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1 **Dextran-shelled oxygen-loaded nanodroplets reestablish a normoxia-like pro-angiogenic**
2 **phenotype and behavior in hypoxic human dermal microvascular endothelium.**

3 *Running head:* Oxygen nanodroplets in hypoxic dermal endothelium.

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49

50 *Conflict of interest disclosure:* Roberta Cavalli, Caterina Guiot and Mauro Prato have a
51 patent no. WO2015/028901 A1 (A nanostructure for the vehiculation of gas and/or active
52 ingredients and/or contrast agents and use thereof) issued. The other authors have nothing to
53 disclose.
54

55 **Abstract**

56 In chronic wounds, hypoxia seriously undermines tissue repair processes by altering the
57 balances between pro-angiogenic proteolytic enzymes (matrix metalloproteinases, MMPs)
58 and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) released from
59 surrounding cells. Recently, we have shown that in human monocytes hypoxia reduces
60 MMP-9 and increases TIMP-1 without affecting TIMP-2 secretion, whereas in human
61 keratinocytes it reduces MMP-2, MMP-9, and TIMP-2, without affecting TIMP-1 release.
62 Provided that the phenotype of the cellular environment is better understood, chronic wounds
63 might be targeted by new oxygenating compounds such as chitosan- or dextran-shelled and
64 2H,3H-decafluoropentane-cored oxygen-loaded nanodroplets (OLNs). Here, we investigated
65 the effects of hypoxia and dextran-shelled OLN on the pro-angiogenic phenotype and
66 behavior of human dermal microvascular endothelium (HMEC-1 cell line), another cell
67 population playing key roles during wound healing. Normoxic HMEC-1 constitutively
68 released MMP-2, TIMP-1 and TIMP-2 proteins, but not MMP-9. Hypoxia enhanced MMP-2
69 and reduced TIMP-1 secretion, without affecting TIMP-2 levels, and compromised cell
70 ability to migrate and invade the extracellular matrix. When taken up by HMEC-1, nontoxic
71 OLN abrogated the effects of hypoxia, restoring normoxic MMP/TIMP levels and
72 promoting cell migration, matrix invasion, and formation of microvessels. These effects were
73 specifically dependent on time-sustained oxygen diffusion from OLN core, since they were
74 not achieved by oxygen-free nanodroplets or oxygen-saturated solution. Collectively, these
75 data provide new information on the effects of hypoxia on dermal endothelium and support
76 the hypothesis that OLN might be used as effective adjuvant tools to promote chronic wound
77 healing processes.

78
79 *Keywords:* oxygen; nanodroplet; matrix metalloproteinase (MMP); tissue inhibitor of
80 metalloproteinase (TIMP); human microvascular endothelial cell (HMEC); skin.

81 **Introduction**

82

83 After injury, skin integrity must be restored promptly to reestablish the homeostatic
84 mechanisms, minimize fluid loss, and prevent infection [Greaves et al., 2013]. This is
85 achieved through wound healing, a complex biological process where multiple pathways are
86 simultaneously activated to induce tissue repair and regeneration. Traditionally, acute wound
87 healing is defined as a complex multi-step and multi-cellular process, distinguished in four
88 phases involving different cell types: i) hemostasis, involving platelets; ii) inflammation,
89 involving neutrophils, monocytes, and macrophages; iii) proliferation, involving
90 keratinocytes, endothelial cells, and fibroblasts; and iv) matrix remodeling, involving
91 keratinocytes, myofibroblasts, and endothelial cells. [Diegelmann et al., 2004]. In particular,
92 during the third and fourth phases, the endothelium plays a pivotal role, since wound
93 microvasculature is rebuilt through angiogenesis to restore the supply of oxygen, blood
94 constituents and nutrients to the regenerating tissue, helping to promote fibroplasia and
95 prevent sustained tissue hypoxia [Eming et al., 2014]. Notably, oxygen represents a key
96 regulator of normal wound healing since it is required for collagen deposition,
97 epithelialization, fibroplasia, angiogenesis, and resistance to infection [Castilla et al., 2012;
98 Sen, 2009]. Once complete, these processes must be shut down in a precise order to prevent
99 exaggerated or delayed responses.

100 In some cases, the combination of systemic (e.g. diabetes, vascular insufficiency, or ageing)
101 or localized (e.g. bacterial infections and dysregulated proteolysis) factors produce persistent
102 pathological inflammation resulting in chronic wound formation [Diegelmann et al., 2004]. A
103 chronic wound is defined as a break in skin epithelial continuity lasting more than 42 days.
104 Its prevalence varies with age, ranging approximately from 1% in the adult population to 3–
105 5% in >65 year-old subjects [Greaves et al., 2013]. Approximately 7 million patients are

106 affected by chronic wounds in the United States, and an estimated \$25 billion dollars is spent
107 annually on the treatment of these wounds [Castilla et al., 2012].

108 A typical feature of chronic wounds is unbalanced proteolytic activity, which overwhelms
109 tissue protective mechanisms [Diegelmann et al., 2004; Pepper, 2001]. Within chronic
110 wounds, activated cells such as endothelial, epithelial, and immune cells display increased
111 production of proteases, including cathepsin G, urokinase and neutrophil elastase [Greaves et
112 al., 2013]. Furthermore, pro-inflammatory cytokines strongly induce the production of matrix
113 metalloproteinases (MMPs) and down-regulate the levels of tissue inhibitors of
114 metalloproteinases (TIMPs), thereby creating an environment with unbalanced MMP/TIMP
115 ratios [Diegelmann et al., 2004; Pepper, 2001]. Consequently, wound repair mediators
116 become targets of proteases, and the resultant matrix degradation contributes to the delay in
117 re-epithelialization, fibroplasia and angiogenesis [Pepper, 2001; Wells et al., 2015].
118 However, the effects of hypoxia on the secretion of MMPs and TIMPs by the cellular
119 environment of the wound are dramatically different depending on the considered cell type.
120 Therefore, it is extremely important to assess carefully the effects of hypoxia on each single
121 cell population participating to the wound healing process, from monocytes and keratinocytes
122 to endothelial cells and fibroblasts. In a couple of recent works published by our group
123 hypoxia was shown to reduce MMP-9 and increase TIMP-1 without affecting TIMP-2
124 secretion by human monocytes [Gulino et al., 2015], whereas in human keratinocytes
125 hypoxia was shown to reduce MMP-2, MMP-9, and TIMP-2 secretion without changing
126 TIMP-1 levels [Khadjavi et al., 2015]. On the other hand, the effects of hypoxia on the
127 secretion of gelatinases and their inhibitors by dermal microvascular endothelium still needed
128 further investigation.

