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Development of a thermoresponsive chitosan gel combined with human mesenchymal stem cells and desferrioxamine as a multimodal pro-angiogenic therapeutic for the treatment of critical limb ischemia

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Abstract

Critical limb ischemia (CLI) is a debilitating ischemic disease caused by vascular occlusion. Pro-angiogenic therapeutics have the potential to produce collateral vasculature, delaying or negating the need for amputation or invasive revascularisation. Thermoresponsive hydrogels can provide an in situ depot for the sustained release of drugs and provide protection and cohesion for encapsulated cells. Human Mesenchymal Stem Cells (hMSCs) have demonstrated strong angiogenic potential in vitro and angiogenic efficacy in vivo. Desferrioxamine (DFO), a pharmacological activator of the pro-angiogenic hypoxia inducible factor-1 α pathway, has shown pro-angiogenic efficacy in vivo. This study combined hMSCs and DFO with a thermoresponsive chitosan/ β -glycerophosphate (β -GP) gel, to function as an injectable, multimodal, pro-angiogenic therapeutic for the treatment of CLI. This gel underwent a thermogelation beginning at 33°C, and provided a sustained, biologically active release of DFO over the space of seven days, whilst permitting the survival, proliferation and migration of encapsulated hMSCs. hMSCs encapsulated in gel containing a 100 μ M concentration of DFO displayed an upregulation in VEGF expression. The combination of hMSCs and DFO within the gel resulted in a synergistic enhancement in bioactivity, as measured by increased VEGF expression in gel-exposed human umbilical vein endothelial cells. This formulation displays significant potential as an injectable pro-angiogenic therapeutic for the treatment of CLI.

Keywords: Chitosan, Desferrioxamine, hMSC, Pro-angiogenic, Thermoresponsive, Hydrogel

Introduction

Critical limb ischemia (CLI) is a severe, end-stage manifestation of peripheral arterial disease (PAD). PAD most commonly refers to a set of disorders in which progressive occlusion of peripheral arteries results in impaired blood flow to the lower limb. Symptoms of CLI include pain at rest, ulceration and gangrene. Amputation of the affected limb is necessary in approximately 30% of cases and prognosis following amputation is bleak, with a mere 30% survival rate within five years [1-2]. Treatment often requires invasive revascularisation procedures, which are not suitable for a significant percentage of patients. A need for new therapeutic strategies which are less invasive and more inclusive is apparent. Therapeutic angiogenic treatments

could facilitate the growth of collateral vasculature, restoring blood flow to affected tissues, and potentially delay or negate the use of revascularisation, and reduce the need for amputation [3].

To date, clinical trials investigating the safety and efficacy of pro-angiogenic therapy in PAD patients including gene, growth factor or cell-based therapies have found that treatments were generally well tolerated but that efficacy was modest or inconsistent, a fact which has been partially attributed to incomplete knowledge of appropriate doses, duration of dosage and delivery methods. Current strategies have not yet resulted in satisfactory clinical efficacy and the use of single pro-angiogenic agents does not appear sufficient to adequately drive the complex vascularisation process in humans [1, 4-5].

The co-delivery of more than one agent has been shown to produce synergistic increases in pro-angiogenic efficacy in pre-clinical models of CLI [6-7]. Similarly, sustained release of pro-angiogenic agents from scaffolds or gels enhances vascular growth when compared to simple bolus delivery [6, 8-10]. hMSC delivery can be enhanced through the use of hydrogel delivery vehicles, in terms of cell engraftment at the injection site. Increased pro-angiogenic activity and ability to promote wound healing have also been reported when delivering hMSCs in a hydrogel matrix [11-12]. The aim of this study was to formulate a multimodal pro-angiogenic therapeutic which capitalised on the efficacious concepts of co-delivery and sustained release, utilising a pro-angiogenic cell source, human mesenchymal stem cells (hMSCs) and a pharmacological pro-angiogenic, desferrioxamine (DFO), suspended within a thermoresponsive chitosan gel, to act as a potential intra-muscular injection for the treatment of CLI.

The administration of therapeutic agents such as drugs or cells via an injectable, thermoresponsive hydrogel delivery vehicle is a concept which is gaining popularity. Such materials can potentially provide a depot for the sustained release of incorporated pharmaceuticals or growth factors and a protective and cohesive environment for encapsulated cells, while permitting a minimally-invasive administration via injection and subsequent sol-gel transition *in situ* [13-15]. Chitosan/ β -GP gels have attracted interest due to their biocompatibility, biodegradation, potential to support the viability of encapsulated cells and produce a sustained delivery of incorporated agents [16-18]. Within this formulation a chitosan/ β -GP gel is conceived as providing support and protection for encapsulated hMSCs while enforcing cohesion at an injection site *in situ* and also permitting a sustained release of DFO.

hMSCs possess an innate angiogenic potential, in terms of the secretion of soluble growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1) and placental growth factor (PLGF), which exert pro-angiogenic actions in a paracrine manner, and the ability to provide direct contact support and extra-cellular matrix components to other vascular cells during vessel development [19-21].

DFO is a prolyl-hydroxylase inhibitor which indirectly facilitates active Hypoxia Inducible Factor (HIF) signalling and is thereby capable of eliciting a multifaceted angiogenic response, effected by the transcriptional targets of HIF -1 α , such as VEGF and angiopoietin-2 (ANG-2) [22]. DFO has been shown to increase nuclear HIF-1 α accumulation, upregulate expression of VEGF in MSCs and increase vascular growth and tissue perfusion in models of ischemic disease [23-26].

Here we present the *in vitro* development and characterisation of this formulation. A multimodal therapeutic such as this must fulfil certain requirements *in vitro* before

consideration as a candidate for further *in vivo* studies. A thermoresponsive delivery vehicle should undergo a rapid sol-gel transition at or below body temperature and be fully syringable at room temperature. The formulation should permit the survival and ideally proliferation of encapsulated cells, while enabling a sustained release of both incorporated drug molecules and cell-secreted paracrine factors and allow cells to migrate into surrounding tissues to exert further therapeutic benefit. Ideally, a multimodal formulation should produce a synergistic enhancement in pro-angiogenic bioactivity when compared to use of a single treatment modality. This study addressed the hypothesis that a chitosan/ β -GP carrier vehicle combined with hMSCs and DFO will fulfil these criteria.

2. Materials and Methods

2.1 Preparation of chitosan/ β -GP gels

Ultra-pure chitosan with a degree of deacetylation > 95% was used in the preparation of all gels (UP CL214 from Pronova Biomedical, Oslo, Norway). For a 2% w/v chitosan, 7% w/v β -Glycerol Phosphate (β -GP) gel, 100 mg of chitosan was dissolved in 4.5mL dH₂O at pH 8-9. 350mg of β -GP (Sigma Aldrich, Ireland) was dissolved in 0.5mL dH₂O, also at pH 8-9, and chilled. The β -GP solution was then added drop by drop to the chitosan solution with stirring on ice to achieve a homogenous gel. Gels were stored on ice until use soon after. Gels containing DFO were prepared by dissolving DFO (Sigma, Ireland) to the correct concentration in the constituent water prior to addition of chitosan or β -GP. Gels containing hMSCs were produced through simple resuspension of cell pellets in gel by pipetting.

