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Extracellular xenogeneic hemoglobin suppresses the capacity for C2C12 myoblast myogenic differentiation

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Abstract: Functional characteristics of satellite cells (SCs) that act as myogenesis initiators and have emerged as a promising target for cell therapy, are dependent on their microenvironment. The aim of this study was to investigate the effect of cell-free hemoglobin, as a part of the microenvironment of SCs, on their functional characteristics. The C2C12 cell line served as the experimental model of SCs; hemoglobin isolated from porcine (PHb) and bovine (BHb) slaughterhouse blood served as the experimental model for extracellular hemoglobin. The proliferation rate of C2C12 cells was assessed by the MTT test, migration capacity by the scratch assay, and myogenic differentiation capacity by histochemical staining and RT-PCR analysis of the expression of genes specific for myogenic lineage. The effect of hemoglobin on the proliferation and migration of C2C12 cells was dependent on its concentration and the animal species it was isolated from, but the effect of BHb was more prominent. Both PHb and BHb decreased the expression levels of myogenin and muscle specific creatine kinase at a 10 μM concentration. While PHb had no effect on the morphometric parameters of C2C12 myotubes, BHb modified the area and length of C2C12 myotubes cultivated in DMEM/2% horse serum and DMEM/10% fetal calf serum. While PHb and BHb had no effect on heme oxygenase 1 (*Hmox1*) expression, they stimulated the expression of hypoxia-inducible factor 1-alpha (*Hif1α*) at a concentration of 10 μM. The mainly inhibitory effect of cell-free hemoglobin on myogenic differentiation suggests that it could be a relevant factor in the outcome of cell therapy of muscle injury.

Keywords: extracellular hemoglobin; C2C12 cells; myogenic differentiation; proliferation; migration

INTRODUCTION

Muscle injuries, often seen in athletes and military personnel, as well as in patients undergoing surgical interventions (e.g. tumor ablation, soft tissue reconstruction, joint arthroplasty) remain an unmet clinical challenge, despite their relatively high incidence rate [1,2]. Aside from the standard RICE (rest, ice, compression and elevation) principle, the treatment for injured skeletal muscle may include medication (nonsteroidal antiinflammatory drugs (NSAIDs) or glucocorticoids), exercise, therapeutic ultrasound and hyperbaric oxygen therapy [1]. Beyond a certain injury severity threshold, the treatment may rely on surgical interventions or cell therapy that has emerged as an attractive alternative therapy for muscle injuries [3,4].

The endogenous response of skeletal muscles to injury or trauma depends on the highly orchestrated series of biological processes that are comparatively well-elucidated and comprised of several phases [2]. Following the rupture and necrosis of myofibers and surrounding blood vessels, hematoma is formed and a proinflammatory response is triggered. In this phase, neutrophils invade the site of injury, degrade muscle membranes and produce free radicals. The second phase is characterized by tissue repair and regeneration and relies on proinflammatory M1 and antiinflammatory

M2 macrophages. M1 macrophages remove debris, execute structural degradation and secrete cytokines that stimulate the proliferation and migration of satellite cells (SCs) [5], whereas M2 macrophages inhibit the deposition of excessive extracellular matrix (ECM) and stimulate the fusion of myoblasts into multinucleated myotubes. The final phase involves the maturation of myotubes into functional, contractile myofibers, remodeling of the connective tissue ECM, and establishment of neuromuscular junctions. The crucial part of this process relies on SCs that are a rare population of muscle-specific progenitor cells (2-7% of all muscle cells), and represent an appealing strategy for treating muscle disorders and injuries because of their intrinsic myogenic potential [3].

Recent reports have provided evidence that the functional characteristics of SCs are essentially dependent on the properties of their microenvironment, i.e. niche [2,3]. Beside resident fibroblasts and fibro/adipogenic progenitor cells, which secrete the muscle tissue extracellular matrix (ECM) [6], the microenvironment of SCs is comprised of different molecules found extracellularly upon rupture of myofibers and surrounding blood vessels, including hemoglobin (Hb). Although SCs have emerged as a promising target for cell therapy, due to their low number, limited ability to proliferate *in vitro*, intrinsic heterogeneity [7] and poor survival rate upon transplantation *in vivo* [3], the majority of *in vitro* studies aimed at the development of skeletal muscle are performed on well-characterized cell lines such as C2C12 [8]. Furthermore, in recent years xenogeneic hemoglobin is attracting attention not only as a substrate simulating the *in vivo* presence of extracellular hemoglobin but also as an agent for tissue and organ preservation [9,10] or as a modulator of mesenchymal cell differentiation [11,12]. We have previously shown that extracellular hemoglobin originating from bovine erythrocytes can decrease the capacity of multipotent mesenchymal cells to differentiate towards chondrogenic, osteogenic and adipogenic lineages [12]. Having enucleated erythrocytes, bovine and porcine blood are interesting xenogeneic hemoglobin sources which show a high degree of homology to human HbA (84% and 85%, respectively) [13,14].

The aim of this work was to investigate the effect of cell-free xenogeneic hemoglobin molecules isolated from bovine and porcine erythrocytes, on the functional characteristics of myoblasts: their proliferation, migration and differentiation. To the best of our knowledge, this issue has not been addressed previously. Data obtained in this study could be of practical importance when treating muscle injuries in patients with hemoglobinemia-related diseases and conditions, as well as when using SCs for cell therapy, since the presence of extracellular hemoglobin could potentially influence its outcome.

MATERIALS AND METHODS

Cell culture

The mouse myoblast cell line C2C12 was purchased from American Type Culture Collection (ATCC, USA). Cells were cultured in growth medium (GM) containing Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA) supplemented with 10% fetal calf serum (FCS; Capricorn-Scientific, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Thermo Fisher Scientific, USA) at 37°C in humidified atmosphere containing 5% CO_2 (standard conditions). The GM was replaced every second day. Upon reaching a confluence of 80%, the cells were detached using a 0.25% solution of trypsin/EDTA (Gibco, Thermo Fisher Scientific, USA).