129 Provided the phenotype of the cellular environment at the milieu of the wound is better
130 understood, new therapeutic approaches addressing hypoxia might help to face chronic

131 wounds. For this reason, the major role played by oxygen in essential wound healing
132 processes has attracted considerable clinical interest and yielded compelling data [Sen, 2009].
133 Additionally, scientific studies targeting the signaling pathways underlying oxygen response
134 within the milieu of the wound tissue are helping to better understand the biochemical
135 pathways involved in hypoxia sensing/response systems. This appears extremely crucial in
136 order to exploit new oxygenating treatments targeting hypoxia-response mechanisms within
137 the healing tissue, thus making them useful in the clinical management of chronic wounds.
138 So far, hyperbaric oxygen therapy remains a well-established, adjunctive treatment for
139 diabetic lower extremity wounds, when refractory to standard care practices [Sen, 2009].
140 However, hyperbaric oxygen therapy is expensive and uncomfortable. Moreover, further
141 rigorous randomized trials are needed to properly validate the outcomes of hyperbaric oxygen
142 therapy on chronic wounds associated with other pathologies (arterial ulcers, pressure ulcers,
143 and venous ulcers). Topical oxygen therapy, based on an O₂ gas emulsion applied to the
144 superficial wound tissue, represents another promising approach to enhance the oxygenation
145 of wounded tissues [Sen, 2009]. Major advantages of topical oxygen therapy appear to be its
146 independence of the wound microcirculation, its lower cost with respect to systemic oxygen
147 therapy, lower risks of oxygen toxicity, and its relative simplicity of handling and application.
148 In this context, intensive research is being pursued to develop new carriers able to release
149 therapeutically significant amounts of oxygen to tissues in an effective and time-sustained
150 manner, such as hemoglobin- or perfluorocarbon-based systems [Cabrales et al., 2013;
151 Schroeter et al., 2010]. Among the options currently under investigation, perfluoropentane
152 (PFP)-based oxygen-loaded nanobubbles have been proposed as efficient and biocompatible
153 ultrasound (US)-responsive tools for oxygen delivery [Cavalli et al., 2009a; Cavalli et al.,
154 2009b]. Furthermore, oxygen-loaded nanodroplets (OLNs), constituted by 2H,3H-
155 decafluoropentane (DFP) as core fluorocarbon and dextran or chitosan as shell

156 polysaccharides, have been recently developed, characterized, and patented by our group as
157 innovative and nonconventional platforms of oxygen nanocarriers, available in formulations
158 suitable for topical treatment of dermal tissues [Magnetto et al., 2014; Prato et al., 2015].
159 Intriguingly, while keeping all the advantages of nanobubbles, OLN_s display higher stability
160 and effectiveness in oxygen storage and release, lower manufacturing costs and ease of scale-
161 up. Encouragingly, chitosan-shelled OLN_s proved effective in counteracting the
162 dysregulating effects of hypoxia on secretion of gelatinases and TIMPs by human
163 keratinocytes [Khadjavi et al., 2015], whereas dextran-shelled OLN_s abrogated hypoxia-
164 dependent alteration of MMP-9/TIMP-1 balances in human monocytes [Gulino et al., 2015].
165 To go beyond the current knowledge on MMP/TIMP dysregulation in the different cell
166 populations within the milieu of chronic wounds and expand the available evidence on OLN_s
167 effectiveness, in the present work we explored the effects of hypoxia and OLN_s on the pro-
168 angiogenic phenotype and behavior of human dermal endothelium. To this purpose, a human
169 dermal microvascular endothelial cell line (HMEC-1) was cultured *in vitro* both in normoxic
170 and hypoxic conditions, in the presence or absence of dextran-shelled OLN_s. Then, cells were
171 challenged for their viability, proteolytic phenotype (secretion of gelatinases and their
172 inhibitors), and wound healing abilities [migration, invasion of the extracellular matrix
173 (ECM), and formation of microvessel-like structures].

174

175

176 **Methods**

177

178 *Materials*

179 All materials were from Sigma-Aldrich, St Louis, MO, aside from those listed below. Sterile
180 plastics were from Costar, Cambridge, UK; MCDB 131 medium was from Invitrogen,
181 Carlsbad, CA; foetal calf serum was from HyClone, South Logan, UT; epidermal growth
182 factor was from PeproTech, Rocky Hill, NJ; Cultrex was from Trevigen, Gaithersburg, MD;
183 LDH Cytotoxicity Assay kit was from Biovision, Milpitas, CA; enzyme-linked
184 immunosorbent assay (ELISA) kit for human MMP-2 was from Abnova, Taipei City,
185 Taiwan; ELISA kits for human MMP-9, TIMP-1 and TIMP-2 were from RayBiotech,
186 Norcross, GA; electrophoresis reagents and computerized densitometer Geldoc were from
187 Bio-rad Laboratories, Hercules, CA; Synergy Synergy 4 microplate reader was from Bio-Tek
188 Instruments, Winooski, VT; recombinant proMMP-9 and MMP-9 were produced and kindly
189 gifted by Prof. Ghislain Opdenakker and Prof. Philippe Van den Steen; ethanol (96%) was
190 obtained from Carlo Erba (Milan, Italy); culture implants for wound healing assay were from
191 Ibidi GmbH (Planegg/Martinsried, Germany); Epikuron 200® (soya phosphatidylcholine
192 95%) was from Degussa (Hamburg, Germany); palmitic acid, DFP, dextran sodium salt (100
193 kDa), and polyvinylpyrrolidone were from Fluka (Buchs, Switzerland); ultrapure water was
194 obtained using a 1-800 Millipore system (Molsheim, France); Ultra-Turrax SG215
195 homogenizer was from IKA (Staufen, Germany); Delsa Nano C analyzer was from Beckman
196 Coulter (Brea, CA); Philips CM10 instrument was from Philips (Eindhoven, The Netherlands);
197 XDS-3FL microscope was from Optika (Ponteranica, Italy); ECLIPSE Ti inverted
198 microscope was from Nikon (Amsterdam, The Netherlands).

199

200 *Dextran-shelled nanodroplet preparation and characterization*

201 OLN, oxygen-free nanodroplets (OFNs), and oxygen-saturated solution (OSS) were
202 prepared as previously described [Prato et al., 2015]. Briefly, 1.5 ml DFP, 0.5 ml
203 polyvinylpyrrolidone and 1.8 ml Epikuron® 200 (solved in 1% w/v ethanol and 0.3 % w/v
204 palmitic acid solution) were homogenized in 30 ml phosphate-buffered saline (PBS) solution
205 (pH 7.4) for 2 min at 24000 rpm by using Ultra-Turrax SG215 homogenizer. For OLN, the
206 solution was saturated with O₂ for 2 min. Finally, 1.5 ml dextran or fluorescein isothiocyanate
207 (FITC)-labeled dextran solution was added drop-wise whilst the mixture was homogenized at
208 13000 rpm for 2 min. For OFN and OSS PBS formulations, OLN preparation protocol was
209 applied omitting O₂ or dextran/DFP addition, respectively. Immediately after manufacturing,
210 nanodroplets were sterilized through ultraviolet (UV)-C ray exposure for 20 min and
211 characterized for: morphology and shell thickness, by optical and transmitting electron
212 microscopy; size, particle size distribution, polydispersity index and zeta potential, by
213 dynamic light scattering; refractive index by polarizing microscopy; viscosity and shell shear
214 modulus by rheometry; and oxygen content (before and after UV-C sterilization) through a
215 chemical assay as previously described [Magnetto et al., 2014;Prato et al., 2015].