2.2 Rheological testing

The rheological properties of chitosan/ β -GP gels were assessed using oscillatory measurements on an AR-1000 cone and plate rheometer (TA Instruments). The thermoresponsiveness of the gels were assessed as a function of temperature and over time at a constant temperature of 37°C, with storage modulus G' being used as an indicator of gel structure. The temperature was increased by 1°C/min using a temperature sweep mode and typically ranged between 20-50°C. Assessments at constant temperature over time employed a solvent trap to prevent dehydration of the sample.

2.3 DFO release from chitosan/ β -GP gels

2% w/v chitosan, 7% w/v β -GP gels were prepared containing DFO at 15, 50 or 100 μ M concentrations. These concentrations were chosen since previous work within our research group has shown that DFO concentrations within this range elicited increased VEGF expression and nuclear HIF-1 α accumulation in hMSCs which were cultured in DFO-containing growth media (data not shown). A defined quantity of each concentration of DFO gel was placed in a glass vial and allowed to gel in a waterbath for one hour at 37°C. 2mL of phosphate buffer (pH 7.1) was added to the vial and the samples were allowed to incubate at 37°C while shaking at 75 rpm for the duration of the release study. The phosphate buffer was completely removed and replaced at 4hrs, 24hrs, day 3, day 5 and day 7 and frozen until analysis. All studies were performed in triplicate. Samples were analysed for DFO content via high-performance liquid chromatography (HPLC) on an Agilent 1120 Compact LC with a Phenomenex Gemini 5u C18 column, mobile phase acetonitrile: phosphate buffer (10%:90% v/v), containing 20mM ethylenediaminetetraacetic acid (EDTA), pH adjusted to 6.5 and UV detection at 440 nm, adapted from the procedure described by van der Horst et al. [27]. All samples were filtered with 0.45 μ M

Durapore PVDF filters (Millipore, Ireland) and combined with 4mM Iron (III) chloride (Sigma Aldrich, Ireland) in a 1:1 ratio immediately before analysis via HPLC. The HPLC assay was validated for linearity, repeatability and reproducibility.

2.4 Assessment of Bioactivity

The bioactivity of the gel formulations was investigated using human umbilical vein endothelial cell (HUVEC) monolayers, since pro-angiogenic agents released from the gel *in situ* are envisaged as acting on nearby blood vessel networks. HUVECs were cultured in adherent 6-well plates at a density of 1×10^5 cells per well in fully supplemented HUVEC growth media (EGM-2 Bulletkit, Lonza, UK), under normal culture conditions. Normal chitosan/ β -GP gels or gels containing DFO at a $100\mu\text{M}$ concentration were prepared. Gels contained hMSCs at a concentration of $1 \times 10^6/\text{mL}$ or were left cell free. $300\mu\text{L}$ of the gels were added to hanging well cell culture inserts with a pore size of $5\mu\text{m}$ (Millipore, Ireland) and allowed to gel at 37°C for 30 minutes. Inserts were then placed in wells along with HUVEC monolayer cultures, where the final volume of media was 3mL basolaterally and 1mL apically. Inserts hung above adherent HUVEC samples whereby substances released from the sequestered gel were permitted to diffuse across the insert membrane and into the growth media (**Fig. 3, A**). Wells containing no inserts, or unmodified, cell-free gels acted as controls ($n=3$). HUVEC samples were harvested via trypsinisation at 24, 48, 72 and 120 hrs. Growth media on 120 hrs samples was removed and replaced with fresh media at 72hrs. RNA was isolated using the RNeasy Mini Kit (Qiagen, Ireland) according to the manufacturer's protocol. RNA quality and quantity was determined using an RNA nanodrop (absorbance: 260 nm). Reverse-transcription of RNA samples was implemented using 400 ng of RNA, according to the manufacturer's instructions (Qiagen, Ireland). Quantitative PCR was carried out using the 7500 Real Time polymerase chain reaction system (Applied Biosystems). A QuantiTect SYBR Green PCR kit (Qiagen, Ireland) was used for this process according to the manufacturer's protocol, utilising the QuantiTect primer for VEGF (Hs_VEGFA_6_SG) and the 18s primer (Hs_RRN18S_1_SG) as a housekeeping gene. VEGF expression was determined using the relative quantification $\Delta\Delta\text{Ct}$ method [28].

2.5 Cell Culture

hMSCs were derived from bone marrow aspirates obtained from human volunteers, with informed consent at REMEDI, the National University of Ireland, Galway. All procedures were 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 (tri-lineage differentiation and a full panel of cell surface markers), as published in Duffy *et al.* [21]. hMSCs were cultured in T175 tissue culture flasks (Sarstedt, Ireland) in low glucose Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma Aldrich, Ireland) at 37°C and in a 5% carbon dioxide environment. Media was replaced every three days and cells were passaged upon reaching 80-90% confluency at a ratio of 1:3.

2.6 Encapsulated hMSC viability

hMSCs were suspended in a 2% w/v chitosan, 7% w/v β -GP gel at a density of 2.5×10^5 cells/mL. This was aspirated into a 1mL syringe and syringed through a 22G needle to form small (2mm diameter) gel droplets on the floor of a 12-well plate. These were allowed to gel at 37°C for 30 minutes before addition of normal hMSC growth media. Cells were cultured in gel for 16 days and droplets were stained with a Live/Dead stain (Molecular Probes, Invitrogen, Ireland) according to the

manufacturer's protocol, at regular intervals throughout this period to assess cell viability over time. Droplets were visualised via fluorescence microscopy on a Leica DMIL microscope (Leica Microsystems, Switzerland). Live cells were stained green with green-fluorescent calcein-AM and dead cells were stained red with red-fluorescent ethidium homodimer-1.

2.7 Encapsulated hMSC proliferation

hMSCs were suspended in a chitosan/ β -GP gel as described above. 150 μ L of this gel was then added into a hanging well cell culture insert with a pore size of 1 μ m, suitable for insertion into a 48-well plate (Scaffdex, Finland). This was allowed to gel at 37°C for 30 minutes. 700 μ L of normal hMSC growth media was then added per well (400 μ L basolaterally, 300 μ L apically). Inserts were cultured up until 24hrs, 48hrs, day 4 and day 7. The gel was then removed from the inserts and homogenised through the addition of 500 μ L a 0.2M NaHCO₃, 1% Triton-X lysis buffer. Lysates were stored at -80°C and underwent three freeze-thaw cycles prior to analysis. Double-stranded DNA (dsDNA) levels within the gel lysates were measured using the Quant-iT PicoGreen dsDNA assay (Molecular Probes, Invitrogen, Ireland) according to the manufacturer's protocol.