Hemoglobin isolation and characterization

The hemoglobin used in this study was isolated from porcine and bovine slaughterhouse blood by the process our group had optimized, involving gradual hypotonic hemolysis, as previously described [15]. Supernatants containing hemoglobin were purified by tangential microfiltration and the protein and lipid contents were analyzed by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) and isoelectric focusing [16], and by thin-layer chromatography (TLC) and gas chromatography [17], respectively. Since UV-Vis spectra and dynamic light scattering analysis showed the absorption bands characteristic for native oxyhemoglobin, and as there were no significant individual variations between hemoglobin samples isolated from different animals of the same species [16], pooled hemoglobin samples were used for the cell culture experiments.

Lipids from hemoglobin samples were extracted with a chloroform-methanol mixture (2:1, v/v). The chloroform phase was vacuum evaporated, and the lipids were dissolved in chloroform [18]. TLC on silica gel GF plates (Merck, Darmstadt, Germany) was used to separate and identify lipids [19]. Petroleum ether-diethyl ether-acetic acid (87:12:1v/v) was used as the solvent system for neutral lipid separation. Lipid bands were visualized using I_2 vapor staining, and the lipid profiles were analyzed by densitometry. Lipids were identified based on their chromatographic mobility, using a standard lipid mixture (C.f.a.s., Calibrator for automated systems; Roche Diagnostics, IN, USA). Individual fatty acids (FA) in phospholipids from hemoglobin samples were determined by gas chromatography by comparing their retention times with those of commercial polyunsaturated fatty acid methyl esters mixture, PUFA-2 (Supelco Inc., Bellefonte, Pennsylvania, USA) [20].

Cell proliferation assays

The C2C12 cell proliferation rate was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) test. Cells were seeded at a concentration of 8×10^3 cells/well in GM and allowed to adhere to plastic overnight. The next day, hemoglobin was added to the cell culture at concentrations of 0.1 μM, 1 μM and 10 μM, and the cells were cultured for additional 24 h, 48 h and 72 h. At the end of the incubation, MTT was added to the cell culture at a concentration of 0.5 mg/mL. The MTT incubation lasted 2.5 h during which formazan crystals were formed and then dissolved in 10% SDS with 0.01 N HCl. Absorbance at 540 nm was measured the next day using an automatic reader for microtiter plates (Labsystems Multiskan PLUS, Finland) [21].

Wound healing assay

Cell migration capacity was analyzed by *in vitro* wound healing, scratch assay. Briefly, C2C12 cells were plated in 24-well plates (5×10^4 cells/well) and cultured in GM until reaching confluence, when a scratch was made in the cell monolayer using a 200-μL pipette tip. After making a scratch in each plate well, the cells were washed two times with PBS and incubated in DMEM with 1% FCS and hemoglobin at concentrations of 0.1 μM, 1 μM and 10 μM for additional 24 h and 48 h. After the incubation period, the cells were fixed in ice-cold methanol for 10 min and stained with 0.1% crystal violet for 10 min. To visualize cell migration into the scratched areas, an inverted light microscope (Olympus BX51, Japan) was used. The scratched areas were photographed and quantified by TScratch software (Computational Science and Engineering Laboratory, Swiss Federal Institute of Technology, ETH Zurich, Zurich, Switzerland) as previously described [22].

Myogenic differentiation and semi-quantitative RT-PCR assay

To induce myogenic differentiation of C2C12 cells for the RT-PCR assay, the cells were cultured in GM, DMEM supplemented with 5% FCS (DMEM/5% FCS), and myogenic differentiation medium (MM) consisting of DMEM supplemented with 2% horse serum (HS) [22]. Upon reaching 70% confluence, hemoglobin was added in MM at concentrations of 0.1 μM, 1 μM and 10 μM, and the cells were cultured for additional 6 days. After the treatment, total cell RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and complementary DNA (cDNA) was synthesized using the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA), with oligo (dT) as a primer. Primers were designed using mRNA sequences of specific genes from the nucleotide database through the National Center for Biotechnology Information (NCBI) online tools and synthesized by Invitrogen (Carlsbad, CA, USA). The primer sets, corresponding annealing temperatures and the number of amplification cycles are provided in Supplementary Table S1. As a control for the amount of cDNA in each sample, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was assessed. PCR products were resolved in 1.5% agarose gel and stained with ethidium bromide. The intensities of the gel bands in the agarose gel were quantified using TotalLab TL120 (TotalLab Ltd, Newcastle Upon Tyne, UK). The expression levels of the analyzed genes were normalized to GAPDH and set as 100% for the control culture. The relative expression of the genes in the presence of hemoglobin was calculated as the percentage of the change compared to the control.