216

217 *Cell cultures*

218 A long-term cell line of dermal microvascular endothelial cells (HMEC-1) immortalized by
219 SV 40 large T antigen [Ades et al., 1992] was kindly provided by the Center for Disease
220 Control, Atlanta, GA. Cells were maintained in MCDB 131 medium supplemented with 10%
221 foetal calf serum, 10 ng/ml of epidermal growth factor, 1 µg/ml of hydrocortisone, 2mM
222 glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin and 20 mM Hepes buffer,
223 pH7.4. Before the experiments, HMEC-1 were seeded at 10⁵ cells/0.5 ml per well in 24-well
224 flat bottom tissue culture clusters and incubated in a humidified CO₂/air-incubator at 37°C in
225 complete medium. After overnight incubation to allow cells adhesion, HMEC-1 were treated

226 for 24 h with/without 10% v/v OLN_s, OFN_s, and OSS, either in normoxic (20% O₂) or
227 hypoxic (1% O₂) conditions. At the end of each treatment, cell supernatants were collected
228 and used for the following analyses.

229

230 *Evaluation of OLN uptake by HMEC-1*

231 HMEC-1 were plated in 24-well plates on glass coverslips and incubated in complete
232 medium for 24 h with/without 10% v/v FITC-labeled OLN_s in a humidified CO₂/air-
233 incubator at 37°C both in normoxic and hypoxic conditions. After 4',6-diamidino-2-
234 phenylindole (DAPI) staining to visualize cells nuclei, fluorescence images were acquired by
235 a LSM710 inverted confocal laser scanning microscope equipped with a Plan-Neofluar
236 63×1.4 oil objective, that allowed a field view of at least 5 cells. Wavelength of 488 nm was
237 used to detect OLN_s, and of 460 nm to detect the labeled nuclei. The acquisition time was
238 400 ms.

239

240 *Cytotoxicity studies*

241 The potential cytotoxic effect of OLN and control formulations was measured as the release
242 of lactate dehydrogenase (LDH) from HMEC-1 into the extracellular medium using the LDH
243 Cytotoxicity Assay kit following the manufacturer's instructions. LDH was measured both in
244 the extracellular medium and in the cells pellet. Briefly, cells were incubated for 24 h
245 with/without 10% v/v OLN_s, OFN_s or OSS, either in normoxic (20 % O₂) or hypoxic (1 %
246 O₂) conditions, in a humidified CO₂/air-incubator at 37°C. Then, cell supernatants were
247 collected and centrifuged at 13000g for 2 min. Cells were washed with PBS and resuspended
248 in 0.5 ml of Triton X100 (2% final concentration) to lyse cells. One hundred microliters of
249 this solution or 100 microliters of supernatant was mixed with 100 microliters of LDH
250 reaction mix, containing the LDH substrate, and incubated for 10 min at room temperature in

251 the dark. Absorbance was then read at 450 nm with a reference wavelength of 650 nm using
252 Synergy 4 microplate reader.

253

254 *Cell viability studies*

255 Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
256 bromide (MTT) assay. HMEC-1 were incubated in complete medium overnight to allow the
257 cells to adhere and then treated for 24 h with/without 10% v/v OLN_s, OFN_s or OSS, either in
258 normoxic (20 % O₂) or hypoxic (1 % O₂) conditions, in a humidified CO₂/air-incubator at
259 37°C in serum free medium. Thereafter, 20 µL of 5 mg/mL MTT in PBS were added to cells
260 for 3 additional hours at 37 °C in the dark. The plates were then centrifuged, the supernatants
261 discarded and the dark blue formazan crystals dissolved using 100 µL of lysis buffer
262 containing 20 % (w/v) sodium dodecylsulfate, 40 % N,N-dimethylformamide (pH 4.7 in 80
263 % acetic acid). The plates were then read on Synergy 4 microplate reader at a test wavelength
264 of 550 nm and at a reference wavelength of 650 nm.

265

266 *Measurement of MMP-2, MMP-9, TIMP-1, and TIMP-2 production*

267 HMEC-1 were incubated overnight in complete medium and then treated for 24 h
268 with/without 10% v/v OLN_s, OFN_s or OSS, either in normoxic (20 % O₂) or hypoxic (1 %
269 O₂) conditions, in a humidified CO₂/air-incubator at 37°C in serum-free medium. Thereafter,
270 cell supernatants were collected, and the levels of MMP-2, MMP-9, TIMP-1, and TIMP-2
271 were assayed in 100 µl of HMEC-1 supernatants by specific ELISA. Standard calibration
272 curves were generated with rhMMP-2, rhMMP-9, rhTIMP-1, and rhTIMP-2, according to the
273 manufacturer's instructions. Of note, ELISA kits could not distinguish between latent and
274 active forms of MMP-2 and MMP-9. For this reason, a complementary analysis by gelatin
275 zymography was performed, as described in the following paragraph.

276

277 *Measurement of the levels of latent and active forms of gelatinases in cell supernatants*

278 The levels of latent and active forms of gelatinases were evaluated by gelatin zymography in
279 the cell supernatants as previously described [D'Alessandro et al., 2013]. Briefly, HMEC-1
280 were incubated overnight in complete medium and then treated for 24 h with/without 10%
281 v/v OLN_s, OFN_s or OSS, either in normoxic (20 % O₂) or hypoxic (1 % O₂) conditions, in a
282 humidified CO₂/air-incubator at 37°C in serum-free medium. Thereafter, 15 µl cell
283 supernatants/lane were loaded on 8% polyacrylamide gels containing 0.1% gelatin under non-
284 denaturing and non-reducing conditions. Following electrophoresis, gels were washed at
285 room temperature for 2 h in milliQ water containing 2.5% (v/v) Triton-X100 and incubated
286 for 18 h at 37°C in a collagenase buffer containing (mM): NaCl, 200; Tris, 50; CaCl₂, 10; and
287 0.018% (v/v) Brij 35, pH 7.5, with or without 5 mM ethylenediaminetetraacetic acid to
288 exclude aspecific bands. At the end of the incubation, the gels were stained for 15 min with
289 Coomassie blue (0.5% Coomassie blue in methanol/acetic acid/water at a ratio of 3:1:6). The
290 gels were destained in milliQ water. Densitometric analysis of the bands, reflecting the total
291 levels of latent and active forms of gelatinases, was performed using a computerized
292 densitometer.

293

294 *In vitro wound healing assay*

295 *In vitro* wound healing assay was performed on HMEC-1 cells using Ibidi's culture inserts
296 according to the manufacturer's instructions. One culture insert per well was placed in a 24-
297 well plate. Then, 70 µl from a suspension of 5x10⁵ cells/ml HMEC-1 cells were plated in
298 each chamber of Ibidi's culture inserts with cell growth medium. After 24 h, culture inserts
299 were detached resulting in two confluent monolayers, divided by a space (scratch) of 500 µm.
300 Thereafter, cells were washed with PBS and incubated in fresh medium for 8 h in the

301 presence or absence of 10% v/v OLN_s or OFN_s, either in normoxic or hypoxic conditions.
302 For each condition, at least two different culture inserts were employed. At the end of the
303 observational period, scratch images were taken using a Nikon Ti-e eclipse microscope.
304 Scratches were also measured and normalized with a time 0 scratch (500 μm).