2.8 Encapsulated hMSC migration

The assay is based on the concept that following serum starvation, cells will migrate towards a serum stimulus [21]. hMSCs were harvested from T175 tissue culture flasks via trypsinisation. Cells were washed twice with serum-free growth media and were suspended in a chitosan/ β -GP gel at a density of 5 X 10⁵ cells/mL. 150 μ L of this gel was added to hanging well cell culture inserts with a pore size of 8 μ m, suitable for insertion into a 12-well plate (Millipore, Ireland). This was allowed to gel at 37°C for 30 minutes. 2mL of serum-free hMSC growth media was then added basolaterally and 0.5mL was added apically. Cells were allowed to incubate in serum free media and were deprived of serum for a total of two hours, at which point basolateral media was replaced with growth media supplemented with 20% FBS. Samples were harvested at 24 hours, day 5 and day 7. Insert membranes were gently rinsed with PBS repeatedly to remove the gel. The inserts were immersed in 4% formalin for 10 minutes to fix adherent cells and stained with hematoxylin for a further 10 minutes. Following a final rinse with PBS the membranes were removed with a scalpel blade. Membranes were mounted on a glass slide, bottom side down. Cell migration was quantified by counting cells from five random fields on the underside of the membrane at 20X magnification.

2.9 Statistical analysis

One way ANOVA followed by pairwise Holm-Sidak analysis was performed unless otherwise stated. Error is reported as standard deviation (SD) and significance was determined using a probability value of P < 0.05. A minimum of N=3 replicates were performed for all experiments.

3. Results

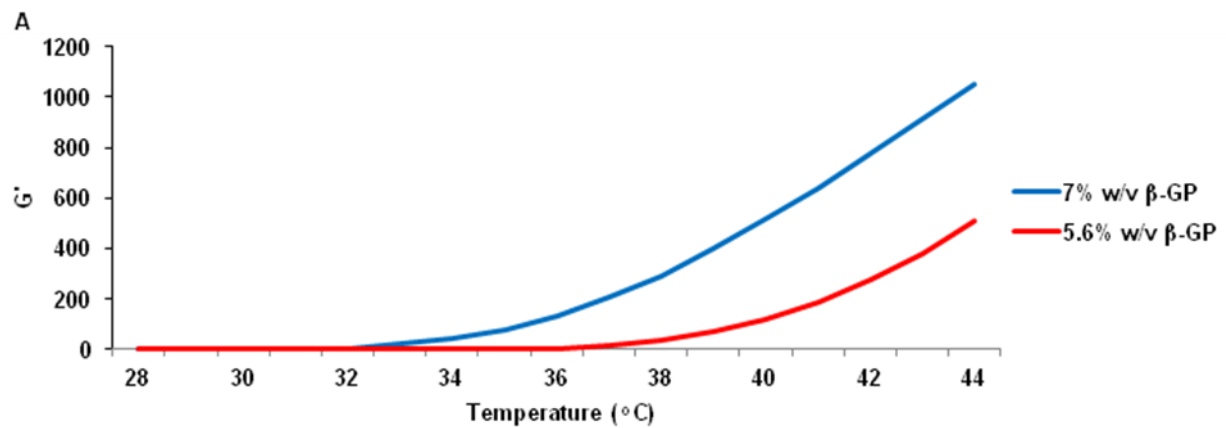
3.1 Rheological testing of chitosan/ β -GP gels

The thermoresponsiveness of chitosan/ β -GP gels was assessed using an oscillatory temperature sweep on an AR-1000 rheometer. The temperature was varied by 1 °C/min and the sweep extended from 20-50°C at a frequency of 0.5 hertz. Two gel formulations were assessed, a 2% w/v chitosan, 5.6% w/v β -GP gel (pH 7-7.2) [16], and a 2% w/v chitosan, 7% w/v β -GP gel (pH 7-7.2). The 5.6% w/v β -GP gel displayed a thermogelation temperature of 37°C, evidenced by an increase in the storage modulus at that temperature. The 7% w/v β -GP gel displayed a lower

gelation temperature of 33°C. Both gels maintained a pH measurement within the physiological range (7-7.2) (**Fig. 1, A**).

Both gels were also assessed for their thermoresponsivity over time at 37°C (7% w/v = 8hrs, 5.6% w/v = 5hrs). The 7% w/v gel displayed a more rapid increase in storage modulus and achieved consistently higher storage modulus figures at all comparable time points. As a result it was decided to continue with a 7% w/v β -GP gel due to these observed enhancements in both gelation temperature and storage modulus (**Fig. 1, B**).

The final formulation would require the incorporation of hMSCs and DFO with the gel. Therefore the gel was combined with hMSCs (2.5×10^5 /mL) or DFO at a 100 μ M concentration and assessed during a temperature sweep from 20-50°C to determine if the addition of either hMSCs or DFO had any effect on the thermoresponsive profile. Results from three independent gels (mean + SD plotted) show that the temperature of thermogelation was unaffected by the addition of hMSCs or DFO. The storage modulus achieved at 37°C was not affected by the addition of DFO or hMSCs (**Fig. 1, C**).



