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# Heme changes HIF-α, eNOS and nitrite production in HUVECs after simvastatin, HU, and ascorbic acid therapies

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ABSTRACT

The sickle cell disease (SCD) is a hemolytic genetic anemia characterized by free heme and hemoglobin release into intravascular spaces, with endothelial activation. Heme is a proinflammatory molecule able to directly activate vascular endothelium, thus, endothelial dysfunction and vascular disease are major chronic events described in SCD. The aim of this study was to evaluate the production of endothelial nitric oxide synthase (eNOS), nitrite and hypoxia inducible factor alpha (HIF-α) in HUVECs (human umbilical vein endothelial cells) activated by heme in response to simvastatin, hydroxyurea (HU), and ascorbic acid therapies. eNOS and HIF-α production were evaluated by ELISA and nitrite was measured by the Griess technique. The production of HIF-α increased when the cells were stimulated by heme ($p < 0.01$), while treatment with HU and simvastatin reduced the production ($p < 0.01$), and treatment with ascorbic acid increased HIF-1a production by the cells ($p < 0.01$). Heme increased eNOS production, ($p < 0.01$) but showed a heterogeneous pattern, and the lowest concentrations of all the treatments reduced the enzyme production ($p < 0.01$). The nitrite production by HUVECs was enhanced by stimulation with heme ($p < 0.001$) and was reduced by treatment with HU ($p < 0.001$), ascorbic acid ($p < 0.001$) and simvastatin ($p < 0.01$). In summary, our results suggest that the hemolytic vascular microenvironment in SCD requires different therapeutic approaches to promote clinical improvement, and that a combination of therapies may be a viable strategy for treating patients.

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## Introduction

Vascular complications are frequent in SCD and one of the main pathophysiological features is chronic endothelial activation. Endothelial cells of SCD patients express adhesion molecules and chemotactic factors when stimulated by the inflammatory stimuli commonly from cytokines, lipid mediators, reticulocytes, normal and irreversibly sickled red blood cells, platelets and even heme (Hebbel et al., 2004; Kumar et al., 1996; Belcher et al., 2000). Endothelial cells are capable of expressing procoagulants, anticoagulants, vasoconstrictors, vasodilators factors, adhesion molecules and cytokines. For that reason they are one of the main regulators of hemostasis (Wakefield et al., 2008).

Heme has many proinflammatory properties including: leukocyte activation and migration, increased expression of adhesion molecules, and cytokine and acute-phase protein induction. It can also activate the endothelial vascular cells (Figueiredo et al., 2007; Graca-Souza et al., 2002; Wagener et al., 1997). After challenge with heme, an increased expression in intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin (Wagener et al., 1997), increased tissue factor expression (Setty et al., 2008), increased mobilization of Weibel–Palade body and von Willebrand factor production (Belcher et al., 2014) were observed in vascular endothelial cells.

The hypoxia-inducible factor (HIF-1 alpha (α), HIF-1 beta (β), and HIF-2α) is known for its influence on vascular genes' transcription. Expression and activity of HIF-1α that forms heterodimers with HIF-1β beta are finely regulated by cellular oxygen concentration, vascularization, and angiogenesis. In a hypoxia environment, the HIF-1α activates gene transcription of erythropoietin, glucose transporters, glycolytic enzymes, vascular endothelial growth factor (VEGF) and its receptors, and other genes whose proteins increase oxygen bioavailability or ameliorate metabolic adaptation to hypoxia. Nitric oxide synthases (NOSs) are an enzyme family (hemeproteins, as p450 cytochrome) responsible for NO synthesis from L-arginine and molecular oxygen (Cokic et al., 2007). NO is generated by the conversion of L-arginine into L-citruline by NOS, that requires nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen, and also the co-factors tetrahydrobiopterin

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(BH4), flavin mononucleotide (FMN) and flavin adenine (FAD) (Palmer et al., 1988; Feng et al., 2011). The endothelial isoform of NOS (eNOS) is constitutively expressed and, as a hemeprotein, is one of the main steps for NO production is the electron transference from FMN to the heme group (Heller et al., 1999; Feng et al., 2011). In SCD there is a large production of ROS that is responsible for a pro-oxidative environment.

The principal therapeutic approach to treat SCD patients is the increase of fetal hemoglobin (HbF) levels, and the only available pharmacological treatment universally accepted is hydroxyurea (HU) (Maluf et al., 2009; Kovacic, 2011), which can reduce clinical manifestations, pain crisis and increases HbF. The HU mechanism is not completely clear, despite the increase of HbF and the percentage of red blood cells (RBC) with HbF, both considered as a beneficial effect (Covas et al., 2004). Statins (inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase) are used to control cholesterol levels in cardiac diseases, and they also have anti-inflammatory properties (Rosch et al., 2010; Collina et al., 2002; Blanco-Colio et al., 2003). A pilot study with simvastatin in SCD showed that the drug decreases serum levels of interleukin-6 (IL-6) and C-reactive protein (CRP) and increases plasma NO (Hoppe et al., 2011). The ascorbic acid is an important exogenous antioxidant in human plasma, it can remove superoxide and other ROS, thus, protecting lipids from peroxidation. It is believed that ascorbic acid has a protective role in atherogenesis, since epidemiological studies show that its plasma concentration is inversely proportional to death by coronary heart disease (Heller et al., 1999; Weber et al., 1996; Retsky et al., 1993; Frei et al., 1989; Som et al., 1983). Although meta-analysis study shows that it does not influence the mortality risk (Bjelakovic et al., 2007). When ROS production overwhelms cellular antioxidants, the cell can suffer oxidative stress (Duarte and Lunec, 2005). Biomarkers of oxidative stress in SCD are useful to identify patients at high risk for oxidative injury, and also to evaluate antioxidant therapies, such as zinc, vitamin C, vitamin E and N-acetylcysteine supplementation (Rees and Gibson, 2012; Nur et al., 2011).

The hemolytic and pro-oxidative vascular environment tends to lead to endothelial dysfunction, which could be improved by different therapeutic approaches. For that reason, we investigated the effect of heme on endothelial cells, simulating the hemolytic milieu described in SCD, and the treatment response to HU, simvastatin and ascorbic acid on HIF-α, eNOS and nitrite production by these cells.