305

306 *Microvessel-like structures formation*

307 HMEC-1 were evaluated for the ability to spontaneously migrate and self-organize in
308 microvessel-like structures when cultured on a basal membrane surface [Prato et al., 2011].
309 Cells were seeded (1×10^5 cells/well) in a 96-well plate previously covered with solidified
310 Cultrex (50μ/well), a growth factor-free basement membrane extract from murine
311 Engelbreth-Holm-Swarm tumor. After 2 h of incubation in the presence or absence of 10%
312 v/v OLN_s, each well was evaluated by optical microscopy. The formation of microvessel-like
313 structures was measured as the number of crosses between microvessel-like structures
314 counted in five randomly selected fields by two independent observers.

315

316 *Statistical analysis.*

317 For each set of experiments, data are shown as means + SEM (LDH, MTT, densitometry,
318 ELISA, and Cultrex assay results) or as a representative image (confocal microscopy and
319 gelatin zymography results) of at least three independent experiments with similar results. All
320 data were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post-
321 hoc test (software: SPSS 16.0 for Windows, SPSS Inc., Chicago, IL) or by Student's *t* test.

322

323

324 **Results**

325

326 *Characterization of dextran OLN preparations*

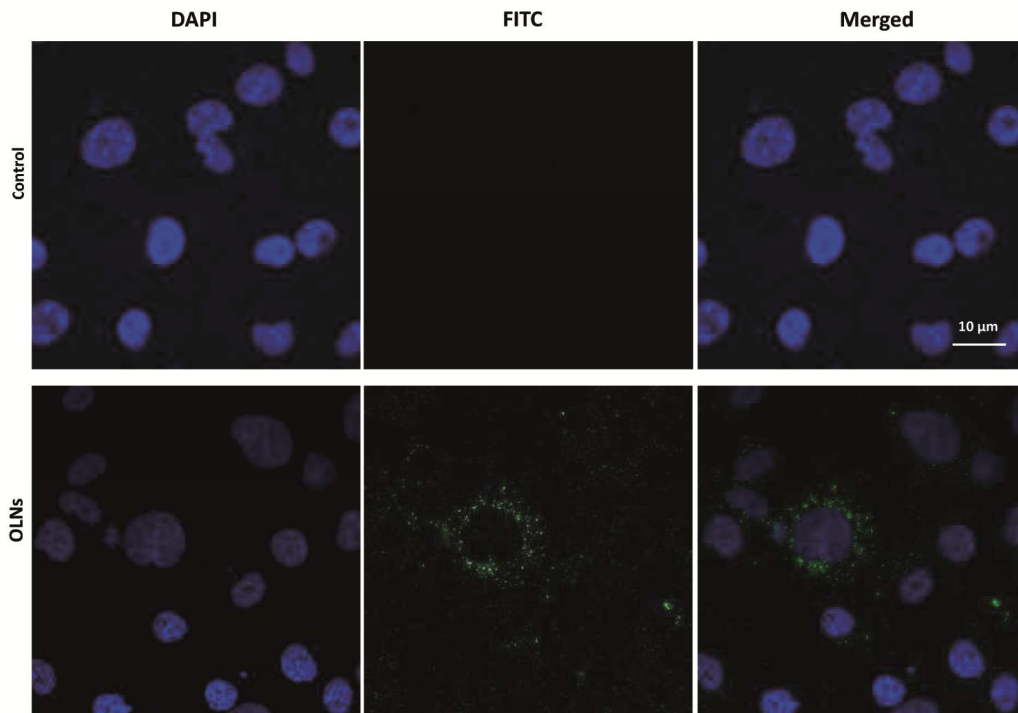
327 Before use, all dextran-shelled OLN preparations were meticulously characterized for
328 physico-chemical parameters. Results were always in line with published data [Prato et al.,
329 2015]: OLN_s displayed spherical shapes, 590 nm average diameters, -25 mV zeta potential,
330 1.33 as refractive index value, 1.59 e-3 Pa·s as viscosity value, and 5.43 e-2 mPa as shear
331 modulus value, calculated at a shear rate value of 150 s⁻¹. OLN_s also showed a good oxygen-
332 storing capacity of 0.40 mg/ml of oxygen either before or after 20-min UV-C sterilization,
333 and such an oxygen amount was comparable with that of OSS. Furthermore, all nanodroplet
334 preparations proved to be stable over time, as confirmed by long-term checking of these
335 parameters.

336

337 *OLN uptake by human dermal microvascular endothelial cells*

338 Confocal microscopy analysis was performed to determine whether OLN_s were internalized
339 by endothelial cells. HMEC-1 were incubated with 10% v/v FITC-labeled dextran-shelled
340 OLN_s or OFN_s for 24 h in normoxic or hypoxic conditions. As shown in Figure 1, confocal
341 microscopy confirmed OLN internalization by normoxic HMEC-1 and their localization in
342 the cytoplasm. Similar results were also obtained upon culturing HMEC-1 cells with OLN_s in
343 hypoxic conditions and with OFN_s both in normoxic or hypoxic conditions (data not shown).

344



345

346 **FIGURE 1. OLN internalization by human dermal microvascular endothelial cells.**

347 HMEC-1 (10^5 cells/0.5 ml MCDB 131 medium) were left untreated (upper panels) or treated

348 with 10% v/v FITC-labeled OLN (lower panels) for 24 h in normoxia (20 % O_2). After

349 DAPI staining, cells were checked by confocal microscopy. Results are shown as

350 representative images from three independent experiments. Left panels: cell nuclei after

351 DAPI staining. Central panels: FITC-labeled OLN. Right panels: merged images.

352 Magnification: 63X.

353

354 *Effects of hypoxia and OLN on HMEC-1 viability*

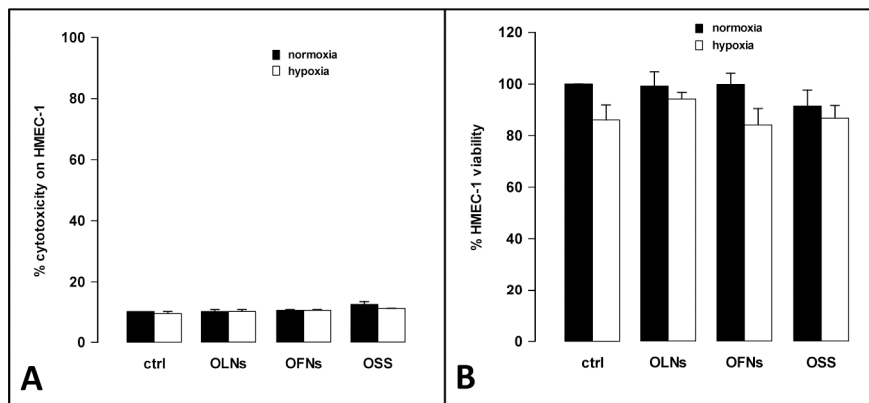
355 After 24 h-incubation of HMEC-1 with or without 10% v/v OSS, OLN or OFN, both in

356 normoxic (20% O_2) and hypoxic (1% O_2) conditions, cytotoxicity and cell viability were

357 analyzed through LDH and MTT assays, respectively (Figure 2). As shown in Panel 2A,

358 OSS, OLN or OFN were not toxic to HMEC-1 both in normoxic (20% O_2) and hypoxic

359 (1% O₂) conditions. As shown in Panel 2B, hypoxia *per se* determined an apparent reduction
360 of the metabolic activity of HMEC-1, however such an effect was not statistically significant
361 and in any case was fully counteracted by OLN.



