased cell viability under ischaemic culture conditions. However, results demonstrate that cell viability and protein release was comparable in both RGD modified and unmodified HA hydrogels. Confocal imaging revealed cellular morphology indicative of weak cell adhesion. Subsequent investigations found that RGD could exert positive effects on encapsulated cells under ischaemic conditions but only if hMSCs were pre-cultured under standard conditions to allow strong adhesion to RGD before exposure. Together, these

results provide novel insight into the value of RGD introduction and suggest that the adhesion of hMSCs to RGD prior to delivery could improve survival and function at ischemic injury sites.

Keywords: Mesenchymal stem cell; Hydrogel; RGD; Myocardial infarction; Hyaluronic acid; Stem cell delivery

Statement of significance

The development of a biomaterial scaffold capable of maintaining cell viability while promoting cell function is a major research goal in the field of cardiac tissue engineering. This study confirms the suitability of a modified HA hydrogel whereby stem cells in the modified hydrogel showed significantly greater cell spreading and protein secretion compared to cells in the unmodified HA hydrogel. A pre-culture period allowing strong adhesion of the cells to the modified hydrogel was shown to improve cell survival under conditions that mimic the myocardium post-MI. This finding may have a significant impact on the use and timelines of modifications to improve stem cell survival in harsh environments like the injured heart.

1. Introduction

Myocardial infarction, is a leading cause of morbidity and mortality worldwide, responsible for 7.3 million deaths annually [1]. It occurs following occlusion of a coronary vessel, typically due to rupture of an atherosclerotic plaque followed by thrombus formation. The sudden inhibition of blood flow to the heart muscle creates an area of ischaemia, where mass death of cardiac myocytes ensues almost immediately. As the myocytes die by both apoptotic and necrotic processes, they initiate an inflammatory response, recruiting a host of white blood cells including neutrophils, macrophages and lymphocytes to the site. Following removal of the debris, cardiac fibroblasts secrete an abundance of extracellular matrix (ECM) proteins, replacing the dead cells with a dense collagen scar. While the fibrotic scar helps to protect the heart wall from rupturing, it also results in contractile dysfunction and

rhythm disorders [2]. Aside from organ transplantation, current treatment options are considered palliative as they improve quality of life and possibly prolong survival but fail to address the underlying damage to cardiac tissue. Therefore there have been significant efforts to develop novel therapies to repair and regenerate the myocardium.

To date, clinical efforts towards cardiac repair have focused on cell-based therapies [3]. Human bone marrow-derived mesenchymal stem cells (hMSCs) have been extensively characterised and commonly used in clinical trials for cardiac repair to date [4]. hMSCs are known to produce and secrete a wide range of factors that have been shown to contribute to cardiac functional recovery by stimulating endogenous repair mechanisms. Soluble factors released by hMSCs can stimulate angiogenesis, as well as induce anti-remodelling, anti-apoptotic and anti-inflammatory effects [5]. Several growth factors have been identified in the conditioned medium of hMSCs including vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), placental growth factor (PGF) and basic fibroblast growth factor (bFGF) [6]. More recently, hMSCs have been shown to release extracellular vesicles and exosomes, which play a role in intracellular signalling [7]. Yet, while the clinical safety profile has been satisfactory, over-all efficacy has been modest, with only a small increase in LV function and a minor reduction in infarct size observed [8–11]. The curative potential of stem cells is hindered by poor engraftment as a result of low cell retention and a high degree of cell death in the harsh ischaemic microenvironment of the infarcted heart. In most clinical trials, stem cells are delivered to the heart suspended in saline, which cannot efficiently retain cells at the injection site due to its low viscosity, nor can it provide a matrix for cell interactions [12,13]. Unsurprisingly, both preclinical [14,15] and clinical studies [16,17] have demonstrated that retention does not exceed 10% of the volume delivered in the heart 24 hours post-delivery.

While the preconditioning, genetic manipulation and co-transplantation of cells has been reported to extend the survival of transplanted cells, these approaches are unable to address the physical factors affecting cellular retention including dispersion from the injection site and anoikis due to a lack of attachment sites [18]. To overcome these limitations, tissue engineering strategies using hydrogels as cell delivery vehicles are currently under investigation. The combination of cells and hydrogel has led to enhanced cardiac repair in both large and small animal models of MI, compared to injection of cells

alone [19–22]. Of the synthetic and naturally derived biomaterials that have been examined, hyaluronic acid (HA) based hydrogels have attracted much interest [23–26]. HA is a linear polysaccharide that consists of alternating units of a repeating disaccharide, β -1,4-D-glucuronic acid- β -1,3-N-acetyl-D-glucosamine. It is a principal component of the ECM with high concentrations typically found in the connective and neural tissues of vertebrates [27]. HA is an attractive material for the fabrication of artificial matrices due to its inherent biocompatibility, biodegradability and non-immunogenic characteristics [28]. While native HA has some clinical applications such as viscosurgery, this unmodified molecule has limited use as a biomaterial due to poor mechanical properties and rapid degradation [29]. To solve this issue, the HA molecule can be modified to generate crosslinkable hydrogels with more robust mechanical properties and an extended biological half-life [23,26,30].

This study reports on HA-based hydrogels capable of covalently crosslinking in the presence of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). This gelation kinetic can be exploited to deliver cells directly into the myocardium minimally invasively using a specially designed catheter by our lab [23,31] with subsequent sol-gel transition *in situ*, trapping cells within a protective matrix and sustaining the release of paracrine factors at the target site. Although clinical trials have demonstrated the safety and feasibility of intramyocardial injections [32], this route may not be suitable for all patients, such as patients with large infarcts where numerous injections would be required. Multiple injection sites maximize the risk of tissue trauma due to the potential dangers of disrupting the myocardium, damaging the coronary vessels or His-Purkinje fibers [33]. To overcome these limitations, the HA hydrogel described in this study can also be delivered minimally invasively using the recently developed **Surface Prone EpicAr dial Delivery System (SPREADS)**, which facilitates delivery of cell-loaded hydrogels to the epicardial surface. This bioresorbable patch enables local application of biomaterial-based stem cell therapy, while also supporting functional recovery of the weakened LV-wall post-MI [26].

Within hydrogels, stem cells are responsive to matrix degradation, topography, mechanical strength and the presentation of growth factors and adherence sites [34,35]. Of these stimuli, cell adhesion is of critical importance as many cells, including hMSCs, are anchorage-dependent and require adhesion to

the matrix in order to survive. Although cells can bind to HA via several surface receptors, such as CD44 and RHAMM, HA is unable to interact with key adhesion receptors such as integrins [36]. Arginylglycylaspartic acid (RGD) is a tripeptide sequence composed of L-arginine, glycine and L-aspartic acid, that is found in many natural adhesive proteins including fibronectin, vitronectin, laminin and collagen type 1 [37]. This physiologically ubiquitous binding motif is one of the most commonly used in tissue engineering [37,38].

RGD plays a role in cellular attachment and spreading as well as in both actin-skeleton and focal-adhesion formation with integrins [39]. The anchorage of cells to the matrix via RGD allows cells to signal bi-directionally across their membrane to engage in a dynamic relationship with the hydrogel [40]. While many studies have included RGD in biomaterials for cell delivery, few have examined its effect on encapsulated cells under the condition of hypoxia and reduced nutrients, as is present in ischaemic injury sites. This dearth in knowledge undermines the therapeutic potential of MSC therapy for ischaemic heart disease [41].