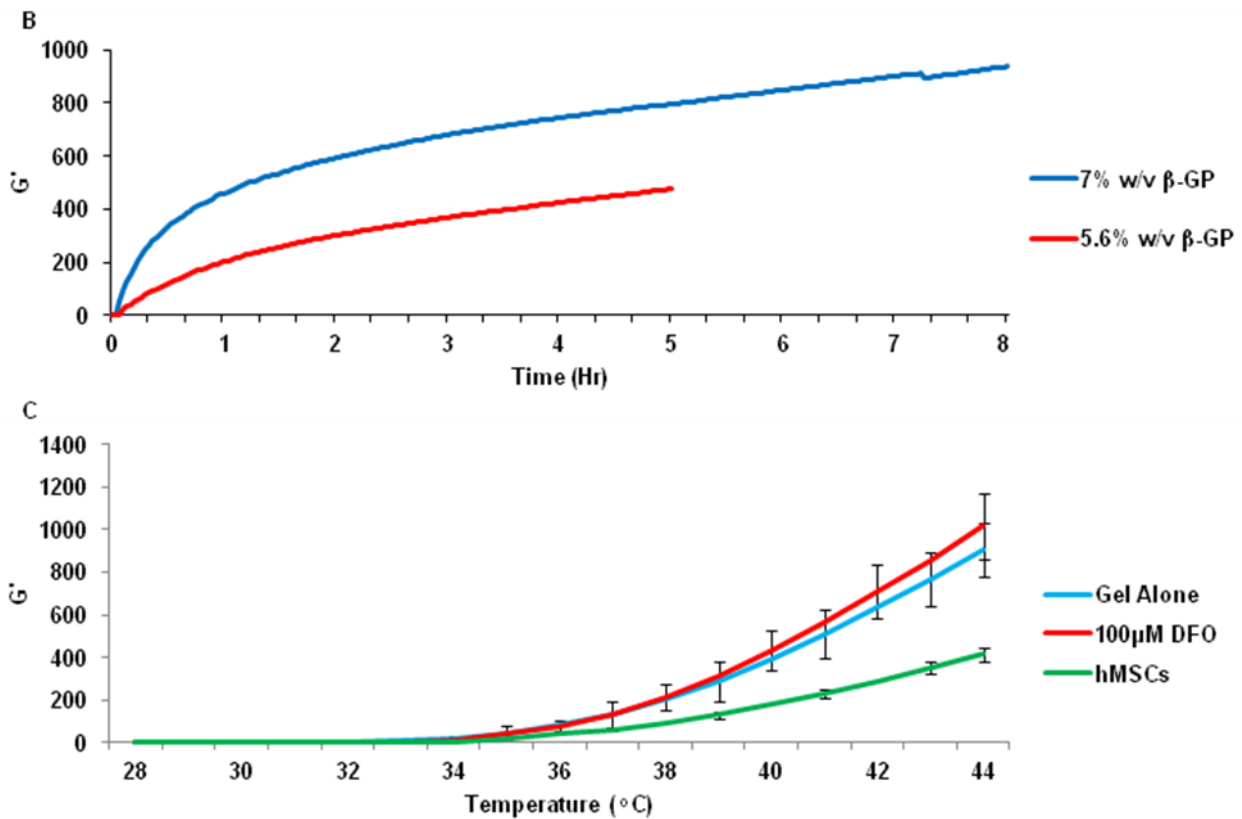


Figure 1 (A) 2% w/v chitosan, 5.6% w/v β -GP gel was shown to undergo an increase in the storage modulus G' , beginning at 37°C, while a 2% w/v chitosan, 7% w/v β -GP gel underwent a comparable increase beginning at 33°C. Oscillatory rheological measurements were taken during a temperature sweep from 20-50°C. Both gels had a final pH of 7-7.2. **(B)** A 2% w/v chitosan, 7% w/v β -GP gel was shown to undergo an increase in the storage modulus G' , when oscillatory rheological measurements were taken over a period of eight hours at a constant 37°C. This increase was most rapid within the first hour and more measured thereafter. A 2% w/v chitosan, 5.6% w/v β -GP gel did not achieve the same increase in storage modulus when placed in the same conditions for 5 hours. **(C)** 2% w/v chitosan, 7% w/v β -GP gels were combined with either hMSCs (2.5×10^5 /mL) or DFO (100 μ M). The storage modulus G' , was assessed via oscillatory rheological measurements taken during a temperature sweep from 20-50°C. The temperature of thermogelation (33°C) was unaltered by the addition of hMSCs or DFO to the gel. The storage modulus recorded at 37°C was unaffected by the addition of DFO or hMSCs when compared to an unmodified gel. Data was collected from three independent gels (mean + SD plotted).

3.2 DFO release from chitosan/ β -GP gels + Bioactivity of released DFO

Chitosan/ β -GP gels were prepared containing 15 μ M, 50 μ M or 100 μ M concentrations of DFO. Release of DFO from the gels into phosphate buffer at 37°C over the course of seven days was measured via HPLC, to assess whether the gel could sustain DFO release for an extended period. The results show that release from the gels at a given timepoint was linearly related to DFO concentration. All gels produced an initial burst release within the first four hours and decreasing but sustained release over the course of seven days. Gels were homogenised on day 8 and the amount of DFO extracted was measured (**Fig. 2, A**).

Cumulative release was calculated from this data. Up to 59% of the DFO initially incorporated in the gel was released over seven days in the case of the 15 μ M gels. Cumulative release from 50 μ M and 100 μ M gels reached 51% and 49% respectively. It was possible to extract an additional 4.5%, 2.5% or 1.7% of the DFO initially incorporated, from the 15, 50 and 100 μ M DFO gels, respectively, via homogenisation on day 8 (**Fig. 2, B**).

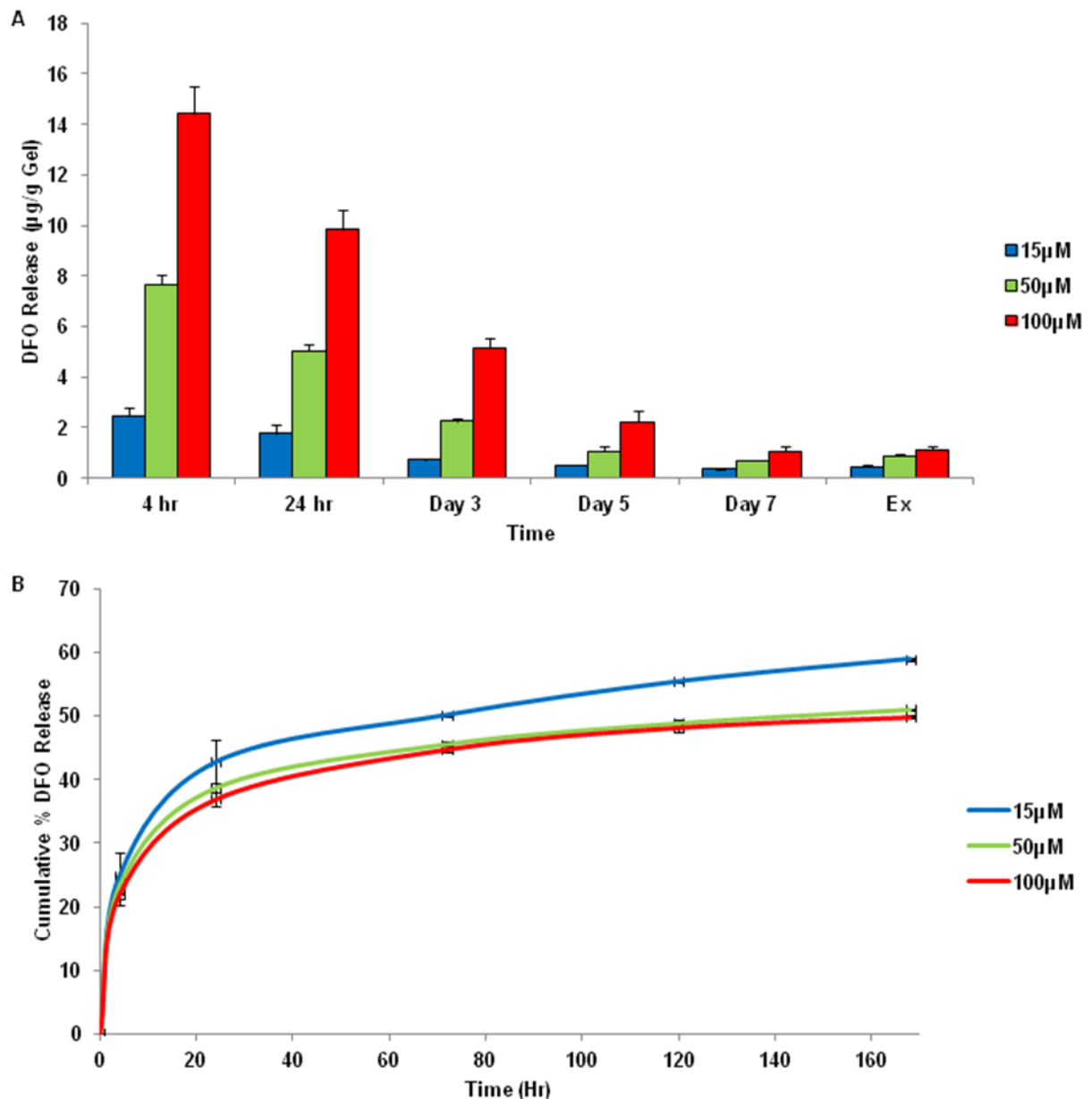
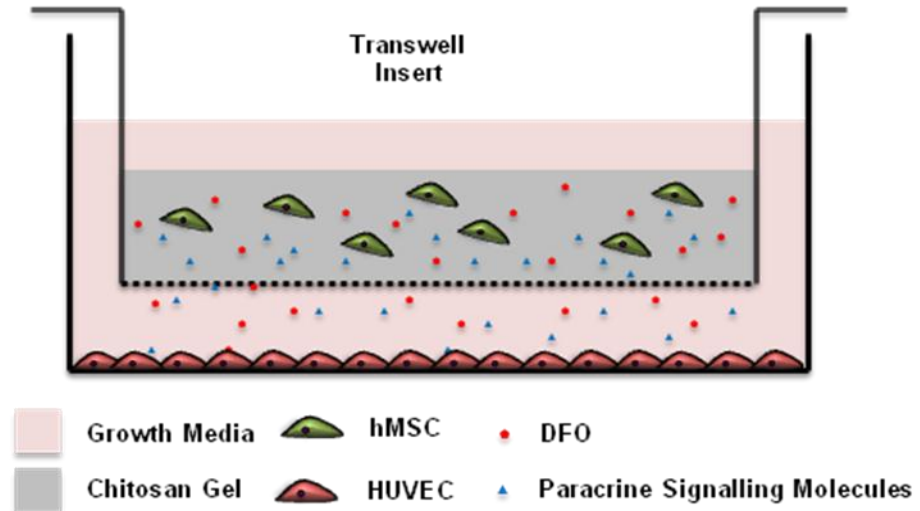