362
363 **FIGURE 2. Hypoxia and OLN effects on human dermal microvascular endothelial cell**
364 **viability.** HMEC-1 (10⁵ cells/0.5 ml MCDB 131 medium) were left untreated or treated with
365 10% v/v OLN, OFN or OSS for 24 h in normoxia (20% O₂, black bars) or hypoxia (1% O₂,
366 white bars). After collection of cell supernatants and lysates, the percentage of cytotoxicity
367 was measured by the release of LDH (panel A), whereas the percentage of cell viability was
368 measured with the MTT assay (panel B). The results are the means+SEM from three
369 independent experiments. Using the ANOVA test, no significant differences between
370 normoxic or hypoxic control cells or between OLN-treated and untreated cells were observed
371 (both panels).

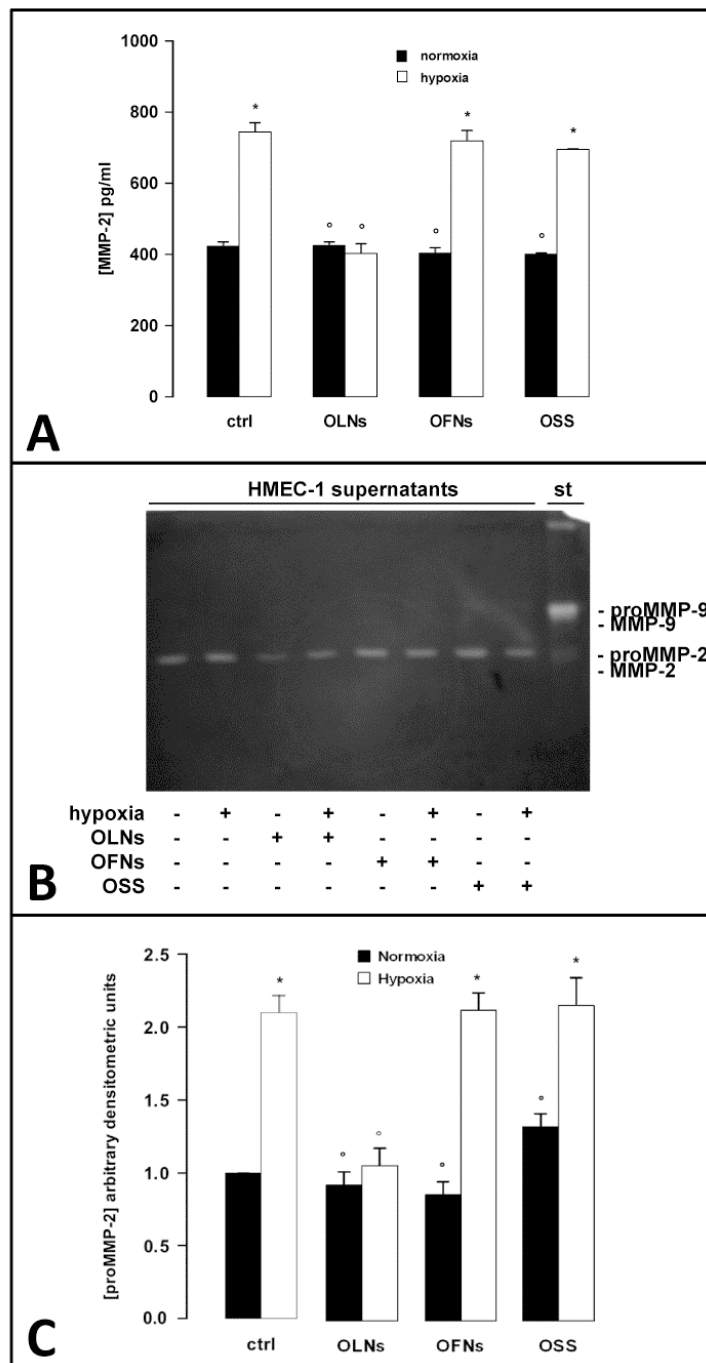
372

373

374 *Hypoxia and OLN effects on gelatinase secretion by human dermal microvascular*
375 *endothelial cells*

376 After 24 h-incubation of HMEC-1 with or without 10% v/v OSS, OLN or OFNs, both in
377 normoxic (20% O₂) and hypoxic (1% O₂) conditions, the secretion of gelatinases (MMP-2
378 and MMP-9) into cell supernatants was evaluated by ELISA as well as by gelatin
379 zymography coupled to densitometry. The results are shown in Figure 3. Untreated normoxic
380 HMEC-1 constitutively secreted ~400 pg/ml of MMP-2 (Panel A). Notably, HMEC-1 only
381 secreted the 72 kDa latent form of MMP-2 (proMMP-2), whereas the 63 kDa active form was
382 not detected in the cell supernatants (Panels B-C). On the contrary, neither ELISA (not
383 shown) nor gelatin zymography analyses detected any MMP-9 protein amounts in endothelial
384 cell supernatants. Hypoxia significantly altered MMP-2 secretion by almost doubling
385 proMMP-2 levels in HMEC-1 supernatants. OLN – but not OFNs or OSS – fully reversed
386 the effects of hypoxia, restoring a normoxia-like secretion of proMMP-2.