Because HA acts as a barrier to cell adhesion and proliferation *in vitro*, it can be used as a blank slate to investigate the influence of RGD on cell behaviour [42]. In this study, hMSC viability, morphology and function is systematically compared in unmodified-HA (HA-TA) and RGD-modified HA (HA-PH-RGD) hydrogel under standard and adverse culture conditions. The tyramine moiety of the HA-TA molecule is a substrate for HRP-mediated oxidation, which leads to the formation of tyramine dimers. These dityramine bridges crosslink the linear HA-TA chains to create a polymer network within the whole volume of the sample. In the case of HA-PH-RGD, the phenolic crosslinkable group (PH) works in the same way as TA, as described in previous publications by our research group [23,26,43]. Herein, we provide a detailed analysis of hMSC behaviour in HA-TA and HA-PH-RGD hydrogel in order to further progress towards identifying the ideal scaffold for cardiac tissue engineering.

2. Materials and Methods

2.1 Materials

This study compares (i) HA-TA and (ii) a mixture of HA-TA and HA-PH-RGD at a ratio of 1:1, herein referred to as HA-PH-RGD. HA derivatives are shown in **Figure 1**. The tyramine moiety of the HA-TA molecule is a substrate for HRP-mediated oxidation, which leads to the formation of tyramine dimers. These dityramine bridges crosslink the linear HA-TA chains to create a polymer network within the whole volume of the sample. In the case of HA-PH-RGD, the phenolic crosslinkable group (PH) works in the same way as TA, as described in previous publications by our research group [23,26]. HA derivatives were synthesised as described in Supplementary Material A and B. Lyophilised HA-TA and HA-PH-RGD with a molecular weight ranging between 240-400 kDa were supplied by Contipro (Czech Republic). The degree of substitution (number of tyramine molecules per 100 repeating units of HA) was 2-3%. The degree of substitution of RGD was 1-1.5% where the final concentration of RGD in formed hydrogels was 0.5-0.75%.

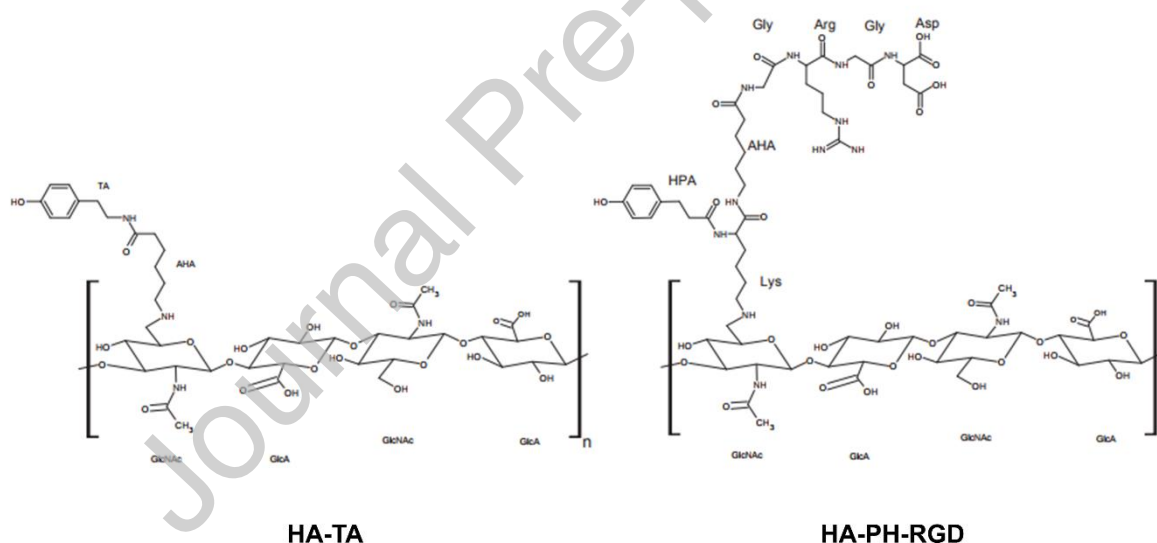


Figure 1 Structures of HA derivatives.

2.2 Hydrogel synthesis

To prepare 2% (w/v) hydrogel, lyophilised HA-TA/HA-PH-RGD was added to phosphate buffer saline (PBS, Sigma Aldrich) at a concentration of 20 mg/mL and rehydrated overnight on a rotational rocker (Stuart, SRT9D). The polymer solution was subsequently divided into two equal volumes and

HRP was added to one solution while H₂O₂ (Sigma Aldrich) was added to the other solution. Equal volumes of the HRP and H₂O₂ polymer solutions were drawn into separate Luer lock syringes before being expelled synchronously through a gel rig containing a static mixer, as previously described [23,26,44]. Hydrogels were injected into custom-made cylindrical polytetrafluoroethylene (PTFE) moulds where resulting hydrogels were 8 mm diameter, 4 mm height and 200 µL volume. The final concentration of crosslinking agents was 0.12 units/mL HRP and 0.0015 %/mL (0.495 µmol/mL) H₂O₂.

2.3 Gelation time analysis

Using a 2-200 µl pipetman, HA-TA-H₂O₂ and HA-TA-HRP were mixed and pipetted up and down until the solutions could no longer be pipetted. The time at which this happened was designated as the gelation time. This was repeated for HA-PH-RGD hydrogel.

2.4 Human mesenchymal stem cell culture

hMSCs were isolated from bone marrow aspirates obtained from human volunteers, at REMEDI, the National University of Ireland Galway. All procedures were carried out with informed consent and ethically approved by the Clinical Research Ethical Committee at University College Hospital, Galway. hMSCs were isolated using standard protocols and stringent analysis of cell phenotype as previously described [45]. hMSCs were cultured using low glucose Dulbecco's Modified Eagle's Medium (DMEM) (D6046, Sigma) supplemented with 10% fetal bovine serum (FBS), 100U/mL Penicillin, 100µg/mL Streptomycin (Sigma Aldrich, Ireland) and 5ng/mL bFGF (R&D Systems) at 37°C in a 5% carbon dioxide (CO₂) environment. Cells were passaged at 80-90% confluency, and were not used beyond passage 5.

2.5 hMSC encapsulation in HA-TA/HA-PH-RGD hydrogel

Solutions of HA-TA/HA-PH-RGD derivative was prepared as before in PBS and filter sterilised using a 0.2 µm filter. The polymer solutions were divided into equal volumes before HRP was added to one solution and H₂O₂ was added to the other solution, as described in Section 2.2. hMSCs were trypsinised and suspended in the HRP polymer solution. Cells were encapsulated at a density of 1 x 10⁶ cells/mL.

2.6 Young's (compressive) modulus analysis

HA-TA and HA-PH-RGD hydrogel samples encapsulating 1 million hMSCs/mL were prepared mechanically tested in unconfined compression between impermeable platens after 4 days and 11 days in culture using a standard materials testing machine with a 5N load cell with an accuracy of $\pm 1\%$ (Zwick Z005, Roell, Germany) as previously described [23,26,46]. Briefly, a preload of 0.01N was applied to ensure that the hydrogel surfaces were in direct contact with the loading platens and to determine the height of the samples. Samples were loaded to 20% compressive strain at 0.01 mm strain per second. The compressive modulus was determined as the slope between 0% and 10% strain of the resulting stress-strain curve [23,26,46].