## Methods

### *Cell culture*

Human umbilical vein endothelial cells (HUVECs) immortalized cells were kindly donated by Ph.D. Ana Moretti and by M.D. Heraldo Possolo de Souza, from the Faculdade de Medicina da USP/São Paulo and cultivated in RPMI medium with 10% fetal bovine serum (FBS, both from Gibco, New York, NY, USA), 1% penicillin (10,000 U/mL) and streptomycin (10,000 μg/mL) (Gibco, New York, NY, USA) and cultured in 25 $cm^2$ flasks (Costar, New York, NY, USA). Cultures were maintained at 37 °C under a humidified 5% $CO_2$ room air atmosphere. Cells used in all assays were until the 5th passage, and a phenotypic characterization of HUVECs was previously made by flow cytometry in order to confirm the presence of ICAM-1 (CD54) (Supplemental Fig. 1). For the activation assays, cells were cultured in 96-well plates with $2 \times 10^4$ cells/mL. First, the cells were grown overnight to form a monolayer, then heme and the drugs were added to each well in the indicated concentrations. Cells were stimulated for approximately 20 h prior to each measurement.

### *Stimuli preparation*

The heme solution was prepared by diluting bovine hemin (Sigma, St. Louis, MO, USA) in 20% NaOH (0.1 N, in endotoxin-free water) and 80% RPMI medium, in the dark, to avoid free radical-formation. We used concentrations of 30 μM, 50 μM and 70 μM of heme to stimulate the cells. The HU solution was prepared by diluting hydroxyurea (Sigma, St. Lous, MO, USA) in RPMI medium. We treated the cells with HU concentrations of 50 μM, 100 μM and 200 μM. This concentration range was chosen based on the plasma concentrations of HU in patients treated with HU therapeutic dose (Lopes et al., 2014; Charache et al., 1992). The sodium simvastatin (Calbiochem, Darmstad, Germany) was solubilized in ethanol PA. The concentrations of 0.1 μM, 1 μM and 5 μM of simvastatin were used to treat the cells. These concentrations are close to those commonly used in similar assays evaluating activation profile in human endothelial cells (Canalli et al., 2011; Kureishi et al., 2000; Zhu et al., 2008). A stock solution of ascorbic acid was prepared by diluting L-ascorbic acid (Sigma, St. Louis, MO, USA) in RPMI base medium, in the dark to avoid early degradation. The concentrations of 30 μM, 60 μM and 120 μM of L-ascorbic acid were used to treat the cells. Acid ascorbic concentrations were used based on the internalization ability of HUVECs and based on recommended therapeutic dose (Montecinos et al., 2007; Linster and Van Schaftingen, 2007; Heller et al., 1999). A new stock solution of each drug was prepared in each new experiment.

### *HIF-α production*

The HIF-α production by the cells was evaluated using the kit Cell-Based ELISA Human/Mouse Total HIF-1α Immunoassay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

### *eNOS production*

The eNOS production was evaluated using the kit Quantikine Human eNOS from R&D Systems (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

### *Nitrite production*

The nitrite production by the HUVECs under the stimuli and treatments was evaluated by the Griess technique with a $NaNO_3$ standard-curve (Bryan and Grisham, 2007).

### *Statistical analysis*

Results were expressed as mean ± standard deviation. All experiments were made in triplicate. Independent t-test was used to analyze two numeric variables, when comparing two values groups within the same variable. ANOVA with Bonferroni's post-hoc test was used to analyze the quantitative variables, with more than three categories

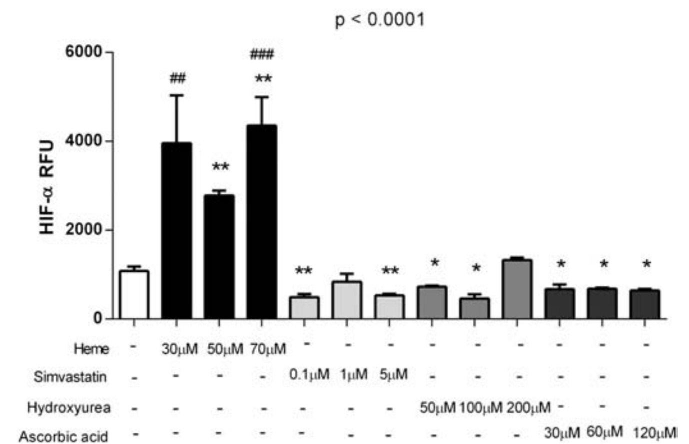


**Fig. 1.** Treatments and heme challenge effects in growing concentrations in the assays on HIF-α production by HUVEC, n = 3, p value is from comparison with negative control, ANOVA p value is shown; ## $p < 0.01$, ### $p < 0.001$ (Bonferroni post-hoc), * $p < 0.05$; ** $p < 0.01$ (independent t-test).

within the experiment groups. Data analysis was performed using Prism 5.01.3 (Graphpad Software, San Diego, CA, USA).

## Results

### *HIF-α production*

The HIF-α is a transcriptional factor involved in the expression of genes that control endothelial vascular cell angiogenesis and proliferation (Lopes et al., 2014). ANOVA results indicated that the drugs induced statistically different production by the cells ($p < 0.0001$). Basal production of HIF-α by the cells in the absence of drugs was 1082 ± 170 relative fluorescence unit (RFU). When we stimulated cells with 30, 50 and 70 μM of heme, the production was 3956 ± 1868; 2775.5 ± 192.5, and 4351 ± 1109 RFU respectively. HIF levels were significantly different from unstimulated controls when all the heme concentrations were used ($p = 0.0003$ and $p = 0.0072$ from t-test, $p < 0.0001$ and $p = 0.0004$ from ANOVA with Bonferroni post-hoc) (Fig. 1).

When treatments were evaluated without heme stimulus, HU at concentrations of 50 and 100 μM reduced the production of HIF-α (727.5 ± 45.5 and 463.5 ± 166.5 RFU respectively) compared with basal production ($p = 0.0251$ and $p = 0.0168$ respectively). Simvastatin at concentrations of 0.1 and 5 μM reduced the production when compared with no treatment, inducing 490 ± 129 and 531 ± 62 RFU respectively ($p = 0.0086$ and $p = 0.0062$). Ascorbic acid also decreased HIF-α production. When we used 30 μM, the cells produced 672 ± 179 RFU ($p = 0.0452$), with 60 μM the production was 681.5 ± 18.5 RFU ($p = 0.0154$), and with 120 μM the production was 644.5 ± 60.5 RFU ($p = 0.0137$) (Fig. 1).