387



388

389 **FIGURE 3. Effects of hypoxia and OLN treatments on MMP-2 secretion by human dermal**
 390 **microvascular endothelial cells.** HMEC-1 (10^5 cells/0.5 ml MCDB 131 medium) were left

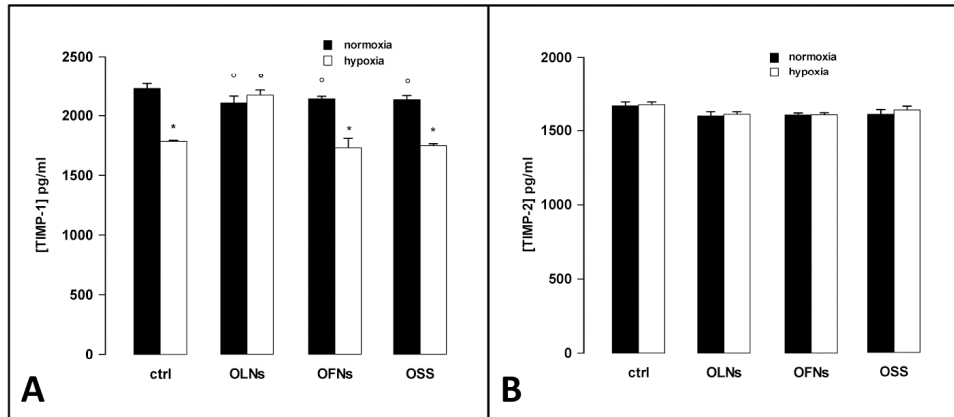
391 untreated or treated with 10% v/v OLN_s, OFN_s or OSS for 24 h in normoxia (20% O₂; panels
392 A and C: black bars; panel B: odd lanes) or hypoxia (1% O₂; panels A and C: white bars;
393 panel B: even lanes). After collection of cell supernatants, MMP-2 protein levels were
394 quantified by ELISA (panel A), whereas MMP-2 latent/active forms were analyzed by gelatin
395 zymography (panel B) and subsequent densitometry (panel C). For gelatin zymography,
396 recombinant human proMMP-9 (92 kDa) was employed as a standard marker (st). Results are
397 shown as means+SEM (panels A and C) or as a representative gel (panel B) from three
398 independent experiments. ELISA and densitometric data were also evaluated for significance
399 by ANOVA: * vs normoxic control cells: $p < 0.0001$ (panel A), $p < 0.0001$ (panel C); ° vs
400 hypoxic control cells: $p < 0.0001$ (panel A), $p < 0.0001$ (panel C).

401

402 *Hypoxia and OLN effects on TIMP secretion by human dermal microvascular endothelial*
403 *cells and MMP-2/TIMP-2 balances*

404 HMEC-1 were incubated for 24 h with or without 10% v/v OSS, OLN_s or OFN_s, both in
405 normoxic (20% O₂) and hypoxic (1% O₂) conditions. Thereafter, the secretion of TIMP-1 and
406 TIMP-2 was evaluated by ELISA. As shown in Figure 4, normoxic untreated HMEC-1
407 constitutively released ~2.2 ng/ml TIMP-1 and ~1.6 ng/ml TIMP-2. Hypoxia significantly
408 lowered by almost 20% the secreted levels of TIMP-1 while TIMP-2 production was not
409 affected. OLN_s – but not OFN_s and OSS - completely abrogated the effects of hypoxia,
410 restoring physiological TIMP-1 amounts also in hypoxic culturing conditions.

411



412

413 **FIGURE 4. Effects of hypoxia and OLNs on protein levels of gelatinase inhibitors**
 414 **(TIMP-1 and TIMP-2) secreted by human dermal microvascular endothelial cells.**

415 HMEC-1 (10^5 cells/0.5 ml MCDB 131 medium) were left untreated or treated with 10% v/v

416 OLNs, OFNs or OSS for 24 h in normoxia (20% O₂; black bars, both panels) or hypoxia (1%

417 O₂; white bars, both panels). After collection of cell supernatants, TIMP-1 (panel A) and

418 TIMP-2 (panel B) protein levels were quantified by ELISA. Results are shown as

419 means+SEM from three independent experiments. Data were also evaluated for significance

420 by ANOVA: * vs normoxic control cells: $p < 0.0001$ (panel A) and p not significant (panel B);

421 ° vs hypoxic control cells: $p < 0.0001$ (panel A) and p not significant (panel B).

422

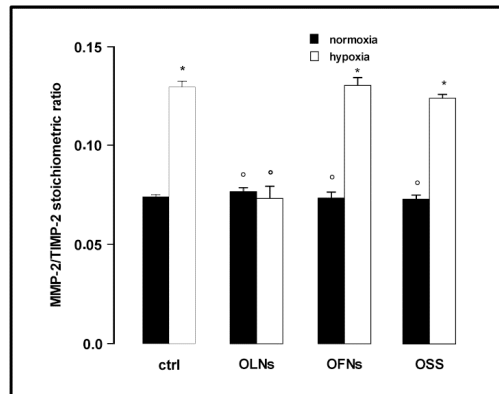
423 Consequently, the balance between MMP-2 and its inhibitor was calculated. As shown in

424 Figure 5, hypoxia significantly affected MMP-2/TIMP-2 stoichiometric ratio, which was

425 almost doubled with respect to cells cultured in normoxic conditions. OLNs – but not OFNs

426 or OSS – effectively counteracted the effects of hypoxia, restoring the MMP-2/TIMP-2 ratio
427 to a value similar to that observed in normoxia.

428



429

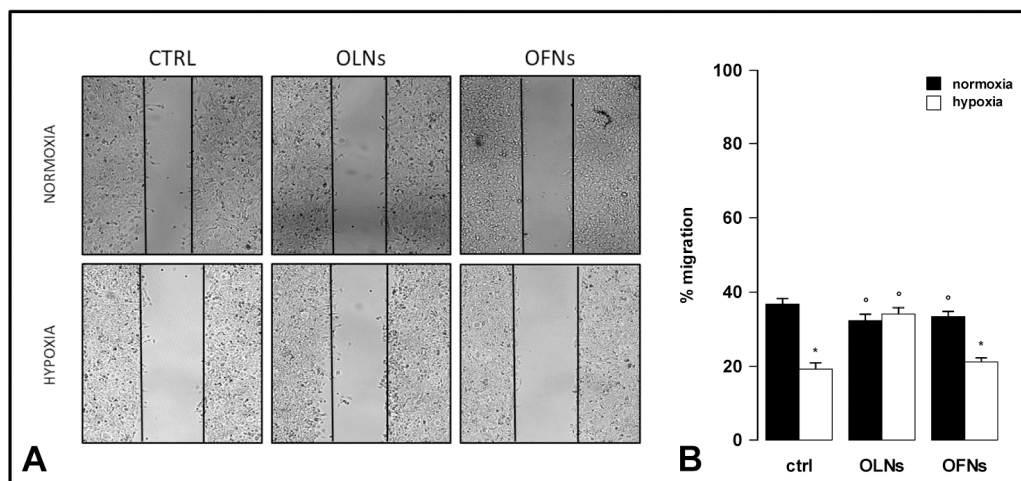
430 **FIGURE 5. Effects of hypoxia and OLN on MMP-2/TIMP-2 balances upon secretion**
431 **by dermal microvascular endothelial cells.** MMP-2/TIMP-2 stoichiometric ratio was
432 calculated from the ELISA data (see Figures 3-4). Results are shown as means+SEM from
433 three independent experiments. Data were also evaluated for significance by ANOVA: * vs
434 normoxic control cells: $p < 0.0001$; ° vs hypoxic control cells: $p < 0.0001$.