2.7 hMSC protein expression

To determine if expression of MSC proteins is maintained within the hydrogels during culture, the cells were stained and visualised using traditional antibodies for MSC markers, CD44, CD90, CD105, CD73. The cells expressing each marker was determined via imaging verses control cells. Control cells included unstained cells and cells that remained in 2D culture over the same passage as the cells in 3D hydrogels. Briefly, HA-TA and HA-PH-RGD hydrogels encapsulating 1×10^6 cells/mL were fixed using 4% paraformaldehyde in 1X PBS for 30 minutes after the culture period. The cells were washed thrice with 1X PBS, incubated in a permeabilisation solution containing 0.25% Triton X-100 (w/v) for 20 minutes and then washed in 1X PBS. Non-specific binding sites were blocked with 10% normal goat serum in PBS for 40 minutes at room temperature and the cells were washed thrice with 1X PBS. Cells were treated with FITC-conjugated primary antibodies from BD Biosciences; CD105 (561443), CD90 (561969), CD73 (561254) and CD44 (560977) for 1 hour at 37°C. Cells were stained simultaneously without addition of primary antibody as control for each antibody. Following a washing step, cells were counter stained with Hoechst (1:1000) for 10 minutes. Immunofluorescence-stained cells were imaged using a spinning disc inverted confocal microscope (CSU22, Yokagawa) combined with Andor iQ 2.3 software.

2.8 Experiments to determine the effect of RGD on hMSC viability, morphology and protein release under various culture conditions

For all experiments described in Sections 2.8.1-2.3 samples were cultured for a period of 11-14 days, with full medium changes at the respective time points. hMSC viability, morphology and protein release was assessed at indicated time points.

2.8.1 Effect of RGD on encapsulated hMSC viability, morphology and protein release under standard culture conditions

hMSCs were harvested and encapsulated in HA-TA and HA-PH-RGD hydrogel and then cultured under standard culture conditions (21% O₂, 5% CO₂) in hMSC medium as described in Section 2.6.

2.8.2 Effect of RGD on encapsulated hMSC viability, morphology and function under ischaemic culture conditions

hMSCs were encapsulated in HA-TA and HA-PH-RGD hydrogels and cultured under reduced nutrient conditions using DMEM no glucose medium (D11966, Thermo Fisher) supplemented with 1% FBS, 100U/mL Penicillin and 100µg/mL Streptomycin. Samples were incubated at 1% O₂ (5% CO₂) using a hypoxia chamber (Model #856-HYPO, Hypoxia Chamber Glove Box, Plas-Labs, Inc™ USA) for 11 days.

2.8.3 Effect of pre-culture with RGD on hMSC viability, morphology and function under ischaemic culture conditions

hMSCs were encapsulated in HA-TA and HA-PH-RGD hydrogel and cultured under standard culture conditions as described in Section 2.6 for 4 days (t-4). Following this, samples were rinsed with PBS and transferred to ischaemic conditions as described in Section 2.7.2.

2.9 Cell counting kit-8 (CCK8) assay

Encapsulated cell viability was determined using the Cell Counting Kit-8 (CCK8, NBS Biological, EU). In the CCK8 assay, a tetrazolium salt, WST-8, is reduced by dehydrogenases in cells to produce a coloured formazan which is soluble in tissue culture medium. The amount of formazan dye generated is directly proportional to the number of living cells. However, under stressful conditions, changes may occur in metabolic and energy homeostasis. Therefore, results are expressed as the metabolic activity of encapsulated cells without correlating this to the number of living cells. 100 µL of the CCK8 solution was added to each well (1 gel/mL medium) and the plates were incubated at 37°C. Following an incubation period of 3 hours, 100 µL of each sample was transferred to a 96-well

plate and optical density (O.D) at 450 nm was determined using a plate reader (Varioskan Flash, ThermoScientific, Ireland).

2.10 Confocal Microscopy-Live/Dead imaging

hMSC-seeded gels were washed with PBS and stained using LIVE/DEAD™ Viability/Cytotoxicity kit (Life Technologies) as previously described [23,26,46,47]. Stained gels were rinsed in PBS and a tiled image of the entire cross-section of the hydrogel was captured by a Carl Zeiss LSM 710 confocal microscope using Zen® 2008 software. Two depths were acquired, one at the surface of the hydrogel construct, 100 µm below the surface to exclude any inconsistencies in the surface topography and 700 µm below the surface of the hydrogel construct. The ‘Analyze Particles’ tool in FIJI was then used to count the number of live and dead cells found within the thresholded overview images [48]. The ratio of live to dead cells was generated based on the total number of cells counted for the combined cross sections.

2.11 Circularity

The degree of cell spreading and attachment from the images described in Section 2.9 was quantified by measuring cell circularity as previously described [49]. Circularity is an index of the compactness of an object, circularity would be one for a circular cell and would decrease as the cell elongated or formed extensions. Cell circularity is reported as the average circularity of live cells within the threshold overview images. Information regarding the number of live cells/mm² analysed for cell circularity calculations reported in **Figures 3A, 4A & 5A** can be found in **Supplementary Figure D, E & F**, respectively.

2.12 Dapi/phalloidin staining

Fluorescent staining of nuclei and F-actin was performed as follows: Medium was removed from the well-plates and hydrogels were washed with PBS before fixing for 1 hour using 1 mL of 4% formaldehyde (Sigma). Samples were washed with blocking solution containing 3% bovine serum albumin (BSA) and 0.5% (w/v) Tween in PBS. Permeabilisation solution consisting of 0.25% Triton X (w/v) in blocking solution was added for 20 minutes. Hydrogels were subsequently stained with phalloidin–tetramethylrhodamine B isothiocyanate (Sigma) at 250 ng/mL for 30 minutes and DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride, Invitrogen) at 100 ng/ml for 10 minutes.

Hydrogels were washed in blocking buffer to remove unbound stains before mounting onto glass slides for analysis by confocal laser scanning [50–52].

2.13 VEGF and MCP-1 detection using enzyme linked immunosorbent assay (ELISA)

After 1, 4, 7 and 11 days in culture, spent medium was collected for enzyme-linked immunosorbent assay (ELISA) assessment. VEGF and MCP-1 ELISAs were carried out according to the manufacturer's instructions (R&D Systems). Using a microtiter plate reader (Varioskan Flash, ThermoScientific, Ireland) the well plate was read at an absorbance of 450 nm with correction at 570 nm. The readings were converted to a concentration using a standard curve generated.

2.14 Statistical Analysis

Statistical analysis of results was carried out using GraphPad Prism software, version 5.01. Normality of distribution was assessed by the Shapiro-Wilk test. Unpaired t-test was performed for comparing between two groups at one time-point and two-way analysis of variance (ANOVA) followed by Bonferroni post-test analysis was performed for multiple time-points. Error is reported as standard deviation (SD) and significance was determined using a probability value of $P < 0.05$. Statistical significance shown as compared to unmodified HA hydrogel.

3. Results

3.1 Physical properties of HA-TA and HA-PH-RGD hydrogel and distribution of cells

HA-TA and HA-PH-RGD hydrogels were found to gelate rapidly, at 12 ± 2 s and 11 ± 3 s respectively (**Figure 2A and B**) with no significant difference between groups. There was no significant difference in the Young's moduli in compression of HA-TA hydrogel and HA-PH-RGD hydrogels encapsulating with 1 million hMSCs/mL at day 4 or day 11 ($p > 0.05$, **Figure 2C**). hMSCs were found to be uniformly distributed throughout the HA-TA and HA-PH-RGD hydrogel constructs over time (**Figure 2D**). Staining demonstrates that hMSCs maintained marker expression over the time course of assessment (**Figure 2E**).

