UNIVERSITY OF TURIN

DOCTORAL SCHOOL IN LIFE AND HEALTH SCIENCES

PhD Programme in Complex Systems for Life Sciences

Cycle XXXII

H_2S INDUCES PRO-ANGIOGENIC EFFECTS AND DECREASES ISCHEMIA/REPERFUSION INJURY IN HUMAN MICROVASCULAR ENDOTHELIAL CELLS

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2016-2019

ABSTRACT

Endothelial cell injury and vascular function strongly correlate with cardiac function following ischemia/reperfusion injury. Moreover, several studies indicate that endothelial cells are more sensitive to ischemia/reperfusion than cardiomyocytes and are critical mediators of cardiac ischemia/reperfusion injury. H_2S is involved in the regulation of the cardiovascular system and can act as a cytoprotectant during ischemia/reperfusion. Activation of ERK1/2 in endothelial cells after H_2S stimulation exerts an enhancement of angiogenesis and its blockage significantly decreases H_2S cardioprotective effects. In this project, we investigated how H_2S preconditioning 24 hours before the ischemic injury could prevent the ischemia/reperfusion injury and promote angiogenesis on microvascular endothelial cells following an ischemia/reperfusion protocol in vitro, using a hypoxic chamber and ischemic buffer to simulate the ischemic event. H_2S preconditioning positively affected cell viability and significantly increased their migration when treated with 1 μM H_2S. Furthermore, mitochondrial function was preserved when cells were preconditioned. ERK1/2 phosphorylation was extremely enhanced in ischemia/reperfusion condition and we inhibited ERK directly or indirectly to see how H_2S triggers this pathway in endothelial cells. Altogether, these data suggest that H_2S can be used 24 hours prior to the ischemic insult to protect endothelial cells from ischemia/reperfusion injury and eventually decreasing myocardial injury.

Contents

1. INTRODUCTION

1.1 Ischemia-reperfusion injury.

Coronary heart disease is the leading cause of death and disability worldwide. The effects of CHD are usually attributable to the detrimental effects of acute myocardial Ischemia-Reperfusion Injury (IRI)[1].

Cardiovascular diseases include different disorders of the heart and blood vessels, majorly represented by Ischemic Heart Disease (IHD). IHD causes 46% of cardiovascular deaths in men and 38% in women worldwide; cerebrovascular diseases represent the second widespread CVD deaths[2].

Nowadays, one of the major challenges in treating the ischemic heart is to avoid IRI, especially in cases of open-heart surgery or primary percutaneous coronary intervention[3,4].

The restoration of blood flow to the ischemic tissue can induce a worse injury than the ischemic event itself. Reperfusion can paradoxically reduce the benefits of blood flow restoration by causing a phenomenon termed reperfusion injury[3].

Lethal reperfusion injury is the injury caused to the myocardium by the restoration of coronary blood flow after the ischemic episode. The outcome of this phenomenon is the death of cardiac myocytes that were still viable at the onset of reperfusion[5]. Cardiomyocytes death can happen through either apoptosis or necrosis, although apoptosis is not as common as necrosis[6].

The process of reperfusion also damages endothelial cells (ECs). Reperfusion alters endothelial structure by inducing a change in the expression of adhesion molecules, thus resulting in capillary leakage[7]. These effects can also lead to alterations in blood cells and micro-embolization, whereas myocyte swelling causes vascular compression[7].