435

436 *Effects of hypoxia and OLN on migration and wound healing abilities of human dermal*
437 *microvascular endothelial cells*

438 The ability of HMEC-1 to spontaneously migrate was investigated through an *in vitro* wound
439 healing assay. As shown in Figure 6, hypoxic HMEC-1 displayed a lower ability to migrate

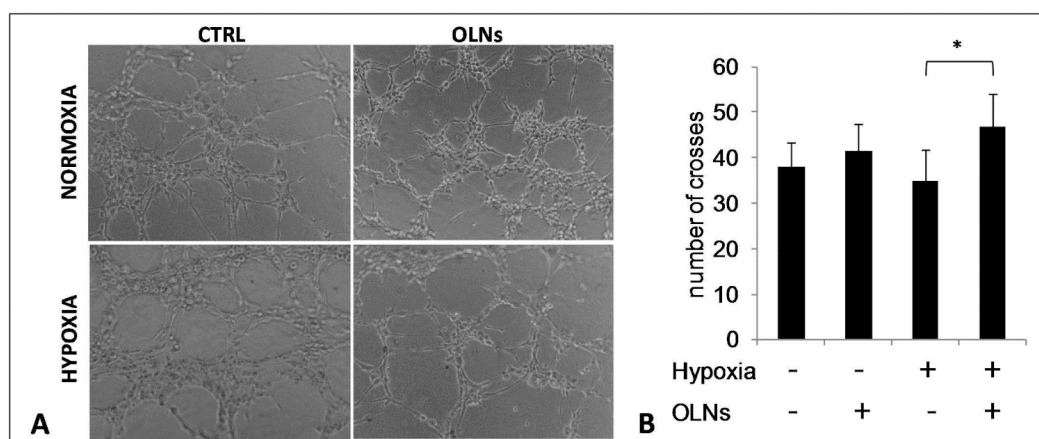
440 compared to normoxic cells. However, the migration ability of hypoxic HMEC-1 was
 441 significantly increased in the presence of OLN. Interestingly, OLN effects were not
 442 reproduced by OFNs, suggesting a peculiar role for oxygen released from the core of OLN.



443
 444 **FIGURE 6. Effects of hypoxia and OLN on migration and wound healing abilities of**
 445 **human microvascular dermal endothelial cells.** HMEC-1 were seeded in two confluent
 446 monolayers, divided by a space (scratch) of 500 μm , and incubated for 8 h in normoxia (20%
 447 O_2) or hypoxia (1% O_2) with/without 10% v/v OLN or OFN. Thereafter, scratch lengths
 448 were measured. A: representative images. B: means \pm SEM of scratch lengths. Results are from
 449 three independent experiments performed in duplicates. Data were also evaluated for
 450 significance by ANOVA: * vs normoxic untreated cells: $p < 0.001$; ^o vs hypoxic untreated
 451 cells: $p < 0.001$.
 452

453 *Effects of hypoxia and OLN*s on abilities of human dermal microvascular endothelial cells to
454 *invade collagen matrix and form microvessel-like structures*

455 The ability of HMEC-1 to invade a collagen matrix and form microvessel-like structures was
456 investigated through an *in vitro* invasion assay. As shown in Figure 7, hypoxic HMEC-1
457 displayed a lower ability to invade matrix and organize in microvessel-like structures
458 compared to normoxic cells. However, the invasion ability (i.e. the number of crosses) of
459 hypoxic HMEC-1 was significantly increased in the presence of OLNs.



460
461 **FIGURE 7. Effects of hypoxia and OLNs on matrix invasion ability of human
462 **microvascular dermal endothelial cells.** HMEC-1 (1×10^5 cells/0.5 ml MCDB 131 medium)
463 were seeded on a Cultrex matrix and incubated for 2 h in normoxia (20% O₂) or hypoxia (1%
464 O₂) with/without 10% v/v OLNs. Thereafter, microvessel-like structures were checked by
465 optical microscopy and the number of crosses between two microvessel-like structures was
466 counted in five fields. A: representative images. B: means+SEM of numbers of crosses.**

467 Results are from four independent experiments. Data were also evaluated for significance by

468 Student's *t* test: * vs hypoxic untreated cells: $p < 0.05$.

469

470 **Discussion**

471

472 During healing processes, the balance between pro- and anti-angiogenic factors determining
473 specific endothelial cell behavior and vessel organization must be spatially and temporally
474 controlled. Among these factors, MMPs appear as pivotal molecules. These evolutionarily
475 conserved and tightly regulated zinc-dependent proteases are expressed either in a
476 constitutive or inducible manner by a broad spectrum of specialized cells, including
477 endothelial cells [Vandenbroucke et al., 2014]. Released as latent zymogens, activated locally
478 by other proteases and inhibited in a 1:1 stoichiometric ratio by their secreted endogenous
479 inhibitors (TIMPs) [Brew & Nagase, 2010], MMPs not only process all the components of
480 the basement membrane and the ECM, but can also cleave cytokines, chemokines, growth
481 factors, enzymes, and membrane-bound proteins, thus promoting their activation, inhibition,
482 degradation or shedding [Cauwe et al., 2007]. As such, they play essential roles in cell
483 survival, proliferation, migration, invasion, hemostasis and inflammation within the cellular
484 milieu of the wound [Gill & Parks, 2008].

485 A long-lasting hypoxic environment represents a critical feature of chronic wounds [4-5].
486 However, the effects of hypoxia on the phenotype and the behavior of the cellular
487 environment of the wound can be dramatically different depending on the considered cell
488 type (monocytes, keratinocytes, endothelial cells, fibroblasts etc). To complement previous
489 data on hypoxia-dependent dysregulation of MMP/TIMP balances in human monocytes
490 [Gulino et al., 2015] and keratinocytes [Khadjavi et al., 2015], the present *in vitro* study
491 aimed at investigating the effects of hypoxia on the pro-angiogenic phenotype and the wound
492 healing abilities of human dermal microvascular endothelial cells. Furthermore, innovative
493 and nonconventional dextran-shelled/DFP-cored OLN_s were challenged for their potential
494 abilities to counteract the effects of hypoxia.

495 Normoxic HMEC-1 constitutively secreted MMP-2, TIMP-1, and TIMP-2 proteins while
496 MMP-9 was not observed. In particular, cells were found to constitutively release only the
497 latent 72 kDa form of MMP-2, whereas its 62 kDa activated form was not detected. These
498 results are in line with previous reports on endothelial cells from both micro- and macro-
499 vascular vessels [Hanemaaijer et al., 1993; Ben-Yosef et al., 2002; Ben-Yosef et al., 2005;
500 Bertl et al., 2006]. Exposure of endothelial cells to prolonged hypoxia led to enhanced MMP-
501 2 and diminished TIMP-2 protein levels in cell supernatants, whereas TIMP-1 production
502 was not altered. The increase of MMP-2 resulted in elevated zymogen secretion but not in the
503 active form of the enzyme. Notably, latent MMP-2 undergoes activation mainly through
504 interactions with membrane-bound MT1-MMP and the $\alpha_v\beta_3$ integrin [Deryugina et al., 2001;
505 Hofmann et al., 2008]. Additionally, low levels of TIMP-2, the main MMP-2 inhibitor,
506 participate in MT1-MMP-mediated activation of MMP-2, while high levels of TIMP-2 can
507 block MMP-2 activation [Brew & Nagase, 2010]. Interestingly, hypoxia-dependent down-
508 regulation of MT1-MMP expression was previously reported for human endothelial cells
509 [Ben-Yosef et al., 2002]. This might justify the absence of the active 62 kDa form of MMP-2
510 in the present hypoxic model.