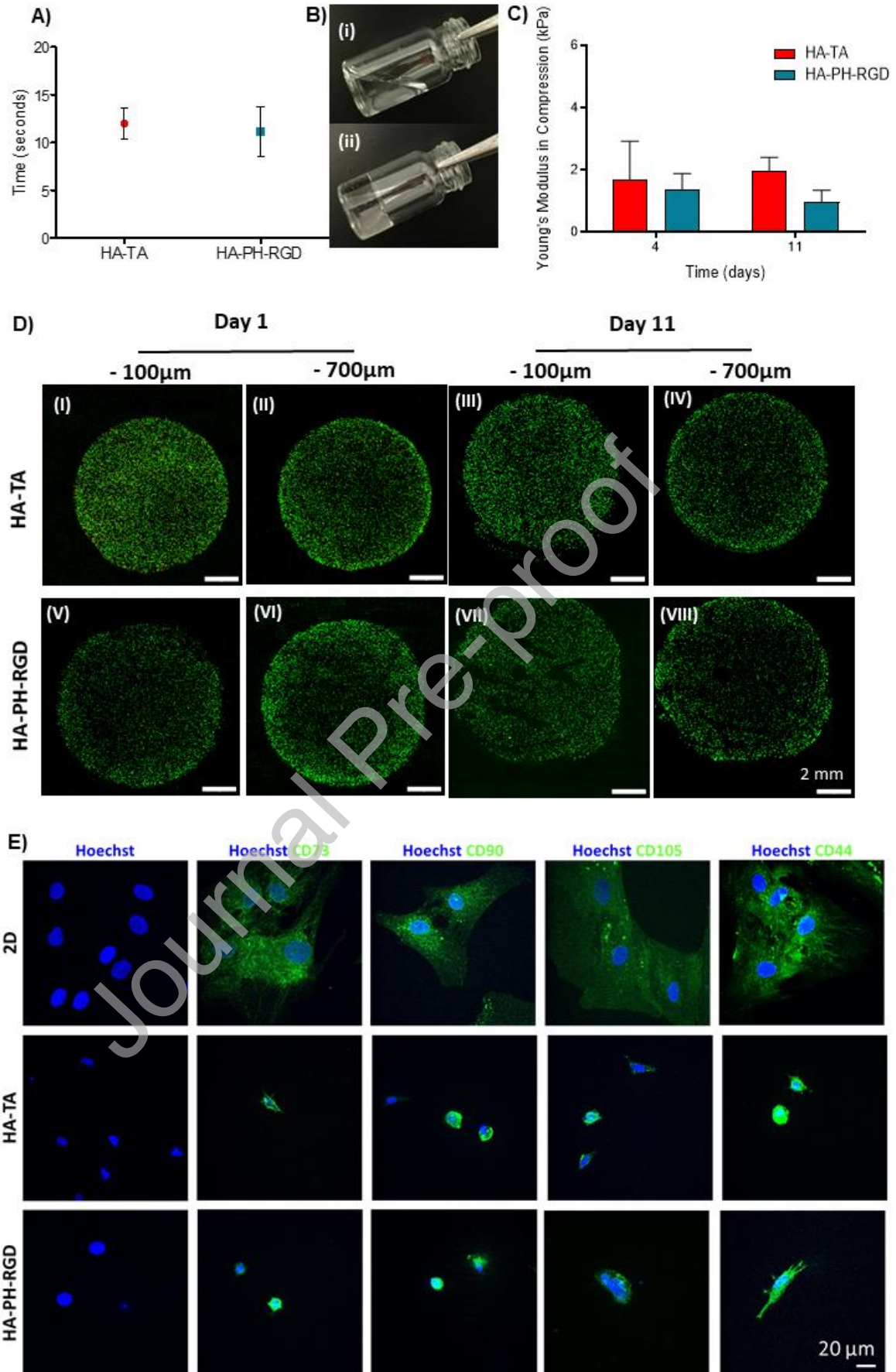


Figure 2 Physical properties of HA-TA and HA-PH-RGD hydrogels. (A) Gelation time of HA-TA and HA-PH-RGD hydrogels. Results are expressed as the mean \pm SD (n=4). (B) Representative photographs of HA-TA hydrogel (i) before and (ii) after gelation. (C) Young's modulus in compression of HA-TA and HA-PH-RGD hydrogels encapsulating 1 million hMSCs/mL after 4 and 11 days in culture. Results are expressed as the mean + SD (n=4/group). (D) hMSC distribution in hydrogel. Representative confocal microscopy images of live/dead stained hMSCs encapsulated in HA-TA and HA-PH-RGD hydrogel at a depth of 100 μ m (I, III, V & VII) and 700 μ m (II, IV, VI & VIII) below the surface over a period of 11 days. Scale bar = 2 mm. (E) hMSCs encapsulated in HA-TA and HA-PH-RGD hydrogels stained positive for stem cell markers (CD105, CD90, CD73 and CD44) after 7 days in culture in normoxic culture conditions. Scale bar = 20 μ m.

3.2 Effect of RGD on hMSCs under standard culture conditions

hMSCs in HA-PH-RGD hydrogel exhibited significantly decreased circularity following 7 and 11 days in culture, compared to cells in unmodified HA-TA hydrogel (**Figure 3A**). This result was confirmed by fluorescent staining of nuclei and F-actin after 11 days, which revealed cells in HA-PH-RGD hydrogel were highly spread, with elongated, spindle-like morphologies (**Figure 3B**). In contrast, hMSCs in HA-TA hydrogel were found to maintain a spherical morphology. Cells remained metabolically active in both HA-TA and HA-PH-RGD hydrogels over a period of 11 days, with no significant difference observed between groups (**Figure 3C**). The percentage of live cells in the HA-TA and HA-PH-RGD hydrogels post-encapsulation was high ($91.57\% \pm 5.89\%$ and $99.97\% \pm 0.05\%$) following 1 day in culture (**Figure 3D**) and remained high for the duration of the study, with $83.9\% \pm 14.16\%$ and $85.7\% \pm 10.48\%$ live cells in HA-TA and HA-PH-RGD hydrogel after 11 days in culture. A significant difference in the percentage of live/dead cells was observed following 4 days in culture (HA-TA vs HA-PH-RGD, $p < 0.01$), with no significant differences observed thereafter. ELISA analysis of spent medium revealed that RGD significantly increased VEGF release after 11 days ($p < 0.05$) (**Figure 3E**) and enhanced MCP-1 release after 7 and 11 days in culture ($p < 0.001$) (**Figure 3F**).