Cell stimulus with 50 μM of heme and 100 μM of HU reduced HIF-α production to 1877 ± 15 RFU ($p = 0.0013$ from t-test) (Fig. 2.A). When we used heme as stimulus at 70 μM concentration only the highest concentration of HU (200 μM) reduced HIF-α production (2505 ± 234 RFU, $p = 0.0478$ from t-test and $p = 0.0398$ from ANOVA with post-hoc Bonferroni) (Fig. 2.B). All the other experimental conditions did not change the HIF-α production profile.

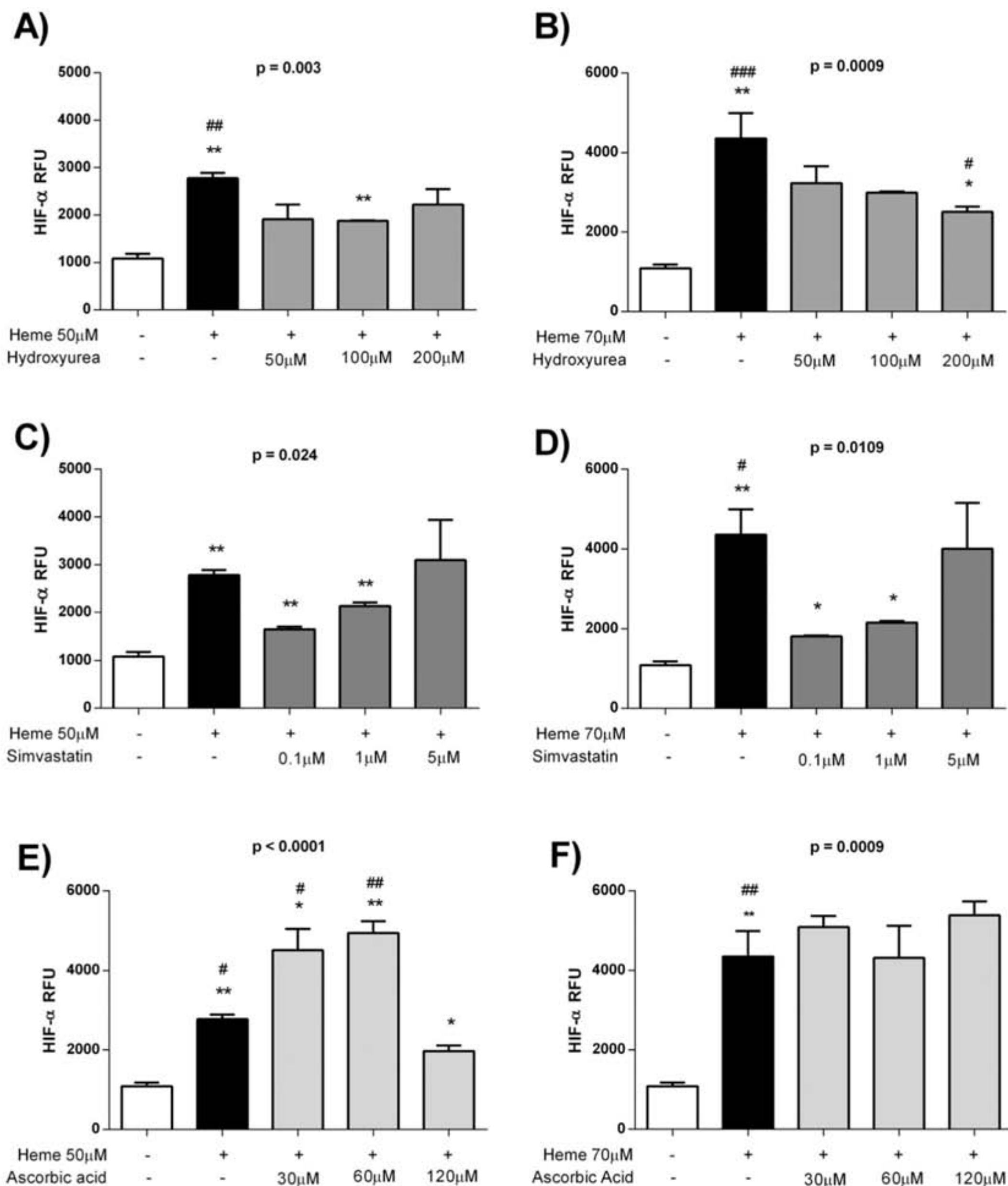


**Fig. 2.** Evaluation of different treatments in association with heme in increased concentrations used in the assays on HIF-α production by HUVEC, n = 3, p value is from comparison with the absence of treatments, ANOVA p value is described for each graphic; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (Bonferroni post-hoc), * $p < 0.05$; ** $p < 0.01$ (independent t-test).

The use of heme at 50 μM and treatment with 0.1 μM and 1 μM of simvastatin HIF-α production reduced to 1654.5 ± 91.5 and 2134.5 ± 129.5 RFU respectively ($p = 0.0008$ and $p = 0.0087$) (Fig. 2.C). The same concentration also showed an inhibitory effect when we stimulated cells with heme at 70 μM (1811 ± 37 and 2155.5 ± 71.5 RFU respectively; $p = 0.0166$ and $p = 0.0267$) (Fig. 2.D).

The ascorbic acid induced HIF-α production pattern only in HUVEC stimulated with 50 μM of heme, in which 30 and 60 μM of ascorbic acid increased HIF-α production (4512 ± 922 and 4942.5 ± 512.5 RFU; $p = 0.0331$ and $p = 0.0024$ from t-test, and $p = 0.0161$ and $p = 0.0033$ from ANOVA with Bonferroni post-hoc respectively), while 120 μM decreased to 1971 ± 238 RFU ($p = 0.0104$) (Fig. 2.E).

Co-incubation of 70 μM of heme and ascorbic acid did not change the production statistically (Fig. 2.F).

Regarding combined treatment, HIF-α production by HUVEC showed a significant difference when we compared the treatment only with 100 μM of HU and all the drugs tested ($p = 0.0016$ from t-test and $p = 0.0007$ from ANOVA with Bonferroni post-hoc), with simvastatin (1 μM) and HU (100 μM) ($p < 0.0001$ from t-test and $p = 0.0009$ from ANOVA with Bonferroni post-hoc), and with HU and ascorbic acid (120 μM) ($p < 0.0001$ from t-test and $p = 0.0014$ from ANOVA with Bonferroni post-hoc) (Fig. 3.A).