Figure 2 (A) Release of DFO ($\mu\text{g/g}$ gel) from chitosan β -GP gels containing 15 μM , 50 μM or 100 μM concentrations of DFO, at 37°C over the space of seven days. The last value (Ex) refers to the remaining DFO that was extractable from the gels by homogenisation on day 8 (mean + SD, $n=3$). **(B)** Cumulative release of DFO from 15 μM , 50 μM and 100 μM chitosan β -GP gels, expressed as a percentage of the total DFO in each gel (mean + SD, $n = 3$).

Bioactivity of the released DFO was assessed through measurements of VEGF expression in HUVECs after exposure to 300 μL of 100 μM DFO chitosan/ β -GP gel sequestered in a trans-well insert. HUVECs displayed a significant increase in VEGF expression after 48 and 72 hours. This had diminished by 120 hrs, after a change of media at 72 hours, but still showed a trend towards increased expression (**Fig.3B**).

A



B

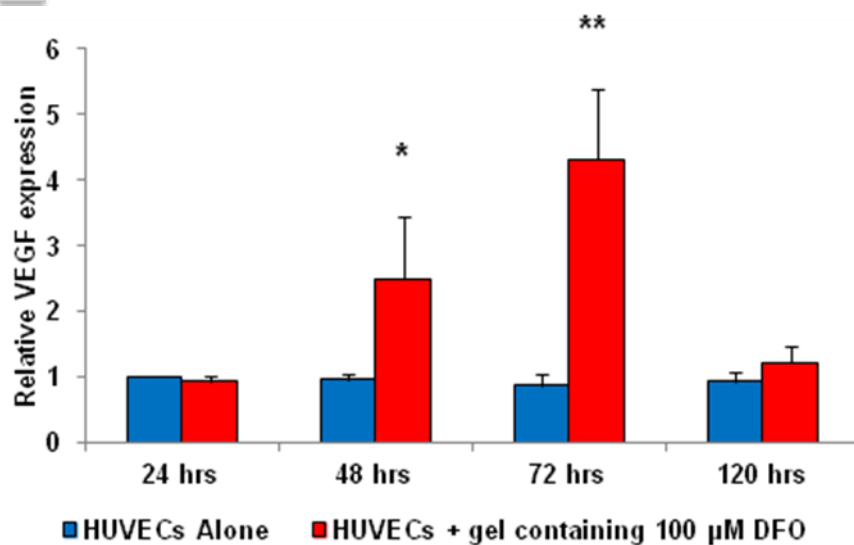


Figure 3 (A) Schematic depicting bioactivity assay and release of DFO and paracrine factors from the gel. **(B)** VEGF expression in HUVECs following exposure to a 100 μ M DFO chitosan/ β -GP gel sequestered in a trans-well insert. Cells showed significantly increased VEGF expression when compared to untreated cells after 48 and 72hrs (two-way ANOVA, $P < 0.05$). This had diminished by 120hrs, after a change of media at 72 hrs, but still showed a trend towards VEGF upregulation (mean + SD, $n=4$).

3.3 Cellular behaviour within chitosan/ β -GP gels

hMSCs were encapsulated in 2% w/v chitosan, 7% w/v β -GP gel droplets at a density of 250,000 cells/mL and cultured under normal conditions. Cell viability was assessed via LIVE/DEAD staining over a period of 16 days, in which live cells are stained green and dead cells stained red. hMSCs were viable and displayed very low levels of cell death throughout this period (**Fig. 4, A**). Measurements of double-stranded DNA (dsDNA) from chitosan-encapsulated hMSCs cultured in 48-well trans-well inserts showed that hMSCs are also capable of proliferation within the gel over the space of seven days (**Fig. 4, B**). Chitosan-encapsulated hMSCs were serum-starved and assessed with regard to ability to migrate through the gel in response to a serum stimulus over seven days, since the ability of hMSCs to leave the gel, and interact with surrounding tissues directly, might enhance any therapeutic benefit the formulation could exert. hMSCs successfully migrated from the gel and levels of trans-gel migration were significantly increased by day 5 and 7 ($P < 0.05$)

(Fig. 4, C).