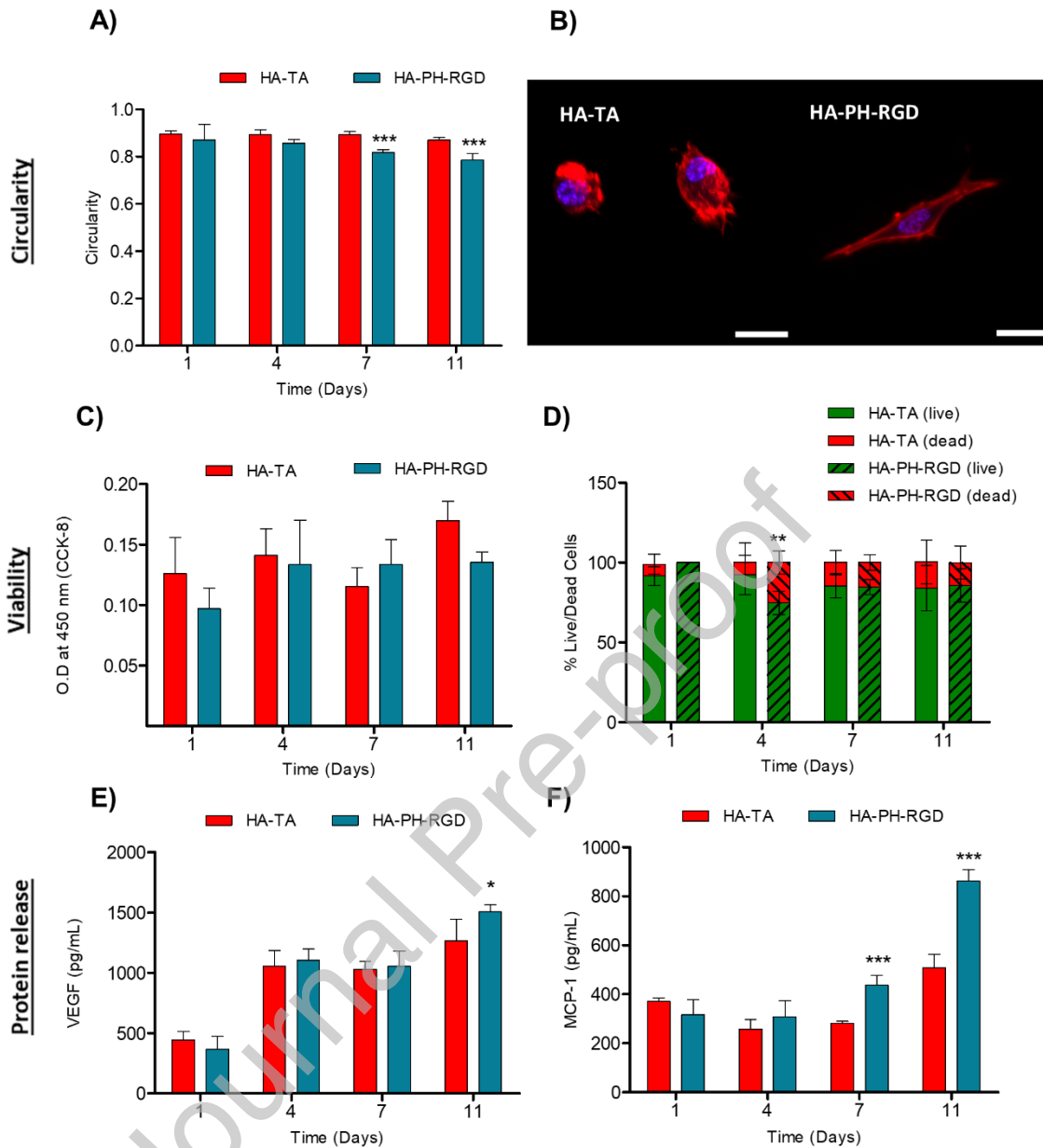


Figure 3 Effect of RGD on hMSCs in HA hydrogel under standard culture conditions. (A) Cell circularity was quantified. A value of 1 indicates a perfect circle and as the value approaches 0, the shape is increasingly elongated. Values expressed as mean + SD ($n \geq 3$). *** $p < 0.001$. (B) DAPI (blue) and phalloidin (red) stained images of hMSCs seeded in HA hydrogel (with or without RGD) following 11 days in culture. Magnification 20x. Scale bar = 20 μm . (C) Metabolic activity of hMSCs determined by CCK8 assay. (D) hMSCs in hydrogels were stained with calcein AM (live-green) and EthD-1 (dead-red). Cell viability is depicted as the percentage of live/dead cells. Results are expressed as mean \pm SD ($n \geq 3$). ** $p < 0.01$. Release of (E) VEGF and (F) MCP-1 from hMSCs encapsulated in HA-TA and HA-PH-RGD hydrogels at various time points determined by ELISA. Results are expressed as the mean + SD ($n = 4$). * $p < 0.05$, *** $p < 0.001$. Statistical significance shown as compared to unmodified HA hydrogel.

3.3 Effect of RGD on hMSCs in HA hydrogel under ischaemic culture conditions

Under ischaemic culture conditions RGD significantly decreased hMSCs circularity at day 4, 7 and 11, compared to unmodified HA-TA hydrogel ($p < 0.001$) (**Figure 4A**). Closer inspection of cell morphology using dapi/phalloidin staining revealed that although cell spreading was evident in HA-PH-RGD hydrogels under ischaemia (**Figure 4B**), it was markedly less pronounced than previously observed under standard culture conditions (**Figure 4B**). When compared to standard culture conditions, cell circularity in HA-PH-RGD hydrogels under ischaemia was found to be significantly increased ($p < 0.001$) (**Supplementary Figure C**). No significant difference was observed in the metabolic activity of encapsulated cells after 7 and 11 days (**Figure 4C**). hMSC metabolic activity was found to be significantly lower in HA-PH-RGD hydrogels compared to HA-TA hydrogels after 4 days, however there was no difference in the number of live/dead cells/mm² at this timepoint (**Supplementary Figure E**). Analysis of cell viability demonstrated that RGD did not increase the percentage of live cells within the hydrogels (**Figure 4D**). Similarly, analysis of spent medium demonstrated that RGD did not improve VEGF and MCP-1 release over 11 days (**Figure 4E & F**).