The comparison between the treatment with simvastatin and all drugs used ($p = 0.0006$ from t-test and $p < 0.0001$ from ANOVA with Bonferroni post-hoc), with simvastatin and HU ($p < 0.0001$ from t-test and $p < 0.0001$ from ANOVA with Bonferroni post-hoc), and with HU and ascorbic acid ($p < 0.0001$ from t-test and $p < 0.0001$ from ANOVA with Bonferroni post-hoc) (Fig. 3.B).

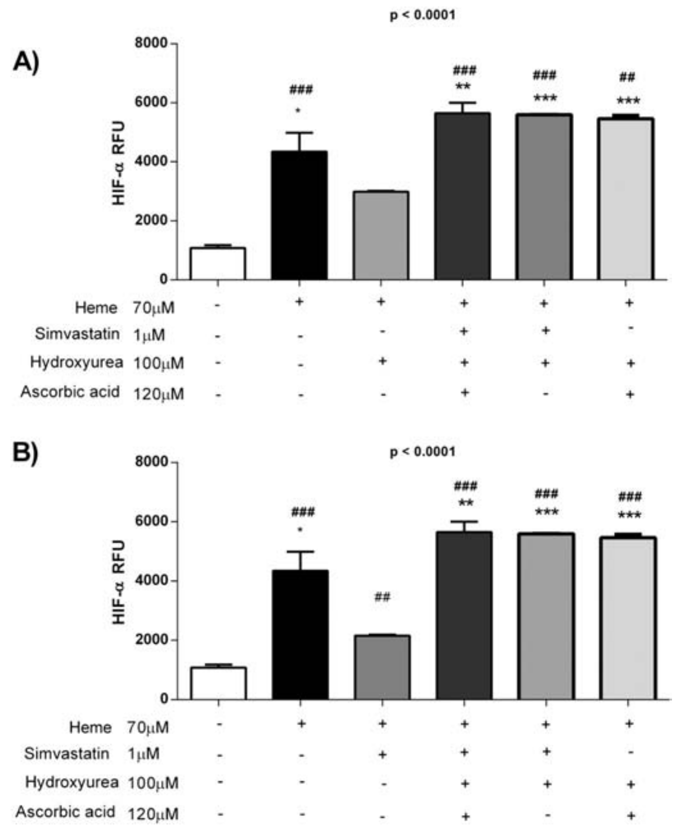


**Fig. 3.** Evaluation of combined treatment on HIF-α production by HUVEC. A) Comparison among treatment only with HU; B) comparison among treatment only with simvastatin, $n = 3$. The black bars (only heme) were compared to the white bars (negative control), and the treatments or their associations were compared. ANOVA p value is described on each graphic; ## $p < 0.01$, ### $p < 0.001$ (Bonferroni post-hoc), * $p < 0.05$; ** $p < 0.01$ (independent t-test).

*eNOS production*

The NOS is an enzymatic group responsible for the NO production by many cell types. In endothelial cells, the gene *NOS3* is responsible for eNOS isoform expression that is the main source of the NO production. The amount of eNOS produced by the cells in the assays was evaluated using the cell lysates. ANOVA results indicated that the drugs induced statistically different production by the cells ($p = 0.0036$). Every heme concentration used induced statistically significant production of eNOS when compared with the negative control when 30 μM of heme was added to the culture 355.07 ± 26.01 pg/mL ($p = 0.0008$) was produced. When 50 μM of heme was used, it increased the production to 518.31 ± 126.04 pg/mL ($p = 0.0050$); and with 70 μM of heme, the production was 251.07 ± 54.23 pg/mL ($p = 0.0153$); the negative control production was 86.95 ± 25.55 pg/mL. (Fig. 4).

In the absence of heme stimulus, HU alone increased eNOS production in a dose-dependent fashion, but only the higher concentration (200 μM) was significantly different from controls (296.88 ± 49.49 pg/mL, $p = 0.0088$). Simvastatin at a concentration of 1 μM also increased eNOS production by the cells to 330.52 ± 34.29 pg/mL ($p = 0.0017$). Ascorbic acid increased eNOS production to 480.89 ± 82.93 pg/mL ($p = 0.0019$) only when the lowest concentration was tested (30 μM). These results were compared with basal production of eNOS by the cells without any drugs in the medium culture (negative control) (Fig. 4).

When the cells were stimulated with heme at 50 μM and treated with HU at 50 μM, the eNOS production was decreased to 8.14 ± 4.51 pg/mL ($p = 0.0022$) and with HU 100 μM the production was 115.64 ± 22.59 pg/mL ($p = 0.0055$) (Fig. 5.A). Cell stimulus with 70 μM of heme and treatment with 50 μM of HU also decreased eNOS production to 33.06 ± 6.96 pg/mL ($p = 0.0023$ from t-test and $p = 0.0005$ from ANOVA with Bonferroni post-hoc), and when HU at 100 μM was used, the production was 96.14 ± 17.67 pg/mL ($p = 0.0093$ from t-test and $p = 0.0067$ from ANOVA with Bonferroni post-hoc) (Fig. 5.B).

The treatment of cells with simvastatin reduced eNOS production when the lowest concentration was applied. Under the stimulus of heme at 50 μM, simvastatin at 0.1 μM eNOS production reduced to 21.89 ± 1.84 pg/mL ($p = 0.0024$ from t-test and $p = 0.001$ from ANOVA with Bonferroni post-hoc), and at 1 μM reduced to 140.31 ± 26.5 pg/mL ($p = 0.0071$ from t-test and $p = 0.0079$ from ANOVA with Bonferroni post-hoc) (Fig. 5.C). When 70 μM of heme was used, the lowest simvastatin concentration induced eNOS production to 34.86 ± 23.5 pg/mL ($p = 0.0032$ from t-test and $p = 0.0046$ from ANOVA with Bonferroni post-hoc) (Fig. 5.D). All the other experimental conditions did not show any statistical difference.