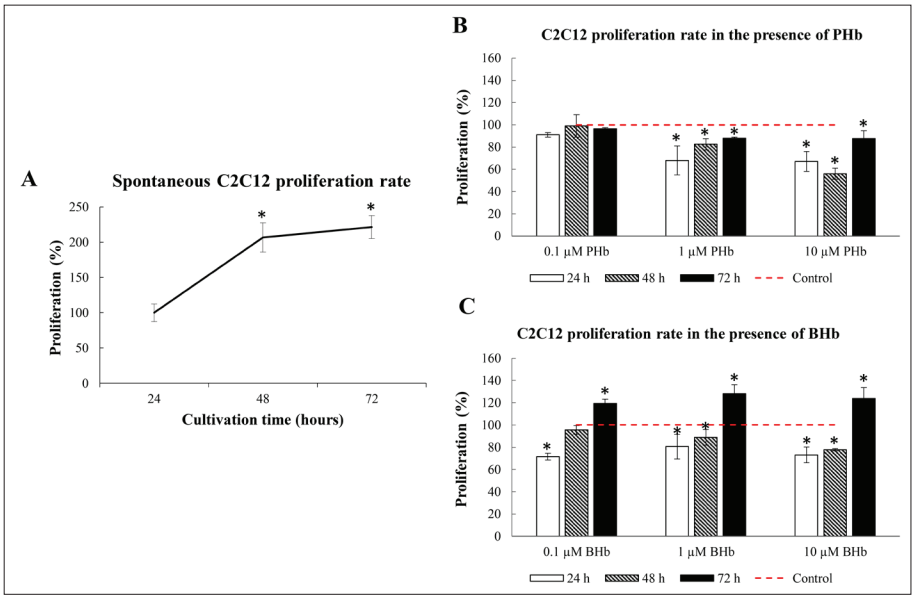


Fig. 1. The effect of porcine (PHb) and bovine (BHb) hemoglobin on C2C12 cell proliferation rates: Spontaneous proliferation of C2C12 cells after 24 h, 48 h and 72 h in culture, assessed by the MTT assay (**A**). The results are expressed as percentages of the cell proliferation rate after 24 h in culture (100%). The effect of 0.1 μM, 1 μM and 10 μM PHb (**B**) and BHb (**C**) on the proliferation rate of C2C12 cells. The results are expressed as relative to corresponding control level (GM=100) and mean±SD (n = 3); * $P<0.05$, PHb/GM vs GM or BHb/GM vs GM.

Myogenic differentiation and morphometric analysis

Myogenic differentiation of C2C12 cells for morphometric analysis was induced by prolonged cultivation (40 days) of cells in DMEM/5% FCS. After this period, the cells were seeded in a concentration of 8×10^3 cells/well in DMEM/5% FCS and upon reaching 70% confluence, they were cultured for an additional 6 days with hemoglobin added to: DMEM/5% FCS, DMEM/2% HS, or DMEM/10% FCS. After these different treatments, the cells were fixed and stained according to the protocol described in the wound healing assay section. Morphometric parameters of the formed myotubes were determined using a light microscope and a digital camera system (Olympus BX51, Japan) as previously described [23]. Namely, C2C12 cells were cultured in different media in 6-well plates; 10 myotubes per well were photographed and measured, or all the observed myotubes for the given treatment if their number was <10. The area, length, average width and hypertrophy index of each myotube were determined using NIH-ImageJ software (National Institute of Health, USA). The average width was calculated by taking 3 short-axis measurements along the length of a given myotube and calculating the average value.

Statistical analysis

Statistical analysis was performed using Student's t-test by Microsoft Office Excel 2010 software (Microsoft Corporation, Wash., USA). Differences were considered significant at a value of *$P<0.05$.

RESULTS

The effect of hemoglobin on C2C12 cell proliferation rate

To assess the effects of PHb and BHb on the proliferation rate of C2C12, we examined 3-day cultures using the MTT assay. The spontaneous cell proliferation rate (cells grown in GM only) increased over time (Fig. 1A). PHb significantly reduced the viability of C2C12 cells at concentrations of 1 μM and 10 μM after 24 h, 48 h and 72 h, while having no effect at the concentration of 0.1 μM (Fig. 1B). BHb reduced the viability of C2C12 cells after 24 h at all 3 examined concentrations. After 48 h this inhibitory effect was manifested only at concentrations of 1 μM and 10 μM. In contrast, BHb increased the viability of C2C12 cells at all 3 tested concentrations after 72 h in culture (Fig. 1C).

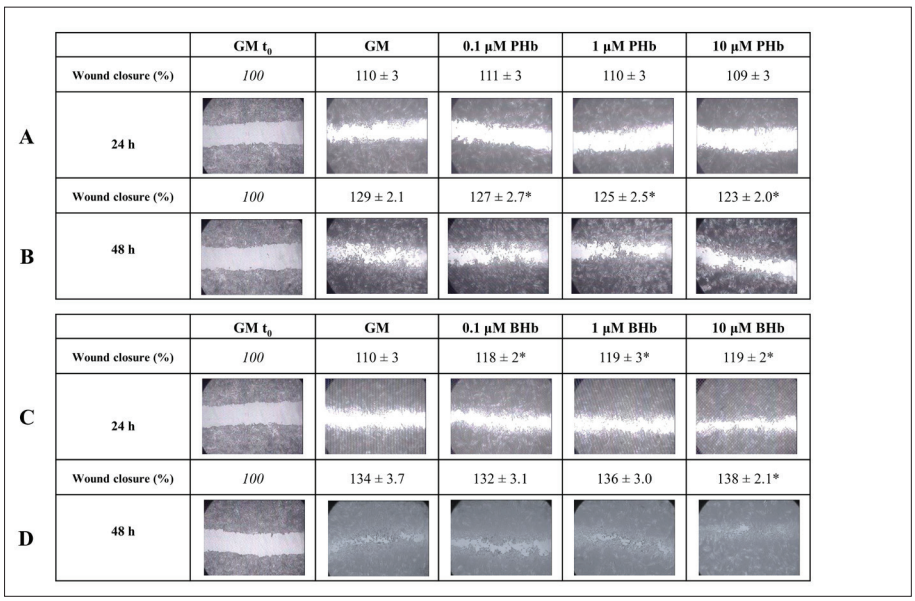
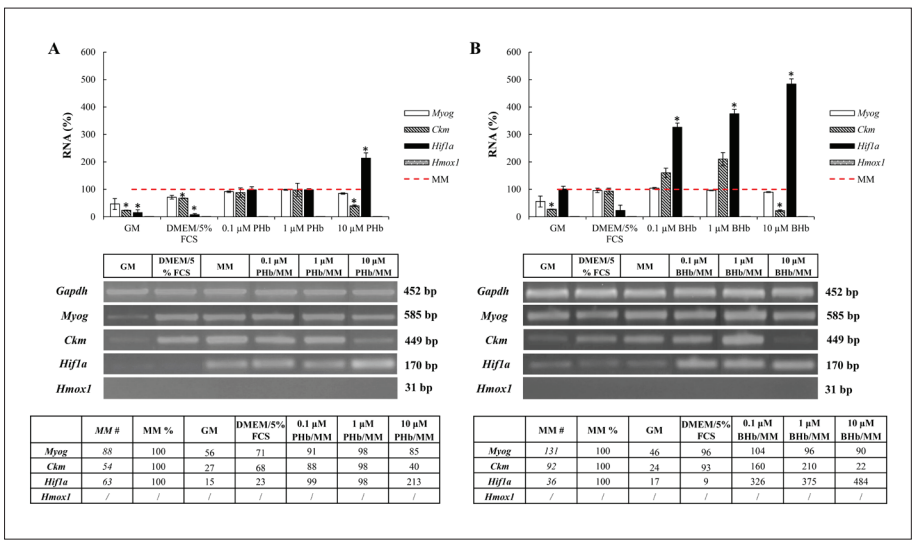