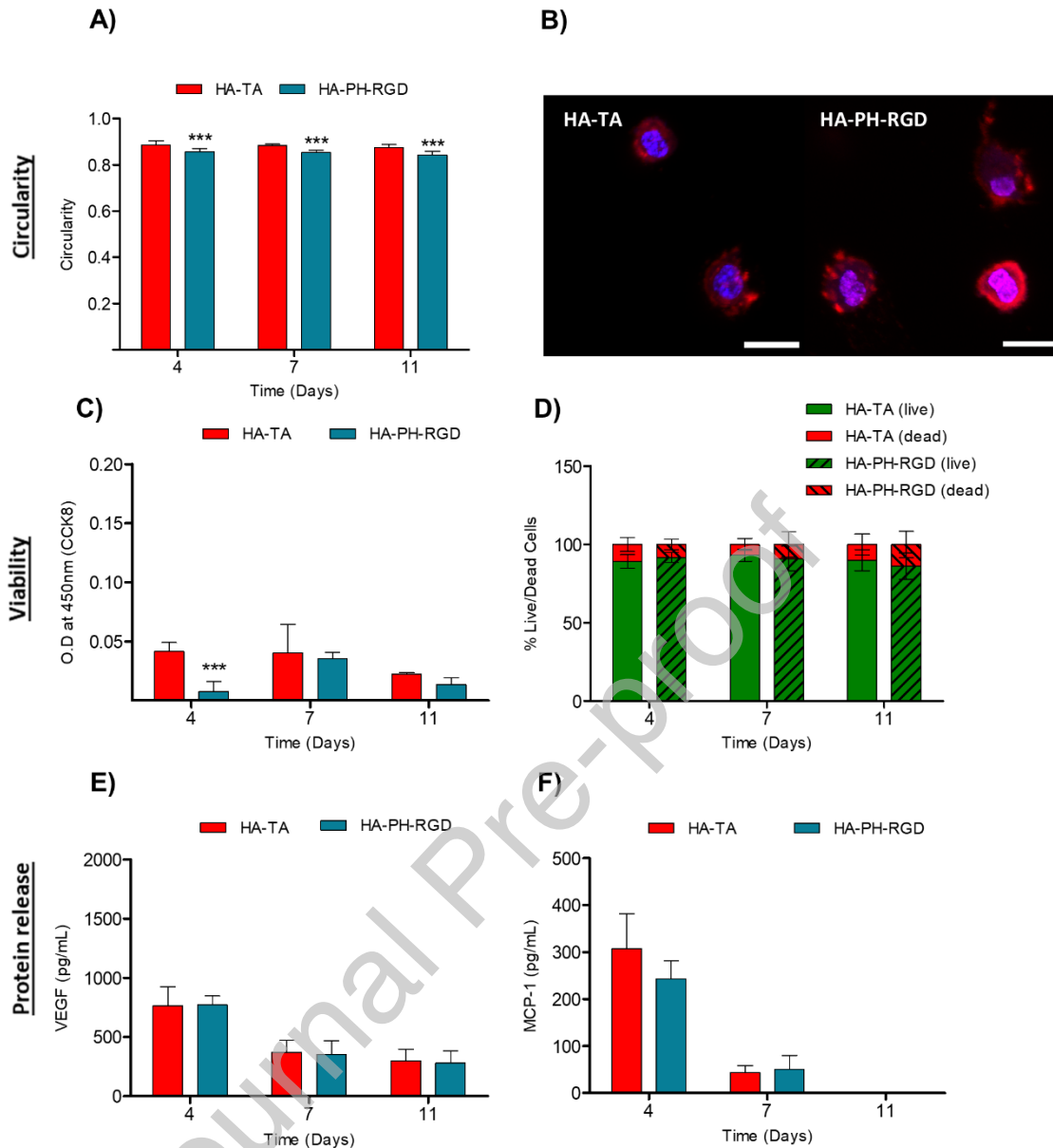


Figure 4 Effect of RGD on hMSCs in HA hydrogel under ischaemic culture conditions (A) Cell circularity was quantified. A value of 1 indicates a perfect circle and as the value approaches 0, the shape is increasingly elongate. hMSC spreading was significantly increased in HA-PH-RGD hydrogel, compared to HA-TA hydrogel. Results are expressed as the mean + SD ($n \geq 3$). *** $p < 0.001$. (B) DAPI (blue) and phalloidin (red) stained images of hMSCs seeded in HA hydrogel (with or without RGD) following 11 days in culture. Magnification 20x. Scale bar = 20 μ m. (C) Viability of hMSCs encapsulated in HA-TA and HA-PH-RGD hydrogels after 4, 7 and 11 days determined by CCK8 assay. *** $p < 0.001$. (D) Quantification of live/dead hMSCs in HA-TA and HA-PH-RGD hydrogel. Data shown as mean \pm SD ($n \geq 3$). Release of (E) VEGF and (F) MCP-1 from hMSCs encapsulated in HA-TA and HA-PH-RGD hydrogels at various time points determined by ELISA. Results are expressed as the mean + SD ($n = 4$). Statistical significance shown as compared to unmodified HA hydrogel.

3.4 Effect of pre-culture with RGD on hMSCs under ischaemic culture conditions

Given the association of cell adhesion and survival, we sought to further examine the effect RGD on hMSCs in ischaemia using a 4-day pre-culture, to enable cell interaction with the RGD peptide before transfer to ischaemic culture conditions. As shown in **Figure 5A**, upon transfer to ischaemic conditions at T0, hMSCs in HA-PH-RGD hydrogel were significantly less circular than those in HA-TA hydrogel, indicating cell spreading had occurred during the pre-culture period ($p < 0.001$). Cells in HA-PH-RGD remained significantly less circular over time, as shown at days 3, 7 and 11 compared to the control. Representative images of dapi/phalloidin staining show hMSC spreading in HA-PH-RGD hydrogel after 11 days (**Figure 5B**). When transferred to ischaemic culture conditions, hMSCs remained metabolically active in both HA-TA and HA-PH-RGD hydrogels, with no significant differences observed between groups (**Figure 5C**). However, RGD significantly increased the percentage of live cells following 11 days in ischaemia ($p < 0.01$) (**Figure 5D**), with quantification of live/dead cells/mm² demonstrating a significant reduction in the number of dead cells in HA-PH-RGD hydrogel, compared to HA-TA ($p < 0.001$) (**Supplementary Figure F**). Following pre-culture, RGD had no effect on VEGF release (**Figure 5E**). However, RGD significantly increased early MCP-1 release (**Figure 5F**). Staining demonstrates that hMSCs maintained marker expression over the 15 day (4 days normoxia followed by 11 days in adverse culture conditions) assessment period (**Figure 5G**).