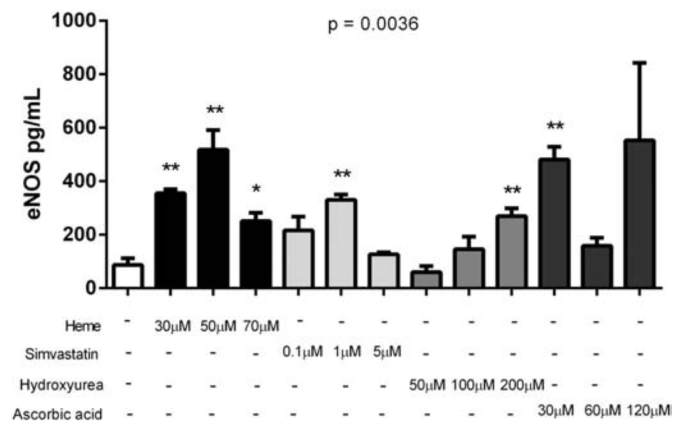


**Fig. 4.** Treatments and heme effects in increasing concentrations used in the assays on eNOS production by HUVEC, $n = 3$, p value is from comparison with negative control, ANOVA p value is shown; * $p < 0.05$; ** $p < 0.01$ (independent t-test).

The ascorbic acid treatment showed a heterogeneous result. When cells were stimulated with heme at 50 μM, treatment with 30 μM of ascorbic acid induced eNOS production to 68.98 ± 46.9 pg/mL ($p = 0.0044$ from t-test and $p = 0.0005$ from ANOVA with Bonferroni post-hoc). With ascorbic acid at 60 μM, the production was 241.59 ± 112.69 pg/mL ($p = 0.0471$ from t-test and $p = 0.0207$ from ANOVA with Bonferroni post-hoc), and with 120 μM was 98.51 ± 32.68 pg/mL ($p = 0.005$ from t-test and $p = 0.0009$ from ANOVA with Bonferroni) (Fig. 5.E). All the other experimental conditions did not show any statistical difference (Fig. 5.F).

### *Nitrite production*

Nitrite production by endothelial cells is an important therapeutic target in SCD. Cells produce the gas through enzymatic conversion of L-arginine in citruline, mediated by NOS action (Steinberg, 2008). ANOVA results indicated that the drugs induced statistically different production by the cells ($p < 0.0001$). In our results, the nitrite production by the cells with increasing amounts of heme showed a dose-dependent response. When we stimulated cells with 30, 50 and 70 μM of heme, the production was 34.45 ± 2.67; 37.76 ± 0.18, and 41.56 ± 1.99 μM of nitrite respectively. In the absence of heme, the nitrite production was 25.11 ± 0.048 μM; and all heme concentrations used in the assays increased the production of nitrite ($p = 0.0038$; $p < 0.0001$; $p = 0.0001$ from t-test and $p = 0.0013$; $p < 0.0001$; $p < 0.0001$ from ANOVA with Bonferroni post-hoc respectively) (Fig. 6).

The effects of drug treatments on nitrite production by the HUVEC were also evaluated. None of the HU concentrations used here affected nitrite production. Only 0.1 μM simvastatin induced a slight production of nitrite (25.54 ± 0.12 μM, $p = 0.0077$). The ascorbic acid at 30 μM reduced nitrite production by the cells (24.75 ± 0.12 μM, $p = 0.0161$); however, when 120 μM of ascorbic acid was used, the production was increased to 27.08 ± 0.77 μM ($p = 0.0116$). Results are significant when compared with nitrite cell production in the medium culture without any of the drugs (Fig. 6).

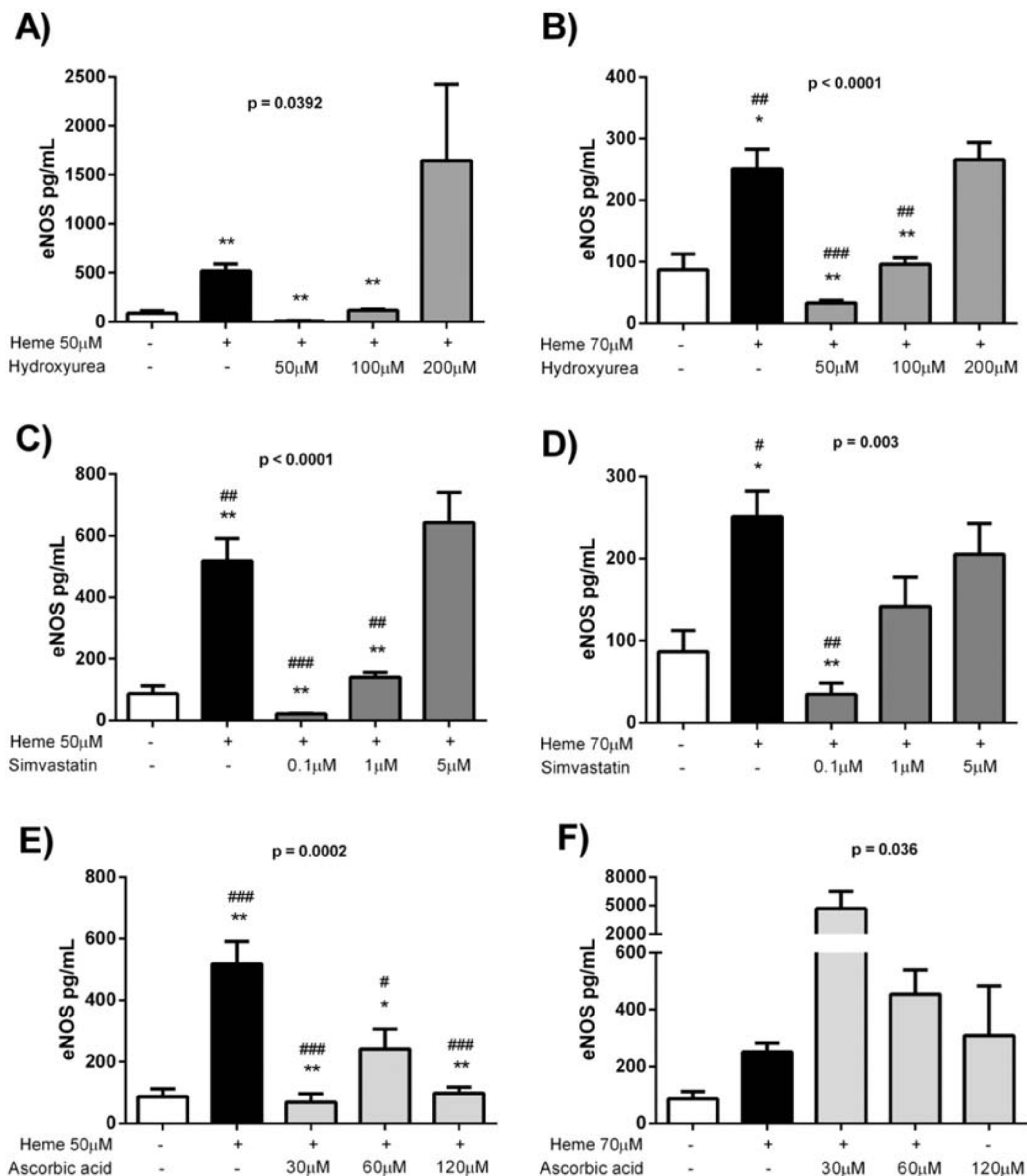


**Fig. 5.** Evaluation of treatments in association with heme in growing concentrations used in the assays on eNOS production by HUVEC, $n = 3$, p value is from comparison with the absence of treatments. ANOVA p value is described on each graphic; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (Bonferroni post-hoc), * $p < 0.05$; ** $p < 0.01$ (independent t-test).