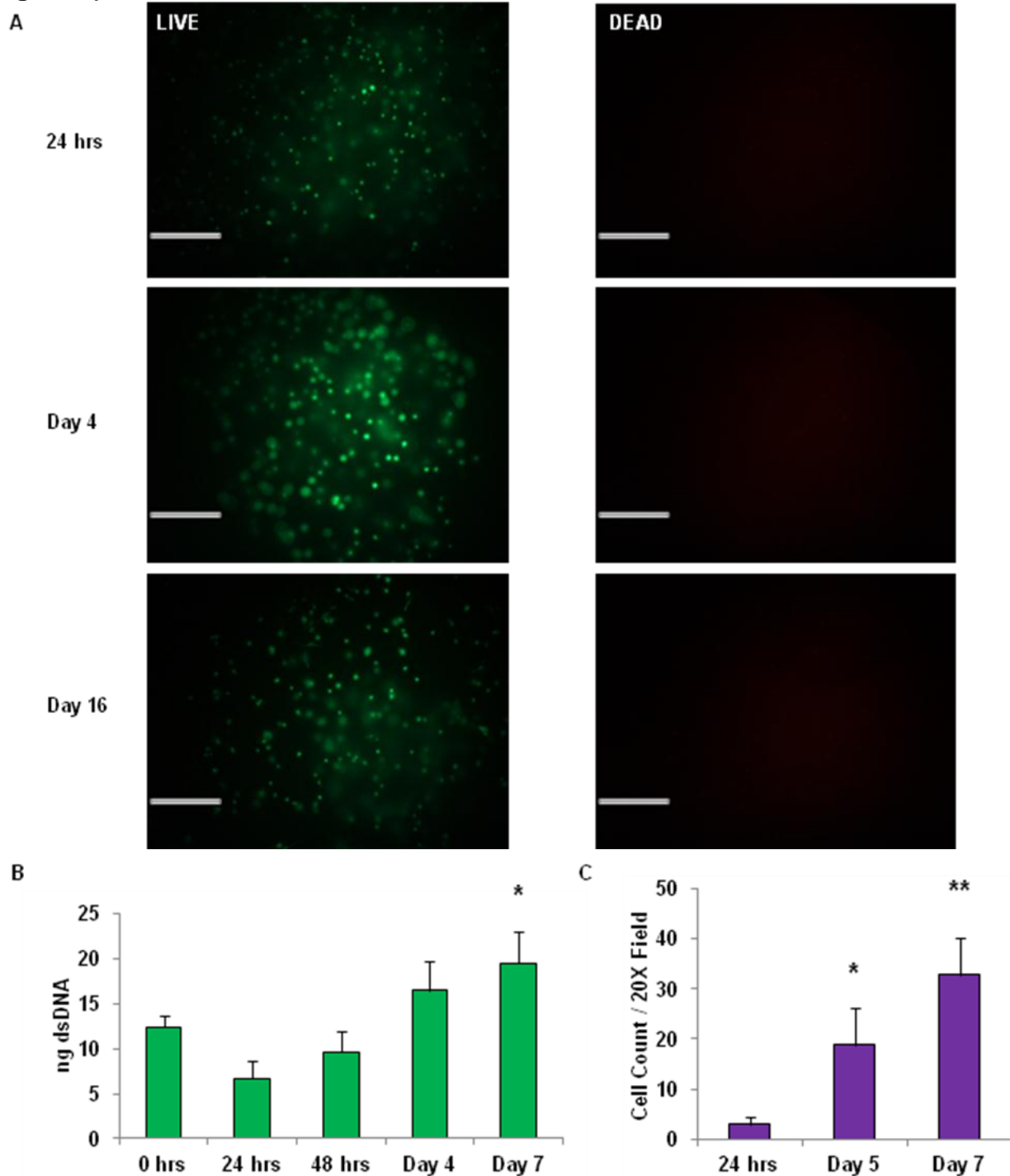


Figure 4 (A) LIVE/DEAD staining of hMSCs encapsulated in thermoresponsive chitosan droplets at 24 hrs, Day 4 and Day 16. Live cells are stained green and dead cells are stained red. **(B)** A measurement of double stranded DNA (dsDNA) from hMSCs cultured in thermoresponsive chitosan. Levels of dsDNA were significantly higher by day 7 ($P < 0.05$). Cells display proliferation over the course of seven days. **(C)** A measurement of cellular migration of hMSCs cultured in thermoresponsive chitosan. Cells were serum starved and then permitted to migrate through the gel in response to a serum stimulus. Cells collected on the membrane of a 12-well transwell cell-culture insert which was stained with haematoxylin. Cells were counted at 5 random fields of view at 20X magnification per membrane. Cells display significantly increased trans-gel migration by day 5 and day 7 ($P < 0.05$) (mean + SD, $n=3$)

3.4 DFO action within chitosan/ β -GP gels

hMSCs were encapsulated in either unmodified, 50 or 100 μ M DFO chitosan/ β -GP gels and cultured under normal conditions for 24 or 48 hours, after which they were assessed for VEGF expression via qPCR, to determine if DFO present in the gel could exert pro-angiogenic effects on encapsulated hMSCs. hMSCs displayed increased VEGF expression in 100 μ M DFO gel at 24 hours (**Supplementary Fig. 1 A, B**) and in 50 μ M DFO gel at 48 hours.

3.5 Bioactivity of chitosan/ β -GP gels containing hMSCs or both hMSCs and DFO

VEGF expression in HUVECs was assessed after exposure to 300 μ L of gel sequestered in a trans-well insert, to determine the effect of each treatment modality on *in vitro* pro-angiogenic bioactivity, alone or in combination. Groups included unmodified gel, gel containing 1 X 10⁶ hMSCs/mL or a multimodal gel containing 1 X 10⁶ hMSCs/mL combined with DFO at a 100 μ M concentration. HUVECs exposed to gel containing either hMSCs or hMSCs + DFO displayed significant increases in VEGF expression after 24 and 48 hrs. By 72 hrs, HUVECs exposed to gel containing hMSCs continued to display a significant 8-fold increase in VEGF expression when compared to HUVECs alone. However, HUVECs exposed to the multimodal gel displayed a 24-fold increase in VEGF expression which was significantly higher than all other treatment groups. All increases in VEGF expression produced by the multimodal gel or gels containing hMSCs alone were significantly higher than increases produced by gels containing DFO alone, as in **Fig. 3**, across all time points. These increases had diminished by 120 hrs, after a change of media at 72 hours. HUVECs exposed to unmodified gels did not show a significant increase in expression at any time point (**Fig. 5**)