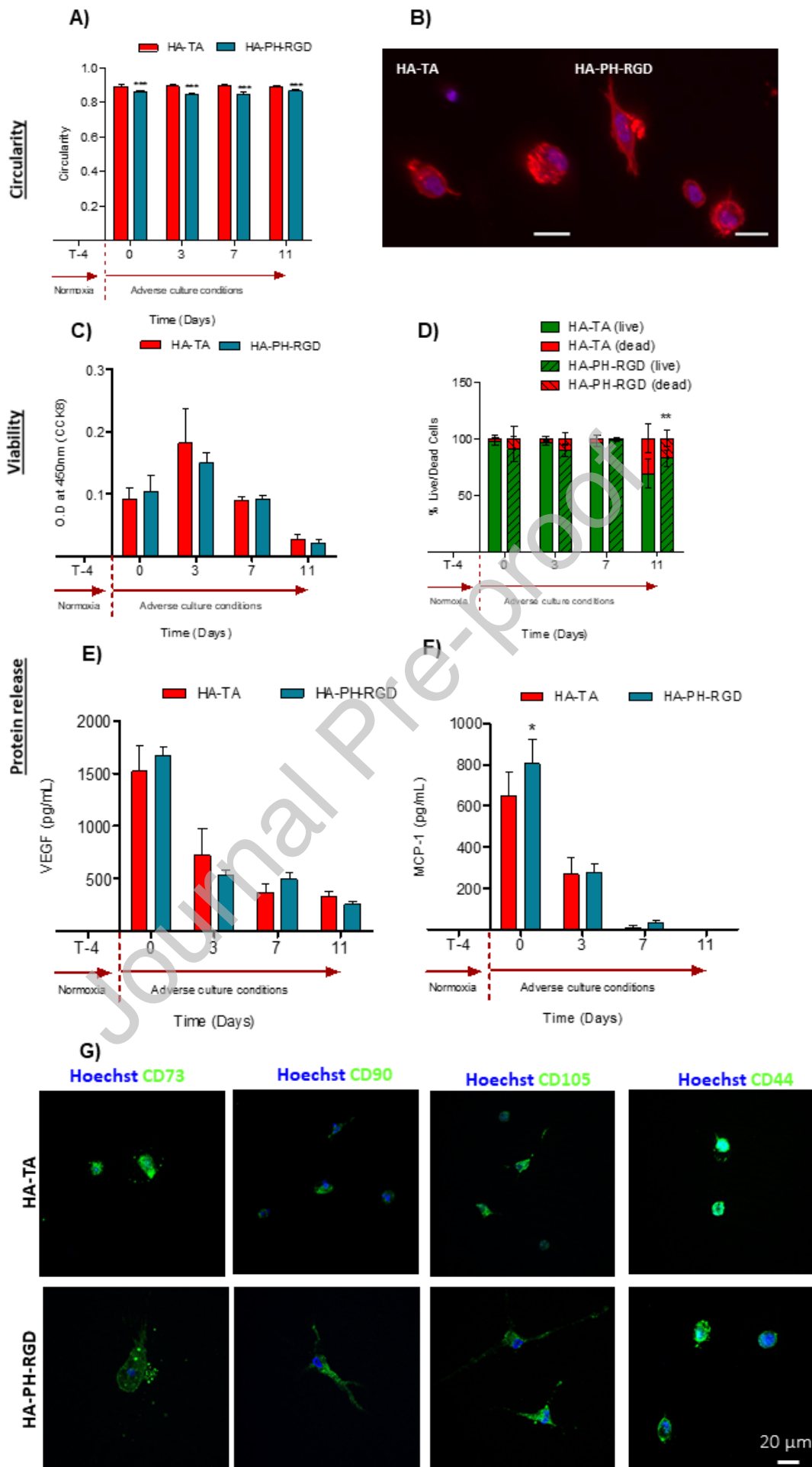


Figure 5 Effect of pre-culture with RGD on hMSCs in HA hydrogel under ischaemic culture conditions. (A) Cell circularity was quantified. A value of 1 indicates a perfect circle and as the value approaches 0, the shape is increasingly elongate. hMSC spreading was significantly increased in HA-PH-RGD hydrogel, compared to HA-TA hydrogel. Results are expressed as the mean + SD ($n \geq 3$). *** $p < 0.001$. (B) DAPI (blue) and phalloidin (red) stained images of hMSCs seeded in HA-TA hydrogel (with or without RGD) following 11 days in culture. Magnification 20x. Scale bar = 20 μ m. (C) Viability of hMSCs encapsulated in HA-TA and HA-PH-RGD hydrogels after 3, 7 and 11 days determined by CCK8 assay. (D) Quantification of live/dead hMSCs in HA-TA and HA-PH-RGD hydrogel showing the percentage of total live/dead cells where results are expressed as the mean \pm SD ($n \geq 3$). ** $p < 0.01$. Release of (E) VEGF and (F) MCP-1 from hMSCs encapsulated in HA-TA and HA-PH-RGD hydrogels at various time points determined by ELISA. Significant increase in MCP-1 from hMSCs in HA-PH-RGD hydrogels, compared to HA-TA hydrogel. Results expressed as mean + SD ($n = 4$). * $p < 0.05$. Statistical significance shown as compared to unmodified HA hydrogel. (G) hMSCs encapsulated in HA-TA and HA-PH-RGD hydrogels stained positive for stem cell markers (CD105, CD90, CD73 and CD44) after 15 days in culture (4 days in normoxia followed by 11 days in adverse culture conditions). Scale bar = 20 μ m.

4. Discussion

While progress has been made in cardiac stem cell therapy, significant clinical hurdles remain. One of the greatest challenges in translating cell therapy into routine clinical use is the limited retention and survival of transplanted cells in the harsh post-MI environment. The delivery of cells in hydrogels is a promising strategy to physically retain cells at the target site while also providing structural support to protect cells and maintain their viability [53]. Although RGD-functionalised hydrogels are commonly employed as cell delivery vehicles to ischaemic injury sites like the heart, few studies have systematically quantified the effect of RGD under ischaemia. In this study, we provide a detailed analysis of hMSC behaviour in RGD-functionalised HA hydrogel. We show that RGD-functionalised HA can significantly reduce encapsulated cell death under ischaemia, compared to unfunctionalised HA; but a pre-culture period is necessary to allow cells to interact with RGD before exposure to harsh conditions.

Interestingly, conflicting results have been obtained from studies which compare RGD-modified and unmodified scaffolds *in vivo* [54] When compared with unmodified alginate, RGD-alginate demonstrated a significant increase in angiogenesis in an animal MI model (12.6 ± 2.7 versus 9.3 ± 4.2 arteriole/ mm^2 , respectively [55]. However, a comparative study revealed unmodified scaffolds promoted better left ventricular fractional shortening, greater fractional area changes, more

attenuation of left ventricular dilatation, a lower left ventricle expansion index, and more scar thickness than RGD-modified alginate scaffolds [56]. In light of these observations, further experiments are necessary to elucidate under what conditions RGD has beneficial effects over unmodified scaffolds and its effectiveness in improving cardiac function in order to determine the added value of RGD introduction.

Evaluation of a biological scaffold typically involves two key aspects; whether the mechanical strength of the hydrogel material is suitable for practical applications and if the hydrogel can support cell growth and adhesion [57]. Our results demonstrate that both HA-TA and HA-PH-RGD hydrogels formed rapidly. Rapid gelation is a desirable property for *in vitro* work as it prevents the settling of cells during the encapsulation process [58]. For clinical translation, the ability to control gelation is of great importance as a long gelation time could result in leakage of the polymer solution from the injection site and dispersion into non-target organs, while a short gelation time may lead to needle blockage and make delivery via multiple injection sites unachievable [23,59]. The gelation kinetic of HA-TA hydrogels has been extensively researched and can be easily modified by adjusting the concentration of HRP, with a higher concentration leading to a decreased gelation time [60–62]. Similarly, the mechanical strength of HA-TA hydrogels can be easily manipulated by adjusting the catalysts or by increasing polymer concentration to alter the crosslinking density of the polymer networks [60,62,63]. The incorporation of the RGD peptide did not significantly alter the compressive modulus of hydrogels compared to unmodified hydrogel controls over time, see Figure 2D. The compressive moduli of HA-TA and HA-PH-RGD hydrogels encapsulating 1 million hMSCs/mL was approximately 2 kPa over the culture period of 11 days, which is in the range of Algisyl-LVR™ (3-5 kPa) but lower than both normal (10-15 kPa) and pathological cardiac tissue (35-70 kPa) [32,64]. For bulking purposes, stiff materials are advantageous as they may provide structural support to the damaged myocardial wall and improve function [26,65]. However, as reported by Lei *et al.*, stiff

materials may not be favourable for cell delivery as increased crosslinking and polymer concentration can negatively affect hMSC spreading, migration and proliferation, compared to softer matrices [66]. Mechanical properties have been shown to strongly modulate stem cell fate [67]. In addition to stiffness; nanotopography, protein composition, and stress-strain can influence stem cell lineage commitment. Hearts beat in rhythmic contraction-relaxation cycles, thus the stress and strain applied to culture environments are important considerations for ECM models [68]. Future studies on the mechanical aspects of engineered materials in combination with the mechanical forces of the *in vivo* environment can be used to understand stem cell fate.