Fig. 2. The effect of porcine (PHb) and bovine (BHb) hemoglobin on the migratory capacity of C2C12 cells assessed by the scratch assay. Upon making a scratch in the cell monolayer of each well the cells were seeded on, the cells were cultured in the presence of 0.1 µM, 1 µM and 10 µM PHb or BHb for 24 h (**A**) and 48 h (**B**) and stained with crystal violet. Cells in wells labeled GM t_0 were fixed immediately after making the scratch. The numbers represent percentages of the scratch areas covered with migrating cells relative to GM t_0 (100%). Representative micrographs are shown; magnification ×40. The results are expressed as the mean±SD (n=3); **P*<0.05, PHb/GM vs GM or BHb/GM vs GM.



	MM #	MM %	GM	DMEM/5% FCS	0.1 µM PHb/MM	1 µM PHb/MM	10 µM PHb/MM
Myog	88	100	56	71	91	98	85
Ckm	54	100	27	68	88	98	40
Hif1a	63	100	15	23	99	98	213
Hmox1	/	/	/	/	/	/	/

	MM #	MM %	GM	DMEM/5% FCS	0.1 µM BHb/MM	1 µM BHb/MM	10 µM BHb/MM
Myog	131	100	46	96	104	96	90
Ckm	92	100	24	93	160	210	22
Hif1a	36	100	17	9	326	375	484
Hmox1	/	/	/	/	/	/	/

Fig. 3. The effect of porcine (PHb) (**A**) and bovine (BHb) (**B**) hemoglobin on the expression of specific myogenic markers, and HIF-1 and HO in C2C12 cells after 6 days of cultivation in myogenic medium (DMEM/2% HS, MM). Using RT-PCR, the expression of *Myog*, *Ckm*, *Hif1a* and *Hmox1* genes in C2C12 cells was assessed. GAPDH was used as a gel loading control. Representative RT-PCR bands are shown as well as quantitative data of RT-PCR results. The expression level of each gene in MM was normalized to the corresponding expression level of GAPDH (marked with an #) and presented as 100%. The results are shown as the mean±SEM (n=3) **P*<0.05, PHb/MM vs MM or BHb/MM vs MM.

The effect of hemoglobin on C2C12 cell migratory capacity

The effect of medium supplementation with porcine and bovine hemoglobin on the migratory capacity of C2C12 cells was analyzed by the scratch assay. After making a scratch in C2C12 cell monolayers, PHb and BHb were added at concentrations of 0.1 µM, 1 µM and 10 µM. Cells were allowed to migrate for 24 h and 48 h since after 24 h no changes in scratch closure were observed for cells cultured with PHb (Fig. 2A). In contrast to this, BHb stimulated the migratory capacity of the cells after 24 h at all tested concentrations (Fig. 2B), and only at the concentration of 10 µM after 48 h (Fig. 2D). The same positive effect on the migratory capacity of C2C12 cells was observed at all examined PHb concentrations in the 48-h cultures (Fig. 2C).

The effect of hemoglobin on C2C12 myogenic differentiation capacity

To further investigate the effect of PHb and BHb on the functional activity of C2C12 cells, we analyzed their influence on the ability of C2C12 cells to differentiate towards the myogenic lineage. Gene expression analysis of C2C12 cells after 6 days of myogenic differentiation

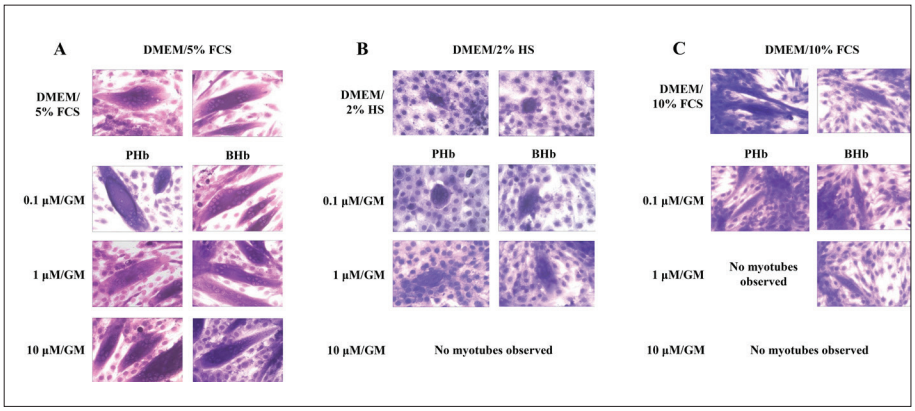


Fig. 4. Representative micrographs of C2C12 myotubes formed after prolonged cultivation (40 days) in DMEM/5% FCS prior to 6 days cultivation in DMEM/5% FCS (**A**), DMEM/2% HS (**B**) and DMEM/10% FCS (**C**) supplemented with 0.1 μM, 1 μM and 10 μM porcine (PHb) or bovine (BHb) hemoglobin. Myotubes were analyzed and photographed using a light microscope and a digital camera system (Olympus BX51, Japan), magnification ×40.