When the cells were stimulated with 50 μM of heme, all the HU concentrations used reduced the nitrite production. When HU at 50 μM was used, the nitrite production was 23.76 ± 0.28 μM ($p < 0.0001$ from t-test and $p < 0.0001$ from ANOVA with Bonferroni post-hoc), at 100 μM of HU the production was 33.4 ± 0.16 μM ($p < 0.0001$ from t-test and $p < 0.0001$ from ANOVA with Bonferroni post-hoc), and with 200 μM of HU the production was 34.65 ± 0.63 μM ($p = 0.0012$ from t-test and $p < 0.0001$ from ANOVA with Bonferroni post-hoc) (Fig. 7.A).

The association of the highest heme concentration (70 μM) and 100 μM of HU also decreased the nitrite production to 36.38 ± 0.25 μM ($p = 0.011$ from t-test and $p = 0.0170$ from ANOVA with Bonferroni post-hoc) (Fig. 7.B).

The simvastatin effect on nitrite production under heme stimulus was also evaluated and only the association with 50 μM of heme showed significant results. When the cells were treated with 0.1 μM of simvastatin, the production was 35.72 ± 0.4 μM ($p = 0.0013$), and with 1 μM the production was 32.98 ± 2.58 μM ($p = 0.0328$ from t-test and $p = 0.0089$ from ANOVA with Bonferroni post-hoc) (Fig. 7.C).

Ascorbic acid modulated nitrite production by HUVEC in all heme concentrations used. Under the stimulus of 30 μM of heme, when we treated cells with 60 μM of ascorbic acid, the nitrite production was 29.39 ± 0.67 μM ($p = 0.0337$ from t-test and $p = 0.0072$ from ANOVA with Bonferroni post-hoc), and when 120 μM was tested, the production was 28.73 ± 0.09 μM ($p = 0.0209$ from t-test and $p = 0.0029$ from ANOVA with Bonferroni post-hoc) (Fig. 7.D).

When we used heme at 50 μM in association with 30, 60, and 120 μM of ascorbic acid, the nitrite production was 33.45 ± 0.15 μM ($p < 0.0001$ from t-test and $p < 0.0001$ from ANOVA with Bonferroni post-hoc), 34.06 ± 0.57 μM ($p = 0.0004$ from t-test and $p < 0.0001$ from ANOVA with Bonferroni post-hoc) and 33.25 ± 0.35 μM ($p < 0.0001$ from t-test and $p < 0.0001$ from ANOVA with Bonferroni post-hoc) respectively (Fig. 7.E).

The highest heme concentration tested was also affected by the ascorbic acid treatment. When we used concentrations of 30, 60 and 120 μM, the nitrite production was 37.1 1 ± 0.05 μM ($p = 0.0179$ from t-test and $p = 0.0133$ from ANOVA with Bonferroni post-hoc), 36.89 ± 1.13 μM ($p = 0.0242$ from t-test and $p = 0.003$ from ANOVA with Bonferroni post-hoc) and 37.81 ± 0.67 μM ($p = 0.0359$ from t-test and $p = 0.0396$ from ANOVA with Bonferroni post-hoc) respectively (Fig. 7.F).

We also verified the effect of combined therapy on nitrite production. A significant difference was only found when we compared an exclusive treatment with HU (100 μM) with the combination of HU (100 μM) and simvastatin (1 μM), when the nitrite production was increased ($p = 0.0101$) (Fig. 8).

## Discussion

In our study we observed that the lowest heme concentration tested did not contribute with our *in vitro* model of endothelial dysfunction, suggesting that concentrations close to plasma levels indeed cause modifications on HIF-α, eNOS and nitrite production by the cells. The HU treatment was able to reduce HIF-α, eNOS and nitrite production, which all was increased by heme. Cells treated with simvastatin also showed decreased HIF-α, eNOS and nitrite production when the lowest concentration was tested. The ascorbic acid was able to increase HIF-α production, decrease nitrite production and produced a heterogeneous pattern on eNOS production by the cells. In the SCD murine model, in steady state, the plasmatic concentration of heme was 52.6 ± 18.3 μM in HbSS-Townes and 28.8 ± 3.1 μM in HbAA-Townes (Belcher et al., 2014). These concentrations were similar to those our group found in human plasma (HbAA 28.5 μM; HbSC 43 μM; HbSS 68.6 μM, Seixas et al., 2014; Carvalho, 2014). The heme released from Hb can cause injury to cell membrane integrity through low-density lipoprotein (LDL) oxidative modifications (Jeney et al., 2002), DNA modifications and protein denaturation (Kumar and Bandyopadhyay, 2005).

The HIF-α is a transcriptional factor that controls enzyme genes and proteins involved in glycolysis, iron transport, angiogenesis, cell proliferation and apoptosis (Vissers et al., 2007; Wenger, 2002; Lando et al., 2003; Kaelin, 2005). Endothelial cells treated with HU modulate HIF-α production. We found a decrease in HIF-α production when the lowest concentrations of HU were used. When cells were stimulated with heme at 50 μM and treated with HU, the production was also reduced. Importantly, when the highest heme concentration was tested, which corresponds to average heme plasma levels found in SCD, the reduction was in a dose-dependent fashion. The *HIF1A* gene expression was evaluated by Lopes et al. (2014) who found a decreased expression of *HIF1A* when HUVECs were treated with 100 μM of HU. In the same study, the authors evaluated the *in vivo* effect of HU in plugs with the drug adsorbed and they found an inhibitory effect in neovascularization. This emphasizes the need to study the interaction of HU and free heme in order to verify pro- or anti-angiogenic mechanisms in SCD.