511 HMEC-1 were also challenged under hypoxic conditions for their ability to migrate, invade
512 the ECM and form tube-like structures. Indeed, ECM structure and composition provides a
513 scaffold and signals for cell adhesion and migration during tissue restoration [Li et al., 2005].
514 ECM effect on angiogenesis appears highly variable over time, strictly depending on protein
515 constituents, protease actions, and ECM ability to sequester growth factors and bioactive
516 molecular fragments [Wells et al., 2015]. Significantly, MMP-mediated degradation of ECM
517 can promote endothelial cell migration through exposure of pro-migratory matrix molecule
518 binding sites [Pepper, 2001; Hangai et al., 2002]. However, in the present work hypoxic
519 HMEC-1 displayed lower abilities to migrate and promote wound healing, as well as to

520 invade a collagen matrix and organize in tube-like structures compared to normoxic cells,
521 despite increased MMP-2 levels. Interestingly, similar results were obtained by Ben-Yosef
522 and colleagues in a previous work using endothelial cells from large caliber vessels, where
523 hypoxia led concurrently to an increase in proMMP-2 secretion and to a significant reduction
524 in the number of tube-like structures spontaneously formed in the culture [Ben-Yosef et al.,
525 2005]. Since specific MMP-2 inhibitors did not restore the normal tube-like formation, the
526 authors concluded that hypoxia-induced anti-angiogenic effects responsible for the observed
527 reduction in tube-like formation were not mediated by MMP-2. Consistently, in another *in*
528 *vitro* model, tube-like formation in human microvascular endothelial cells was shown to
529 depend directly on membrane-bound MT1-MMP and not on secreted MMPs such as MMP-2
530 [Koike et al, 2002]. Therefore, the compromised migration and invasion abilities of HMEC-1
531 highlighted here might be secondary to hypoxia-induced reduction of MT1-MMP, previously
532 reported for endothelial cells [Ben-Yosef et al., 2002]. On the other hand, in chronic wounds,
533 reduced protein levels compared to acute wounds have been described for several growth
534 factors including FGF, EGF, PDGF, VEGF, and TGF- β , secondary to trapping by ECM
535 molecules or excessive degradation by MMPs [Greaves et al., 2013]. Importantly, many of
536 these growth factors are MMP-2 substrates, including TGF- β , released after decorin cleavage
537 [Cauwe et al., 2009; Imai et al., 1997].

538 Once ascertained that hypoxia hampers HMEC-1 pro-angiogenic phenotype and behavior by
539 increasing MMP-2/TIMP-2 stoichiometric ratio and reducing cell migration and ECM
540 invasion abilities, new dextran-shelled OLN [Prato et al., 2015] were challenged for their
541 therapeutic potential to counteract the effects of hypoxia. The core structure of these
542 innovative and nonconventional gas nanocarriers is constituted by DFP, a stable and
543 biologically inert liquid fluorocarbon which carries molecular oxygen without actually
544 binding it, thus favoring gas exchange [Cote et al., 2008]. On the other hand, OLN shell is

545 constituted by dextran, a well-known polysaccharide classified as class 4 (low-toxicity)
546 substance [Bos et al., 2005]. OLN_s are able to release significant amounts of oxygen into
547 hypoxic environments in a time-sustained manner, opposite to OSS, which releases oxygen
548 only transiently, and to OFN_s, not releasing oxygen at all [Prato et al., 2015]. All sterile
549 nanodroplet preparations employed here displayed spherical shapes, nanometric sizes,
550 negative charges, high stability over time, and good oxygen-storing and -releasing abilities, in
551 accordance with literature data [Prato et al., 2015].

552 OLN_s were internalized by HMEC-1 into the cytoplasmic region, not entering the nuclei.
553 This evidence complements previous data on the uptake of OLN_s by other eukaryotic cells,
554 including human keratinocytes [Prato et al., 2015, Khadjavi et al., 2015] and monocytes
555 [Gulino et al., 2015]. OLN_s did not display cytotoxic effects on HMEC-1. Even more so, OLN_s
556 fully abrogated hypoxia-dependent dysregulating effects on proteolytic activity, restoring
557 normoxia-like balances between MMP-2 and TIMP-1/2 and improving migration and ECM
558 invasion abilities. These effects were specifically dependent on time-sustained oxygen release
559 from the inner core of OLN_s, since they were not reproduced after treatment with OFN_s or
560 OSS. These results are in full agreement with those obtained from parallel works with
561 dextran-shelled OLN_s, able to restore normoxia-like MMP-9/TIMP-1 ratio in hypoxic human
562 monocytes [Gulino et al., 2015], and chitosan-shelled OLN_s, effective in abrogating hypoxia-
563 dependent dysregulation of balances between gelatinases and their inhibitors in human
564 keratinocytes [Khadjavi et al., 2015]. Therefore, the findings proposed here appear extremely
565 relevant to reach a global vision of the pro-angiogenic phenotype of the chronic wound, since
566 endothelial cells play relevant roles during healing processes in concert with both monocytes
567 and keratinocytes [Eming et al., 2014].

568 In conclusion, the present work shows that prolonged hypoxia significantly alters the
569 phenotype and behavior of human dermal microvascular endothelium, enhancing MMP-2 and

570 reducing TIMP-1 secretion, and compromising cell abilities to migrate, promote wound
571 healing, invade the ECM and form tube-like structures. These findings enlarge the available
572 knowledge on the effects of hypoxia on the pro-angiogenic profile of single cell populations
573 actively involved in wound healing processes, thus helping to better understand the dynamics
574 occurring at the milieu of the hypoxic chronic wound. Intriguingly, dextran-shelled/DFP-
575 cored OLN_s proved effective in counteracting hypoxia, reestablishing normoxia-like pro-
576 angiogenic features in hypoxic microvascular endothelial cells. As such, these results support
577 the proposal that OLN_s should be tested as innovative, nonconventional, cost-effective, and
578 nontoxic adjuvant therapeutic tools for chronic wound treatment, in order to promote or
579 accelerate tissue repair and the regeneration processes. Based on the present *in vitro*
580 evidence, future preclinical studies to translate OLN technology to clinical practice are
581 envisaged.

582

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595

596 **List of Abbreviations**

597

598 ANOVA, analysis of variance; DAPI, 4',6-diamidino-2-phenylindole; DFP, 2H,3H-
599 decafluoropentane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
600 ECM, extracellular matrix; FITC, fluorescein isothiocyanate; LDH, lactate dehydrogenase;
601 MMP, matrix metalloproteinase; OFN, oxygen-free nanodroplet; OLN, oxygen-loaded
602 nanodroplet; OSS, oxygen-saturated solution; PBS, phosphate-buffered saline; PFP,
603 perfluoropentane; TIMP, tissue inhibitor of metalloproteinase; US, ultrasound; UV,
604 ultraviolet.

605

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