induction showed that in the myogenic differentiation medium (MM = DMEM/2% HS) C2C12 expressed both myogenin (MYOG, encoded by the *Myog* gene) and creatine kinase specific for muscle tissue (CKM, encoded by the *Ckm* gene). Both PHb and BHb at concentrations of 0.1 μM and 1 μM did not influence the expression levels of *Myog* in C2C12 cells, but slightly decreased it at a concentration of 10 μM (Fig. 3A, B). At concentrations of 0.1 μM and 1 μM, BHb increased the expression of *Ckm*, while PHb had no effect on the expression of this myogenic marker at these concentrations. In contrast, both PHb and BHb significantly decreased the expression of *Ckm* at a concentration of 10 μM (Fig. 3A, B). While the expression of heme oxygenase-1 (HO-1) mRNA was not detected by RT-PCR, the influence of PHb and BHb on the expression pattern of hypoxia-inducible factor 1 subunit α (HIF-1α) mRNA was dose-dependent and more pronounced in cells cultured with BHb. While PHb had no effect on *Hif1a* expression at concentrations of 0.1 μM and 1 μM, it stimulated its expression at a concentration of 10 μM. The effect of BHb on *Hif1a* expression was stimulatory at every tested concentration. Our results also showed that C2C12 cells express *Myog* and *Ckm* at a low basal level even in GM (DMEM/10% FCS) and in a medium containing two-fold less serum (DMEM/5% FCS) than the GM. As a result, we decided to test the effect of PHb and BHb supplementation of these 3 different media on the morphometric parameters of C2C12 myotubes after their cultivation in DMEM/5% FCS.

The effect of hemoglobin on the morphometric parameters of C2C12 myotubes

Myotube formation was observed in cells cultured in MM and DMEM/5% FCS after 6 days, but not in GM (Supplementary Fig. S1). Since the expression patterns of myogenic markers in C2C12 cells showed that DMEM/5% FCS can also act as a medium for the induction of myogenic differentiation of these cells, we decided to analyze the effects of prolonged cultivation (40 days) of C2C12 cells in this medium, and to see whether adding PHb/BHb to this medium, or whether switching back to the conventionally used MM (DMEM/2% HS) or GM (DMEM/10% FCS) media with or without PHb or BHb, would affect the formation and morphology of C2C12 myotubes.

After growth in DMEM/5% FCS, C2C12 cells formed large hypertrophied myotubes (Fig. 4A) and the addition of PHb or BHb did not influence the morphometric parameters (area, length, average width and the hypertrophy index (H.I.)) of the analyzed myotubes (Fig. 5A, D). When switching back to the routinely used MM for the induction of myogenic differentiation of C2C12 cells, DMEM/2% HS, the myotubes were significantly smaller (Fig. 4B). PHb at concentrations of 0.1 μM and 1 μM had no influence on the morphometric parameters of C2C12 myotubes in this experimental setup (Fig. 5B), while the addition of BHb to DMEM/2% HS increased the area of the formed myotubes at the concentration of 0.1 μM, and decreased the length at the concentration of 1 μM (Fig. 5E). In the medium supplemented with 10 μM PHb or BHb, no myotubes were formed (Fig. 5B, E).

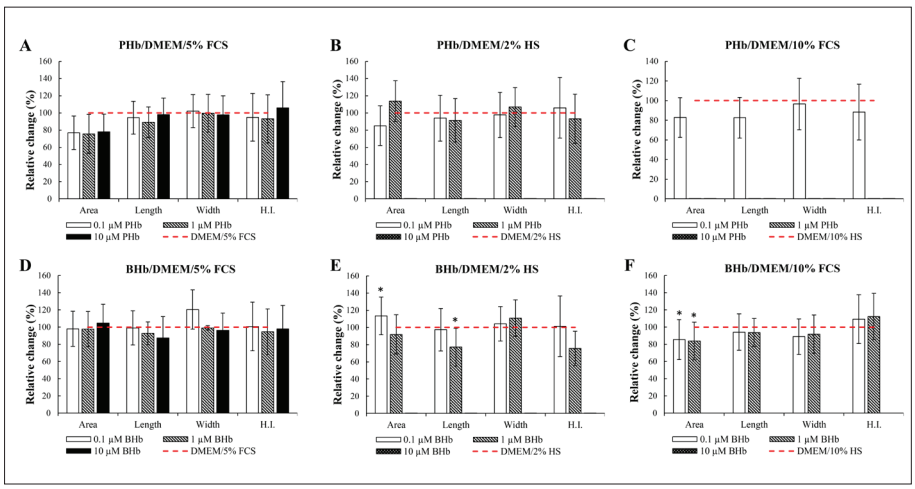
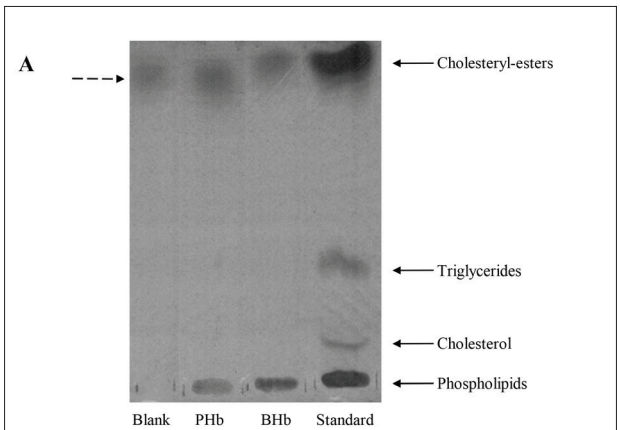