Statins are also able to interfere in angiogenesis; they interact with HIF-α in a different way, once in smooth muscle cells and endothelial cells they can inhibit HIF-α (Dichtl et al., 2003). However, in a study undertaken by El-Azab et al. (2012), they verified that simvastatin and vitamin C promoted angiogenesis *in vivo* by up-regulating VEGF. In a different study (Zhu et al., 2008), it was reported that simvastatin may inhibit or promote HIF-α depending on the nature of the stimulus that endothelial cells receive. They found that when the cells were in hypoxia and then are re-oxygenated in the presence of simvastatin, HIF-α is increased; however, when the noxious stimulus tested was TNF-alpha, in the presence of simvastatin, the HIF-α production was inhibited.

Angiogenesis, chronic inflammation and cell response to oxygen tension are co-dependents (Lopes et al., 2014). Hypoxia-reperfusion injury is a strong inflammatory mechanism, and hypoxia by itself is a potent HIF-α activator. In SCD, hypoxia and inflammation are chronic. In our study, simvastatin reduced HIF-α production in all concentrations tested, in the absence of heme. When heme was added to the cells, along with simvastatin, an inhibitory effect was verified. We can suggest that simvastatin may have an inhibitory effect in the vascular hemolytic microenvironment in SCD. However, the inflammatory characteristics in the disease are complex, and pro-angiogenic factors increase systemically in patients (Brittain et al., 2010).

The transcriptional factor HIF-α is regulated by hydroxylases that can lead to its proteasomal degradation or to abolish its assembly (Vissers et al., 2007). Ascorbic acid can increase hydroxylase activity

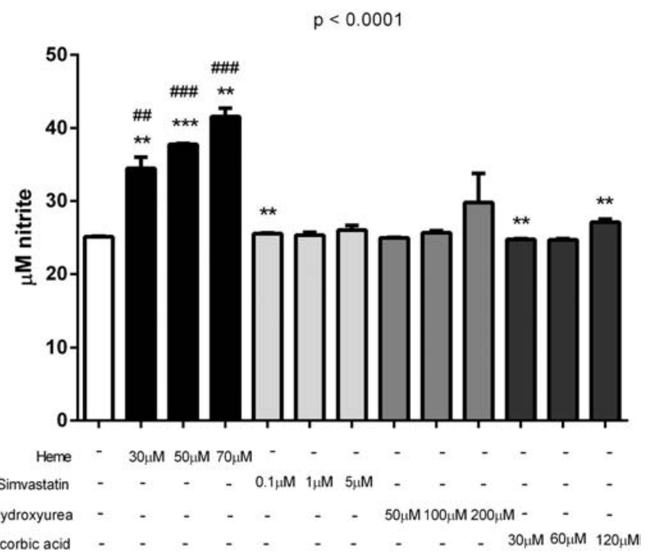


**Fig. 6.** Treatments and heme effects in growing concentrations used in the assays on nitrite production by HUVEC, $n = 3$, p value is from comparison with negative control, ANOVA p value is shown; ## $p < 0.01$, ### $p < 0.001$ (Bonferroni post-hoc), * $p < 0.05$; ** $p < 0.01$ (independent t-test).

(Schofield and Ratcliffe, 2004), which can contribute to reduce *in vitro* HIF-α production by HUVEC. When cells were treated only with ascorbic acid without heme, there was a decrease in HIF-α production. When heme was added to the assays, HIF-α production was increased, suggesting that pro-oxidant or pro-inflammatory heme stimulus was higher than ascorbic acid capacity to reduce HIF-α. In only one experimental condition, with the highest ascorbic acid concentration, the HIF-α production was reduced. Ascorbic acid used in *in vitro* assays depends on the intracellular accumulation rate and its ability to act as pro-oxidant in the presence of free transition metals (as iron) (Carcamo et al., 2002; Clement et al., 2001; Cai et al., 2001). It is thought that vascular endothelial cells more easily internalize oxidized ascorbic acid. The reduced L-ascorbate needs specific receptors, and it is less frequent in endothelial cells, so the beneficial effect may be underestimated (Dhar-Mascareno et al., 2005).

Although our results have not found an effect of HU on nitrite production by HUVEC, the treatment increased eNOS production. When the culture was treated only with HU without heme, we observed the improvement of eNOS production in a dose-dependent way. In the assays in which cells were stimulated with heme and treated with HU, we also observed an increase of eNOS. This response is in accord with a previous report (Cokic et al., 2007), suggesting that HU acts to increase eNOS by protecting it from proteasomal degradation, in even lower concentrations than we used.

It was verified that statins are able to increase eNOS phosphorylation (Kureishi et al., 2000), they are also able to improve endothelial dysfunction by the stabilization of the eNOS mRNA, as well as to activate the protein kinase Akt which leads to increase eNOS activity, resulting in increased NO bioavailability (Kavalipati et al., 2015). In our results, when we used simvastatin in the lowest concentration and in the