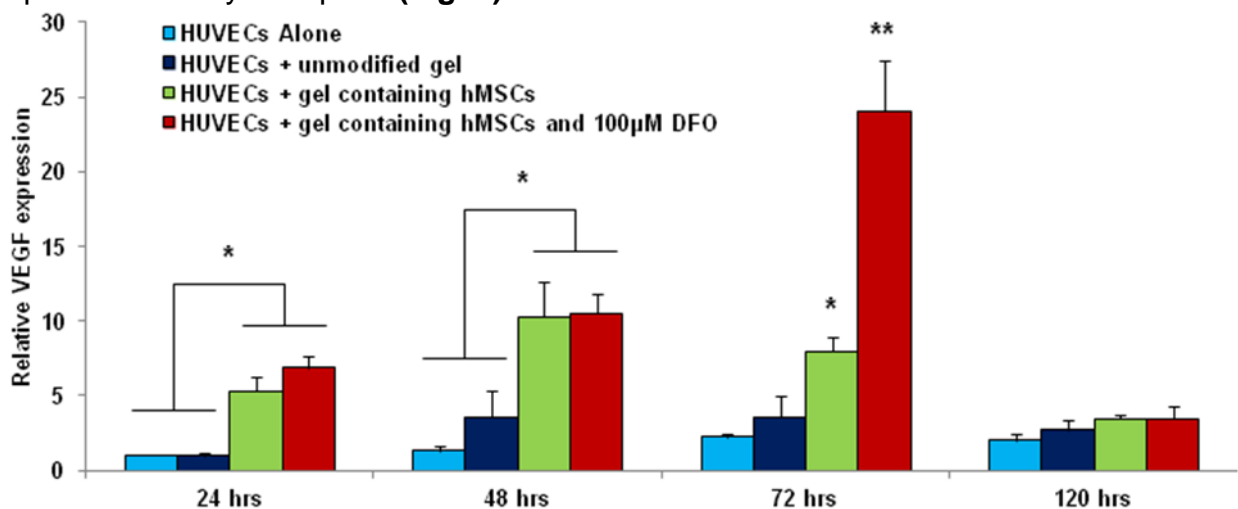


Figure 5 VEGF expression in HUVECS after exposure to unmodified gels, gels containing hMSCs or multimodal gels containing hMSCs and DFO together. Expression was significantly increased in HUVECs exposed to gels containing hMSCs or multimodal gels at 24, 48 and 72 hours. However, this expression was significantly greater in HUVECs exposed to multimodal gels than any other group at 72 hours (two-way ANOVA, $P < 0.05$) (mean + SD, $n=3$).

4. Discussion

The use of single pro-angiogenic agents as mediators of therapeutic angiogenesis in CLI patients has proved largely ineffective. Use of multimodal therapies, which combine more than one therapeutic agent, has resulted in synergistic enhancements in pro-angiogenic efficacy in pre-clinical models. The multimodal approach holds promise in terms of bringing therapeutic angiogenesis closer to a clinical reality for CLI sufferers. The aim of this study was to develop an injectable, multimodal, pro-angiogenic therapeutic for the treatment of CLI, utilising a thermoresponsive chitosan

hydrogel, hMSCs and DFO. This study has identified a novel combination of pro-angiogenic agents which are deliverable in a minimally invasive manner and exploit the efficacious phenomena of sustained release and co-delivery, to produce significant increases in pro-angiogenic bioactivity *in vitro*.

A growing body of pre-clinical evidence suggests that pro-angiogenic efficacy which has been not been forthcoming in a clinical setting may be achievable through strategies which exploit co-delivery and sustained release of pro-angiogenic agents. For example, Layman et al. showed that simultaneous administration of bFGF and granulocyte-colony stimulating factor within a fibrin matrix along with unfractionated bone marrow cells produced an enhanced therapeutic effect in the ischemic hindlimb when compared with delivery of growth factor or cell alone [7]. Jeon et al. showed that bone marrow mononuclear cells (BMMCs) suspended in a fibrin matrix produced greater increases in capillary density than unencapsulated cells when administered to the murine ischemic hindlimb. They found that sustained release of bFGF from the same matrix produced superior results than a direct injection, before finally showing that the combination of BMMCs and bFGF within the fibrin matrix produced synergistic increases in efficacy [11]. The formulation presented in this study has advantages with regard to clinical translation when compared with formulations which deliver only pro-angiogenic growth factors, since DFO has pre-existing FDA approval for the parenteral treatment of haemochromatosis [29]. This formulation also circumvents problems such as the limited half-life of delivered proteins since the incorporated hMSCs are envisaged as a self-sustaining source of such molecules [19].

An injectable, thermoresponsive gel must be able to undergo a rapid gelation once injected to slow dissolution in tissue fluid and reduce excessive mechanical disruption and dispersion due to muscle contraction. It was possible to create a gel which underwent a more rapid gelation by increasing the concentration of β -GP to 7% w/v. This had no effect on the final pH of the gel yet reduced the thermogelation temperature to 33°C and subsequently produced a more rapid thermogelation when held at 37°C. This gel also had a higher storage modulus at all comparable temperatures, indicating a stronger gel structure. Hence this 7% β -GP formulation was chosen for further investigation. Several studies have determined that chitosan/ β -GP gels display prolonged retention and stability, when injected into various tissues, prior to a well characterised enzymatic degradation, demonstrating the suitability of this gel as an injectable therapeutic delivery vehicle [16, 30-32].

The 2% w/v chitosan, 7% w/v β -GP gel was then combined with either 2.5×10^5 hMSCs/mL or DFO at a 100 μ M concentration and assessed during a temperature ramp from 20-50°C. DFO or hMSCs had no effect on the thermogelation temperature of the gel or the storage modulus achieved at physiological temperature. However, the presence of hMSCs in the gel did cause a drop in the maximum storage modulus achieved at 50°C. This may be due to the physical presence of a cellular bolus within the gel preventing aggregation of chitosan chains to the same degree as within an empty gel. However, upon visual inspection, gels containing hMSCs behaved in a similar manner to empty gels, with regard to resistance to disruption upon handling and, importantly, resisted dissolution to a similar degree during prolonged culture periods.

The release of three concentrations of DFO (15, 50 and 100 μ M) from the gel was then investigated over the space of seven days. It was found that after an initial burst release within the space of four hours that sustained release was present up until day 7, albeit decreasing steadily over that time. When delivered as a bolus, DFO has

been shown to increase vascular growth in the ischemic hindlimb and ischemic skinflaps in mice. However, these approaches required the administration of large, repeated doses of DFO (100mg/kg per day over 28 days, and 100mg/kg administered twice before induction of ischemia, respectively) [25, 33]. The formulation developed in this study is conceived as a locally administered, sustainable DFO depot which would not entail large, repeated, systemic administrations of drug to achieve efficacious concentrations at the site of action, for a sustained duration, thereby limiting possible off-target effects while potentially achieving comparable or greater efficacy. DFO has previously been incorporated into an injectable fibrin gel which successfully increased vascular growth in the rabbit ischemic hindlimb or ischemic skeletal muscle in sheep. However, authors did not assess DFO release from this formulation or compare the activity of the fibrin/DFO combination to that of either component alone [26, 34].