Fig. 5. Morphometric analysis of C2C12 myotube parameters after prolonged cultivation (40 days) in DMEM/5% FCS followed by 6 days cultivation in DMEM/5% FCS (**A** and **D**, respectively), DMEM/2% HS (**B**, **E**), and DMEM/10% FCS (**C**, **F**), supplemented with 0.1 μM, 1 μM and 10 μM porcine (PHb) (**A**, **B** and **C**, respectively) and bovine hemoglobin (BHb) (**D**, **E** and **F**, respectively). The area, length, average width and hypertrophy index (HI) are shown. The results are expressed as relative to the corresponding control level (medium without hemoglobin = 100%) and the mean±SD ($15<n<60$); *$P<0.05$, PHb/GM vs GM or BHb/GM vs GM.

When cultured in GM for an additional 6 days, the myotubes assumed their usual long and narrow morphology (Fig. 4C). Myotubes were detected in the medium supplemented with either 0.1 μM PHb or 0.1 μM and 1 μM BHb. PHb had no effect on the morphometric parameters of these myotubes (Fig. 5C), while BHb decreased the area of myotubes at concentrations of 0.1 μM and 1 μM (Fig. 5F). No myotubes were detected in GM supplemented with 1 μM and 10 μM PHb or with 10 μM BHb (Fig. 5C, F).

Lipid content in hemoglobin samples

Using TLC, both porcine and bovine hemoglobin samples were analyzed for the presence of inevitable impurities originating from erythrocyte membranes during the isolation process. Phospholipids were the only lipids identified in hemoglobin samples of both species (Fig. 6A). Ten FA were identified in phospholipids originating from PHb, and 6 in phospholipids present in the BHb (Fig. 6B). Saturated FA (palmitic and stearic acid) were the most dominant FA in the hemoglobin samples of both species (>50%), but monounsaturated (MUFA) and polyunsaturated FA (PUFA) were also detected. The amount of palmitic acid was higher in PHb samples compared to BHb samples, while the percentage of stearic acid was approximately equal in the hemoglobin samples of both species. The MUFAs detected in PHb samples were palmitoleic, oleic and vaccenic, while oleic acid was the only MUFA detected in the BHb sample. Five PUFA were detected in PHb samples (linolenic, γ-linolenic, α-linolenic, dihomo-



B

	Fatty acids	Number of carbon atoms	PHb	BHb
Saturated fatty acids	Palmitic	16:0	30.4 ± 0.4	24.1 ± 1.0
	Stearic	18:0	29.2 ± 5.2	31.2 ± 3.7
Monounsaturated fatty acids	Palmitoleic	16:1	0.4 ± 0.1	/
	Oleic	18:1 (n-9)	22.2 ± 1.0	27.6 ± 2.5
	Vaccenic	18:1 (n-7)	1.6 ± 0.1	/
Polyunsaturated fatty acids	Linoleic	18:2	12.2 ± 1.4	13.6 ± 1.3
	γ-linolenic	18:3 (n-6)	1.7 ± 0.1	/
	α-linolenic	18:3 (n-3)	0.3 ± 0.1	/
	Dihomo-γ-linolenic	20:3	1.7 ± 0.1	5.4 ± 0.1
	Arachidonic	20:4	6.3 ± 0.1	1.9 ± 0.1

Fig. 6. TLC analysis of the lipid content in PHb and BHb isolates. The dashed arrow points to the non-specific butyl-hydroxy-toluene band (**A**). The fatty acid (FA) content in phospholipids in PHb and BHb isolates determined by gas chromatography (**B**). The results are expressed as the mean±SD (n=3).

γ-linolenic, arachidonic and linoleic acid), and three (dihomo-γ-linolenic, arachidonic, and linoleic acid) in the BHb sample.

DISCUSSION

While sequestered within the confines of the erythrocytes, hemoglobin has a role in the transport of gaseous molecules, maintaining cellular bioenergetic homeostasis and signaling, and modulating inflammatory responses [24]. When present in the extracellular environment, hemoglobin can acquire numerous functions that exceed its roles while sequestered inside of erythrocytes. Extracellular hemoglobin is a potentially dangerous redox-sensitive molecule with a high potential for producing reactive oxygen species (ROS) that pose a threat to cellular integrity [25]. Additionally, hemoglobin and its degradation products are defined as alarmins or indicators of different pathological states and conditions in organism. Also, hemoglobin can interact with microbial ligands, such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), and immune system receptors, such as Toll-like receptors (TLRs), modulating the host immune response to pathogens [26]. Numerous studies have confirmed the role of hemoglobin in the pathophysiology of various diseases, such as paroxysmal nocturnal hemoglobinuria (PNH), sickle cell disease (SCD), Alzheimer's disease, multiple sclerosis, cerebral intraventricular hemorrhage and preeclampsia [24,27-30].