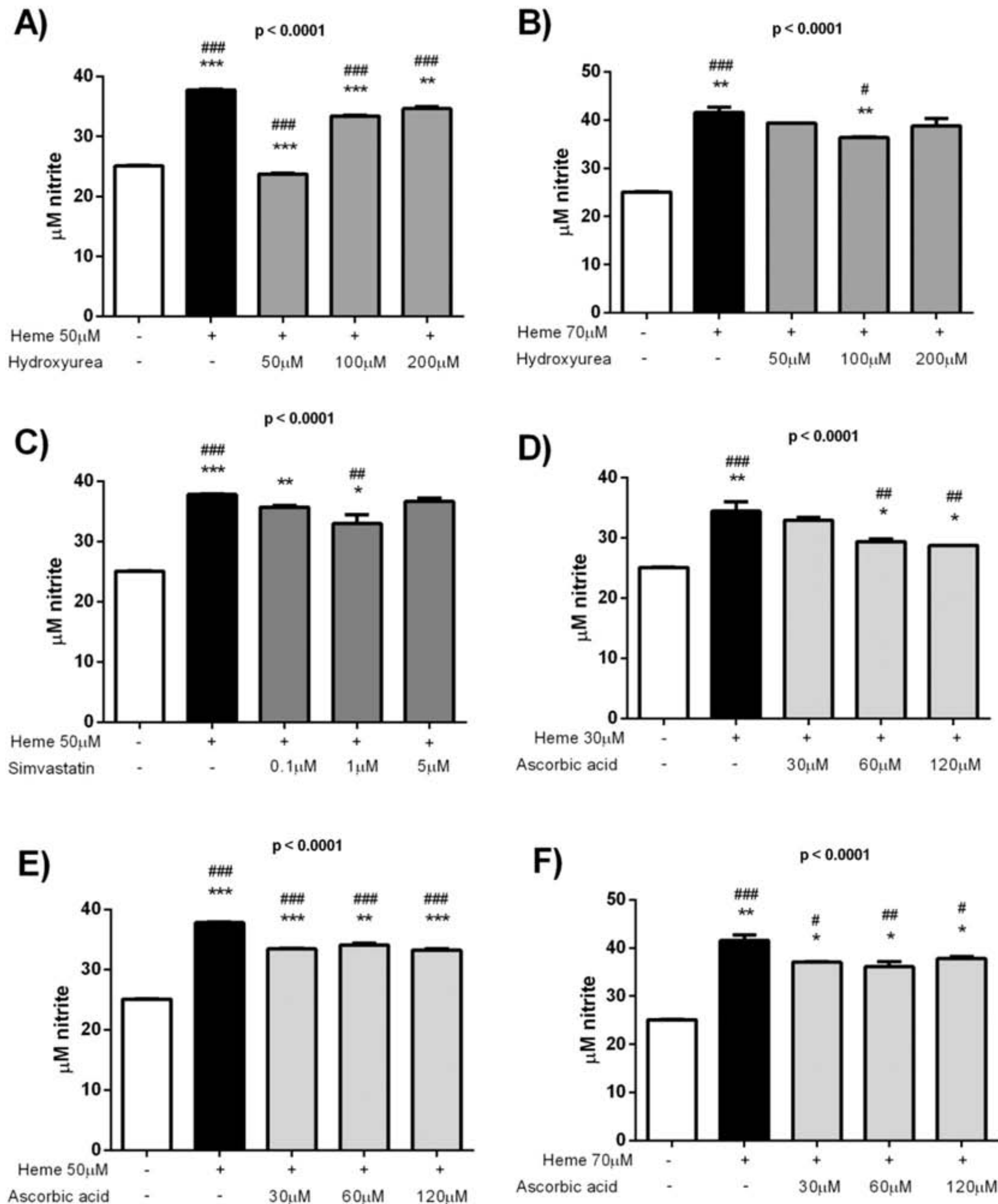


**Fig. 7.** Evaluation of treatments in association with heme in growing concentrations used in the assays on nitrite production by HUVEC, n = 3, p value is from comparison with the absence of treatments. ANOVA p value is described on each graphic; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (Bonferroni post-hoc), *$p < 0.05$; **$p < 0.01$ (independent t-test).

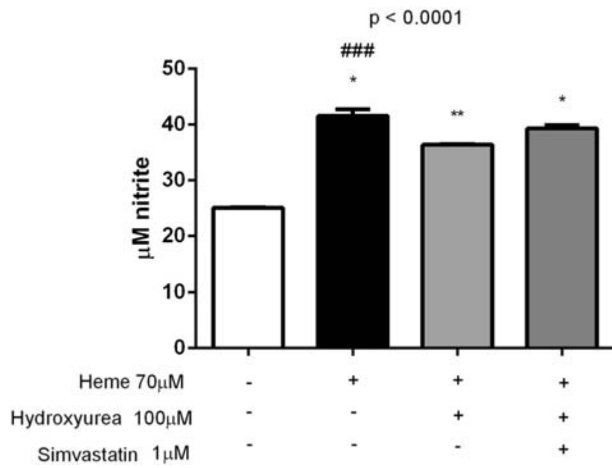


**Fig. 8.** Evaluation of combined treatment on nitrite production by HUVEC, comparison with HU as the only treatment, n = 3. The black bar (only heme) was compared to the white bar (negative control), and the treatments or their associations were compared. ANOVA p value is described on each graphic; ### $p < 0.001$ (Bonferroni post-hoc), *$p < 0.05$; **$p < 0.01$ (independent t-test).

absence of heme, eNOS production was increased. In the assays with heme, eNOS production was also increased. Meantime, even with an increase in eNOS production, nitrite production did not change. This may happen by a compensatory mechanism that was not sufficient to promote a biological effect in the cell culture.

In different studies (Heller et al., 1999; Kuzkaya et al., 2003) it has been verified that ascorbic acid might increase eNOS production. Among ascorbic acid antioxidant properties, its ability to scavenger free radicals, which could also protect NO from degradation, has been described. In the assays without heme, ascorbic acid increased eNOS production, and when heme was added, the enzyme production was reduced. This may have happened due to pro-oxidant nature of heme and to the presence of iron, which may overwhelm ascorbic acid scavenger properties.

In the present study, HU did not modulate nitrite production by HUVEC in the absence of heme in the cultures. On the other hand, when heme was added to the cultures, there was a significant reduction on nitrite production by the cells upon HU treatment. It is known that the chemical interaction between HU and heme can lead to NO production (Rupon et al., 2000; Pacelli et al., 1996), which partly explains the beneficial effects in SCD treatment.

The nitrite production by HUVECs without heme was increased when the cells received the ascorbic acid treatment. In the assays with heme, there was a small decrease in nitrite production. The ascorbic acid has been associated with a protection feature of tetrahydrobiopterin (BH4), which can maintain eNOS activity and, consequently, increase NO (Kuzkaya et al., 2003). However, in our results, heme interaction with ascorbic acid did not increase nitrite production by the cells.

## Conclusions

In summary, we found that HUVEC challenged by heme increased nitrite production, which decreased with HU treatment. eNOS production was heterogeneous with all drugs tested; thus the mechanism associated with the enzyme production needs to be better understood. HIF-$\alpha$ production increased with heme, however, simvastatin and ascorbic acid treatments need further evaluation since these drugs have different therapeutic targets. The search for new therapeutic strategies to improve the clinical profile of SCD patients is important in order to improve the chronic inflammatory status described in this disease, and a combination of therapies may be a viable strategy for treating these patients.

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## Authors' contribution

CCG and RPS did the experiments, analyzed the data and co-wrote the manuscript.

TNP and SSS provided technical support.

DLZ, VMG and MSG analyzed the data, provided academic support and co-wrote the manuscript.

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