HUVECs exposed to 100 μ M DFO chitosan/ β -GP gel displayed an increased VEGF expression at 48 and 72 hrs showing that the released DFO was bioactive and that sustained release from the gel contributed to upregulated VEGF expression up to 72 hrs. This had diminished by 120hrs, following a change of media at 72 hours. The amount of gel used per well in this *in vitro* study was quite small (300 μ l) when compared with the amount of media present in the well (3mL basolateral, 1mL apical), meaning that released DFO might be substantially diluted when compared to an IM injection, where an injected gel bolus would be cradled by, and in direct contact with target tissues. Therefore DFO release from the gel *in vivo* could potentially exert a greater effect on surrounding tissues for a longer period.

hMSCs were fully viable in the gel, as assessed by Live/Dead staining. Indeed cellular proliferation was evidenced by the fact that dsDNA levels increased over the space of seven days during gel culture. This indicates the suitability of this material as not only an agent of cellular delivery but of cellular growth, permitting the administration of a given cellular payload which will then proliferate over the duration of therapy. This evidence of cellular viability is consistent with several studies where various cell types were successfully cultured in this material or variants thereof, and adds to the body of evidence which indicates the potential of chitosan/ β -GP thermoresponsive gels as agents of cell delivery [35-37].

hMSCs displayed an ability to migrate from the gel in response to a serum stimulus over seven days in a sustained manner. MSCs have been shown to home to sites of injury and inflammation [38]. Therefore it may be possible for encapsulated cells to migrate from the gel in response to cues derived from the necrosis of ischemic tissues.

hMSCs encapsulated in gel containing DFO showed an upregulation in VEGF expression. VEGF is an established transcriptional target for HIF-1 α and VEGF upregulation is a commonly reported outcome upon DFO treatment *in vitro*. Therefore it is possible to interpret the action of DFO as measurable by changes in VEGF expression [22-23]. This is of particular interest with regard to encapsulated hMSCs since the significant action of DFO on these cells shows that the formulation can act to prime encapsulated cells for pro-angiogenic action. Therefore this multimodal formulation does not merely encompass the additive effect of both pro-angiogenic components but promotes a synergistic interaction between them. HUVECs exposed to gels containing either hMSCs or a multimodal formulation containing hMSCs combined with DFO both displayed large increases in VEGF expression from as early as 24 hrs up to 72 hrs. These increases had diminished by 120 hrs, following a change in media at 72 hrs. The action of hMSCs within the gel

on the HUVEC monolayer below was likely paracrine in nature since direct contact between HUVEC and hMSC cultures was not permitted by the trans-well insert. At 72 hrs the multimodal gel produced a 24 fold increase in HUVEC VEGF expression, which was significantly greater than the increases produced by any other groups, which demonstrates a synergistic interaction between the DFO and hMSC components of the gel to produce a tangible enhancement in pro-angiogenic bioactivity. The presence of hMSCs and DFO in the multimodal group meant that HUVECs were likely exposed to both DFO and pro-angiogenic paracrine secretions from hMSCs, which may have been increased or enhanced by the action of DFO on hMSCs within the gel. The synergistic action of DFO in combination with hMSCs, when delivered in a chitosan thermoresponsive matrix which facilitates sustained release, could provide a greatly enhanced pro-angiogenic efficacy in ischemic tissues, when compared to use of either component alone.

5. Conclusions

These results are consistent with a growing body of evidence which suggests that the delivery of multiple pro-angiogenic agents in a sustained fashion provides superior efficacy to the administration of single agents or bolus delivery. This multimodal formulation fulfils important criteria for use as an injectable pro-angiogenic. A 2% w/v chitosan, 7% w/v β -GP gel displayed a suitable thermoresponsive profile for use as an injectable therapeutic, which supported hMSC viability, proliferation and migration and is therefore highly suited as a cell delivery vehicle. This gel permitted a sustained release of DFO over the period of seven days, which exerted a bioactive effect *in vitro* for up to 72 hours. DFO also increased pro-angiogenic gene expression within encapsulated hMSCs, demonstrating the synergistic interactions achievable through multimodal therapeutic formulation. This was corroborated by significant enhancements in multimodal bioactivity at 72 hrs. This formulation and multimodal therapies in general, require further application and investigation at a pre-clinical level to advance the fields of therapeutic angiogenesis and CLI therapy, and potentially help alleviate the suffering caused by this debilitating condition.

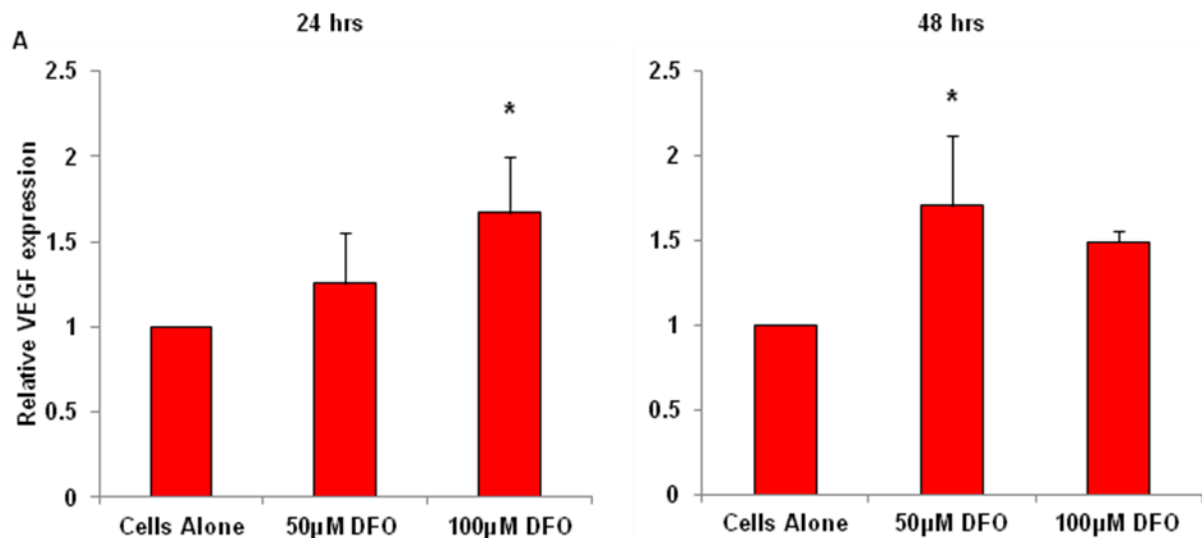
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SUPPLEMENTARY MATERIAL

VEGF expression within encapsulated cells

hMSCs were encapsulated in unmodified gel or gel containing 100 μ M DFO, at a density of 2×10^6 /mL. 800 μ L of this gel was added to hanging cell culture inserts (6-well format, 1 μ m pore size). The gel was allowed to set at 37°C for thirty minutes after which normal hMSC media was added to wells. Gels were cultured for 24 or 48 hours after which they were snap frozen in liquid nitrogen and stored at -80°C. RNA was isolated utilising a modified cetyltrimethylammonium bromide (CTAB) method, followed by sample purification using the Qiagen RNeasy minikit, as described by Wang and Stegemann [36]. RNA quality and quantity was determined using an RNA nanodrop (absorbance: 260 nm). Quantitative PCR was implemented with a one-step SYBR Green RT-PCR kit (Sigma) on a 7500 Real Time polymerase chain reaction system (Applied Biosystems) utilising the QuantiTect primer for VEGF (Hs_VEGFA_6_SG), and the 18s primer (Hs_RRN18S_1_SG) as a housekeeping gene.



Supplementary Figure 1 (A)(B) VEGF expression in hMSCs following encapsulation in either unmodified, 50 or 100µM DFO chitosan/β-GP for 24 or 48 hours. Cells showed significantly increased VEGF expression at 100 and 50µM concentrations of DFO at 24 and 48 hours respectively ($P < 0.05$) (mean + SD, n=3).

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