Muscle tissue growth and regeneration relies on SCs, a well-known muscle-resident cell population, located between muscle fiber and the basal lamina [2,31]. SCs are naturally quiescent until an activation signal arrives from injured myofibers, when these cells migrate from their niche and interact with the components of their environment to promote muscle tissue regeneration through myogenic differentiation [3,32]. Damaged muscle cells express and secrete numerous factors, amongst which is stromal cell-derived factor-1 (SDF-1), also known as chemokine CXCL12, the ligand for CXCR4, which facilitates the migration of SCs to the site of injury [31], enhancing their proliferation and subsequent differentiation into myoblasts. During the proliferation phase, activated SCs secrete factors to promote the survival and differentiation of other cells. By then, SCs have migrated to the site of injury, proliferated and initiated the myogenic differentiation process. Macrophages that will support muscle tissue regeneration usually reach the muscle on about the second day after injury [2]. These macrophages are also involved in the clearance of hemoglobin-haptoglobin complexes (via the CD163 receptor) [33], giving the extracellular hemoglobin and its degradation products enough time to exhibit their effects on the functional characteristics of the surrounding cells. It was demonstrated that in patients with persistently elevated levels of cell-free hemoglobin that exceed the capacities of their scavenging mechanisms, skeletal muscle function and regeneration are impaired due to the presence of hemoglobin [34].

To the best of our knowledge, this is the first study of the effect of extracellular hemoglobin on myoblast cells proliferation. The effect of hemoglobin and its degradation products, heme, hemin and peptides, on the viability/proliferation of cells has been investigated thoroughly on numerous primary cells and cell lines. These results are controversial, as they indicate that this effect depends on cell type, hemoglobin concentration and the species it was isolated from, as we have also previously demonstrated [12].

Our results show that PHb decreased the C2C12 proliferation rate at concentrations of 1 μM and 10 μM but had no effect at a concentration of 0.1 μM. These results are in agreement with the results of several other studies. Namely, HEMOXCell®, extracellular hemoglobin extracted from a marine invertebrate *Nereis virens* [35], decreased the viability of the CHO-S cell line at concentrations of 1 g/L and 2 g/L [36], which correspond to the highest concentrations used in our study. Furthermore, the tetrapeptide derived from BHb exhibited slight cytotoxicity on the human pancreatic cancer MIAPaCa-2 cell line at concentrations of 0.1-0.4 mg/mL after 72 h of incubation [37]. At concentrations of 0.25, 0.5 and 1 mg/mL, human hemoglobin decreased the viability of human umbilical vein endothelial (HUVEC) cells after 24 h, 48 h and 72 h of incubation [38]. At high concentrations (1-5 g/L), human hemoglobin also decreased the viability of human U251 glioblastoma cells in hypoxic culture [39]. The cytotoxic effect of hemoglobin and hemin on neurons and glial cells has also been reported [40-42]. The effect of BHb on the proliferation rate of C2C12 cells was similar to that observed after exposure to PHb for 24 h in culture (i.e. a decreased proliferation

rate at all concentrations tested) and for 48 h (i.e. a decreased proliferation rate at concentrations of 1 μM and 10 μM), but interestingly, BHb stimulated the proliferation of these cells after 72 h at all 3 tested concentrations. A few studies describe cell viability/proliferation stimulation as one of the effects of hemoglobin on the functional characteristics of cells. Namely, human hemoglobin stimulated the proliferation of colon cancer cell lines, HT-29 and Lovo, as well as of the normal colonic fibroblast cell line, CCL-33Co in a dose-dependent manner [43]. Bovine hemoglobin supplementation (15 g/L) supported the maintenance of a higher hepatocyte cell mass in hepatic hollow fiber bioreactors [44] and dose-dependently increased cell viability of rat primary astrocyte cultures [45]. As we are aware of the limitations of the MTT assay in measuring cell viability [46,47], further studies are needed to elucidate the molecular mechanisms that underlie the observed effect of hemoglobin on cell proliferation rate and to investigate whether it is due to heme/hemin, globin chains or iron derived from the hemoglobin molecule.

To the best of our knowledge, no single study has addressed the issue of hemoglobin influence on myoblast mobilization and migration before. After 24 h in culture, PHb did not influence the migratory capacity of C2C12 cells. Thus, we decided to prolong the assay for an additional 24 h. After 48 h, PHb slightly inhibited the migration of these cells, which is in accordance with a previous study that showed that HbA1c, glycated hemoglobin isolated from patients with type 2 diabetes, reduced the migration of HUVEC cells in a dose- and time-dependent manner [48]. Also, it was reported that two peptides derived from BHb blocked about 50% and 30% wound closure in a scratch assay of the MIAPaCa-2 cell line when compared to the control group after treating for 72 h [37]. At variance with the effect of PHb, BHb in our study stimulated the migration of C2C12 cells after 24 h in culture, and since the scratch of cells in the medium without hemoglobin was nearly entirely closed after 48 h, this effect of BHb was observed only at the highest concentration used for 48 h in culture. The stimulatory effect of BHb on cell migration was previously also reported for glioma cells under hypoxic conditions [39].

The C2C12 cell line, a myoblast cell line derived from murine satellite cells, is extensively used as an *in vitro* model to study myogenesis [23,49]. Upon changing sub-confluent C2C12 cultures from high-serum (10% FCS) to low-serum conditions (2–5% HS), these cells exit the cell cycle, commit to myogenic differentiation and fuse with other myoblasts to form multinucleated myotubes. As the process of myogenesis advances, the myotubes become more elongated and start to express proteins such as myosin, a-actin, troponin, MYOG, CKM and other components of the muscle-contractile machinery [49]. Quiescent SCs do not express muscle-specific transcription factors including MYOG [31]. Newly formed, young myoblasts do not express MYOG, and its absence prevents their differentiation into myofibers or fusion with the old ones, instead allowing cell proliferation. When the number of myoblasts increases, the expression of MYOG in SCs is stimulated, leading to myoblast differentiation [6]. Considering this, MYOG is considered an early marker of myogenesis. In contrast, muscle creatine kinase, the enzyme with a crucial role in muscle cell energy management, represents a late marker of myogenesis [50,51]. We therefore decided to test the effect of hemoglobin on the expression patterns of both of these markers to investigate their effects on myogenesis at two points of time, at the beginning of this process and later. Our results show that both PHb and BHb decreased the expression levels of *Myog* and *Ckm* at the concentration of 10 μM. Even though previous reports of the effect of hemoglobin on myogenic cell differentiation do not exist, we showed that BHb decreased the capacity of mesenchymal stem cells and different cell lines towards chondrogenic, osteogenic and adipogenic differentiation [12]. At odds with our results, it was reported that hemin enhanced the fusion and maturation of cultured regenerated skeletal myotubes while the ferrous ion had no effect on these cells [52]. Since in our study neither PHb nor BHb triggered the expression of *Hmox1*, we hypothesized that the hemoglobin molecule remained intact in the C2C12 culture and exhibited an effect that was different from the effect of its degradation products. Nonetheless, without further and more sophisticated analysis of the fate of the hemoglobin molecule in C2C12 cell culture, hemin, globin chains and iron cannot be excluded as the potential inducers of the observed effects on myogenic differentiation of C2C12 cells.