The optical transparency of our HA-PH-RGD hydrogel enabled the visualisation of encapsulated cells in a non-invasive manner and imaging revealed that hMSCs were uniformly distributed throughout the hydrogel. hMSC viability was high (>90%) 24 hour post-encapsulation in HA-TA and HA-PH-RGD hydrogels. While viability was maintained throughout the cultivation period, the RGD peptide did not increase the number of viable cells within the hydrogel. The failure of RGD to increase cell proliferation in a 3D microenvironment has been observed in multiple studies using a variety of cell types including MSCs and osteoblasts [69,70]. Interestingly, RGD is known to increase cell proliferation in 2D studies, whereby cells are cultured on top of RGD-modified hydrogels [38,71]. The effect of RGD on cell morphology has been widely observed [38,66,72–74]. As expected, the RGD peptide significantly increased hMSC spreading, with hMSCs in HA-PH-RGD hydrogels exhibiting a typical spindle shape with protruding pseudopods indicative of strong adhesion, while hMSCs in HA-TA hydrogel retained a spherical morphology. hMSCs in RGD-modified HA hydrogel secreted more VEGF than hMSCs in the unmodified control. VEGF is a potent angiogenic factor frequently investigated for the treatment of ischaemic heart disease [75,76]. Treatment with VEGF protein has been shown to improve coronary blood flow and regional myocardial function in an animal model of chronic myocardial ischaemia [77]. Although several phase I trials demonstrated the safety of recombinant VEGF protein for therapeutic angiogenesis in patients, the phase II VIVA trial reported IC VEGF administration was ineffective [78,79]. This was later attributed to the short half-life of VEGF and low myocardial uptake following coronary infusion [80]. In order to overcome these limitations, researchers are increasingly turning to delivery of the VEGF gene rather than the protein

[81,82]. However, while the IM injection of VEGF plasmid DNA was shown to be safe [83], both the EUROINJET-ONE [84] and NORTHERN [85] trials found no significant clinical benefit. Alternative strategies to enhance the therapeutic effect of VEGF are currently under investigation including delivery of VEGF-transfected MSCs [86], the sustained release of VEGF through PLGA microparticles [87] and conjugation of VEGF to an injectable hydrogel [88]. The success of these approaches in preclinical models provides strong evidence that the controlled and targeted delivery of VEGF is necessary to achieve clinically relevant vascular growth. The HA-PH-RGD hydrogel described in this study could potentially be used to immobilise MSCs in myocardial tissue to facilitate the sustained release of VEGF to enhance cardiac repair in normoxia.

The RGD peptide also significantly enhanced MCP-1 secretion from hMSCs in HA hydrogel. MCP-1 is a chemotactic factor that is released by a variety of cells in response to pro-inflammatory stimuli to attract white blood cells to sites of insult or injury [89]. In addition to recruiting monocytes to the ischaemic myocardium, MCP-1 is known to affect many processes involved in MI. Like VEGF, MCP-1 mediates angiogenesis and has been shown to stimulate the formation of new blood vessels in the infarcted heart [90,91]. MCP-1 has been shown to inhibit apoptosis of cardiomyocytes under hypoxia to enhance their survival [92,93]. Transgenic mouse studies have demonstrated that cardiac overexpression of MCP-1 prevents LV dysfunction and remodelling after MI, providing further evidence of the cardioprotective effects of MCP-1 [94]. Lastly, it has been demonstrated that MCP-1 is involved in the homing of stem cells, including MSCs, to the injured myocardium [95]. Neural crest stem cells were found to migrate and assemble at the ischaemic border zone area of infarcted hearts in response to MCP-1, where they contributed to cardiac regeneration [96]. These findings make MCP-1 a potential target for therapeutic intervention in MI [95,97]. It should be noted that the release of MCP-1 may also have a deleterious effect. Frangogiannis *et al.* [98] reported that MCP-1, identified in the ischemic myocardium after repetitive cycles of brief ischemia/reperfusion, was associated with interstitial fibrosis, LV dysfunction, elevated collagen content and macrophage infiltration. The underlying reason why RGD enhances VEGF and MCP-1 release from hMSCs is unclear and merits further examination. However, these findings are consistent with a recent study by Ho *et al.*, who

reported hMSCs spheroids in RGD-modified alginate hydrogel secreted more VEGF and MCP-1 than spheroids in unmodified gels [99].

As adhesion is known influence cell survival, we sought to examine the effect of hMSC adhesion to RGD on survival and function under ischaemic culture conditions. Cell adhesion occurs in three stages: attachment, spreading and formation of focal adhesions and stress fibres [100]. Focal adhesions are protein complexes which act as transmembrane links between the ECM and the actin cytoskeleton [101]. Following initial cell-ligand attachment, cells increase their surface area contact through the formation of actin microfilaments and spreading. If the appropriate signals are provided, cells continue to organise their cytoskeleton as indicated by the formation of focal adhesions and actin-containing stress fibres [100]. Under oxygen and nutrient deprived conditions, RGD was found to increase hMSC spreading compared to unmodified hydrogel; however, visualisation of hMSCs in HA-PH-RGD hydrogel revealed a spherical morphology indicative of weak adhesion to the ligand. The initial attachment of cell integrins to ECM components is a passive process. However after attachment, the transition from early spreading to late spreading is an active process that requires energy expenditure [100]. Further analysis of cell viability and protein release found that this weak attachment of hMSCs to RGD did not enhance cell viability, as assessed by CCK8 and live/dead staining, or function, as assessed by VEGF and MCP-1 release, under ischaemic culture conditions.

In light of these findings, a new experimental plan was devised whereby hMSCs were given time to adhere strongly to RGD in HA hydrogel before exposure to adverse culture conditions. Multiple reports have identified cell spreading in RGD modified hydrogels after 3-5 days in culture [73,99,102]. Therefore, hMSCs were given 4 days under standard culture conditions (pre-culture) before they were transferred to adverse culture conditions. Following delayed transfer to ischaemic culture conditions, hMSCs in HA-PH-RGD were found to maintain their elongated, spindle-like morphology after 11 days. Adhesion is a reversible process, and such reversal may occur under both normal and pathological conditions [100]. Our results indicate that de-adhesion did not occur. Strong adhesion of hMSCs was found to increase the percentage of live cells within the hydrogel construct. A significant reduction in the number of dead cells was observed in HA-PH-RGD hydrogel after 11 days. Early release of the chemoattractant protein MCP-1 was also increased compared to unmodified

HA. MCP-1 is typically released by cells in response to pro-inflammatory stimuli, growth factors or cytokines in order to attract and activate white blood cells to sites of insult or injury [89]. These observations provide evidence that the adhesion of cells to RGD prior to transplantation may subsequently enhance their longevity and function in the ischaemic myocardium.

RGD-modified hydrogels have been widely used as a method to deliver stem cells to cardiac muscle [19,23,103]. However a recent study from our lab investigating the efficacy of an epicardial device SPREADS loaded with HA-PH-RGD and cells in a chronic porcine study, found no significant difference in % LVEF between the groups treated with and without cells (although both had a significantly higher % LVEF 28 days post-treatment when compared with gold standard). The results presented in this study allow us to further understand this outcome, as we have shown that the viability and function of cells in HA hydrogel is negatively affected under ischaemic conditions and that the presence of RGD attachment sites did not help cells to overcome these effects. Here we report that cells pre-cultured with RGD perform better under ischaemic conditions, suggesting that more beneficial effects can be achieved if stem cells are given time to adhere to the RGD peptide *ex vivo*, prior to transplantation. While the delivery of a preformed hydrogel poses significant challenges, we have designed the SPREADS device which can be prefilled prior to delivery and will allow pre-cultured gels to be delivery to the epicardial surface [26] and as such enabling cell therapy to reach its full potential.

5. Conclusion

The development of a biomaterial scaffold capable of maintaining cell viability while promoting attachment and function represents a major research goal in the field of cardiac tissue engineering. This study confirms the suitability of RGD-modified HA hydrogel for this purpose. hMSCs in RGD-modified HA hydrogels demonstrated significantly greater cell spreading and protein secretion compared to hMSCs in unmodified HA hydrogel. A pre-culture period allowing strong adhesion of hMSCs to the RGD peptide was shown to improve cell survival under adverse culture conditions. This finding may have a significant effect on the use of adhesive ligands to enhance stem cell engraftment in ischaemic injury sites.

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Disclosures

Lenka Kohutova and Martin Pravda are employed by Contipro. Vladimir Velebny is the owner of Contipro.

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