In the elucidation of the molecular mechanism underlying the observed effect of hemoglobin on myo-

genic differentiation, in addition to *Hmox1* expression, we also analyzed *Hif1α* expression. It is known that hypoxia through HIF-1α and Notch signaling can block the differentiation of myogenic progenitors [53]. Given that our results showed that both PHb and BHb at the highest concentration tested (10 μM) stimulated the expression of *Hif1α* in C2C12 cells and that this effect of BHb was more prominent, we concluded that this can potentially explain the observed downregulation of myogenic markers (*Myog* and *Ckm*). Furthermore, hypoxia through HIF-1 can promote the proliferation of skeletal myoblasts [54]. Therefore, we hypothesized that the observed stimulation of the proliferation rate of C2C12 cells in the presence of BHb was at least in part due to the upregulation of *Hif1α* in these cells. We are aware of the limitations of the MTT assay, which depends on the metabolic rate and number of mitochondria in the cells [55]. Hence, the results obtained in this study can be viewed in the context of recently published data that show that upregulation of aerobic metabolism can enhance the proliferative capacity, osteogenic potential and migration capacity of MSCs *in vitro* [56].

Several methods can be applied to quantify the *in vitro* myogenic differentiation of C2C12 cells [8]. Determining the expression levels of myotube-specific genes and proteins provides an overall and unbiased measure of differentiation but does not inform about the shape and size of myotubes or the effect of the tested agents on these parameters. It is for this reason that we decided to perform an analysis of the morphometric parameters of C2C12 myotubes. It is well known that withdrawal of serum from C2C12 cells causes them to exit the cell cycle and fuse into myotubes [8,23]. Furthermore, the extent of differentiation and the morphological characteristics of formed myotubes depend on the type of media used to culture them, the concentration of serum in the media and the length of incubation [8]. C2C12 cells also provide a well-characterized *in vitro* model system for studying the induction of hypertrophy in myotubes that occurs as a result of an increase in size rather than an increase in the number of myotubes [23]. Accordingly, our results showed that prolonged cultivation of C2C12 cells in DMEM/5% FCS led to the formation of the large hypertrophied myotubes and that hemoglobin did not significantly influence their morphometric parameters. This is probably to a large extent due to myotube hypertrophy that masked the subtle potential changes in their biochemical and subsequent morphometric parameters by hemoglobin. Subsequent cultivation of C2C12 cells for only six days in the DMEM/2% HS myogenic differentiation medium that is routinely used for the induction of myogenic differentiation, caused the cells to become smaller and mostly polygonal, which is in accordance with the observed morphology of C2C12 myotubes upon cultivation of these cells in DMEM/2% HS after growth in GM (DMEM/10% FCS) [22]. When switching to cultivation in DMEM/10% FCS after prolonged cultivation in DMEM/5% FCS, the morphology of C2C12 myotubes changes and they become more elongated. While PHb did not affect the morphometric parameters of C2C12 myotubes in any of the experimental frameworks and used concentrations, BHb exerted an effect on the area of myotubes when cultured in DMEM/2% HS and DMEM/10% FCS, as well as on the length when cultured in DMEM/2% HS, once again confirming the potency of BHb over PHb in modifying the functional characteristics of C2C12 cells. When cultured in the presence of the highest concentration of hemoglobin (10 μM), no myotubes were observed after cultivation in DMEM/2% HS and DMEM/10% FCS, which is in accordance with our RT-PCR results that showed that this concentration downregulated the expression of myogenic markers.

The observed differences in the effects of PHb and BHb on the functional characteristics of C2C12 cells can be the consequence of differences in the primary and higher levels of structure of these proteins [57,58]. Additionally, our results showed the presence of phospholipid FAs in hemoglobin samples whose distribution and amounts differ in isolates from different species. Literature data confirm the effect of the FAs on myogenic differentiation. Supplementation with linoleic acid impaired muscle regeneration and increased fibrous tissue deposition, resulting in prolonged contractile function recovery, while oleic acid supplementation reduced fibrous tissue deposition and improved contractile function recovery [59]. Palmitic acid suppressed C2C12 cells myogenic differentiation *in vitro* [60,61], while other studies demonstrated an inconsistent effect of α-linolenic acid [62,63]. Additionally, it is known that the FA compositions of commercially available serum used to supplement cell culture media widely differ and

can alter cellular FA composition and potentially the functional characteristics of cells [64,65].

CONCLUSION

In this study, we showed that extracellular hemoglobin modifies the proliferation and migration of C2C12 cells in a dose- and time-dependent manner, while having a mainly inhibitory effect on myogenic differentiation of these cells. These findings could be of particular importance for treating muscle injuries in patients with hemoglobinemia-related diseases and conditions. This should also be kept in mind when using SCs for cell therapy, since the presence of extracellular hemoglobin can influence its outcome.

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