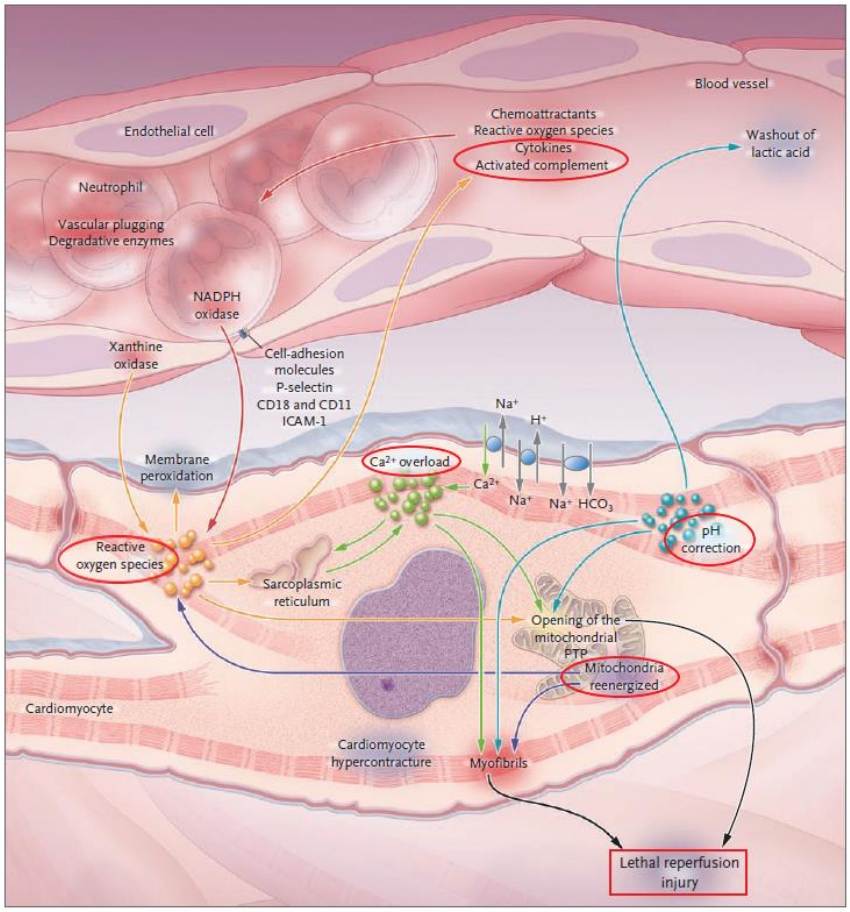


Fig.1. Major mediators of lethal reperfusion injury. Mitochondrial re-energization (purple); generation of Reactive Oxygen Species (ROS) (orange); intracellular Ca^{2+} overload (green); rapid restoration of the physiological pH (blue); inflammation (red) (Modified from Yellon and Hausenloy, 2007[3]).

Re-oxygenation of the ischemic tissue causes oxidative stress (Fig. 1). This happens because during reperfusion, reactive oxygen species are generated leading to an increased myocardial injury. The sudden overload of oxygen is too much to be dealt with and causes a damage greater than the ischemic event alone.

Xanthine oxidase, NADPH oxidase, and the mitochondrial electron transport chain are the main generators of ROS during reperfusion in the myocardium. Moreover, cytosolic xanthine oxidase and the mitochondrial electron transport chain (complexes I and III) provide the greatest amount of superoxide anion also in endothelial cells exposed to I/R[8,9].

It is known that mitochondria have to form and maintain a normal mitochondrial membrane potential, ΔΨm, in order to function properly. The mitochondrial membrane potential is essentially the result of redox transformations, it also serve as an intermediate form to store energy, which is going to be used later by ATP synthase. These reactions generate both a proton gradient and an electrical potential, that together create the transmembrane potential of hydrogen ions[10]. A physiological ΔΨm is necessary both for oxidative phosphorylation and to regulate the mitochondrial inner membrane selectivity and permeability[11].

Since ΔΨm is fundamental to preserve mitochondrial function and structure, its dysregulation is involved in many physiological and pathophysiological states, the most relevant being apoptosis[12,13].

The identification of the reperfusion injury salvage kinase (RISK) pathway[14] and the mitochondrial permeability transition pore (mPTP)[15,16] led the way to research on how to improve the clinical outcomes of acute myocardial infarction and reduce the risk of heart failure after infarction[17].

1.2 Endothelial cells and their role in I/R injury.

In 1980, Furchgott and Zawadski discovered the fundamental role of the vascular endothelium in the vasomotor tone, emerging as an essential component in the cardiovascular system, both structural and functional[18]. In terms of structure, endothelial cells form a monolayer that covers the inner surface of the vascular tree[19]. This unique localization is key for their role in interacting physically with the surrounding tissues and integrate different signals. The endothelium is known to regulate the vascular tone[18], cellular adhesion[20], inflammation[21] and smooth muscle cell phenotype[22].

Since the ‘80s, many important contributions were made regarding endothelium and its importance in the cardiovascular system, redefining the working concepts about the cardiovascular system[23–30].

For this reason, the heart is now seen as a pluricellular, multifunctional organ where the endothelium plays a pivotal role in many functions such as rhythmicity and contractile performance but also cardiac growth and metabolism[31].

Nowadays, it is unthinkable to approach cardiac function only considering cardiomyocytes, leaving out the cardiac microcirculatory system. For this reason, we decided to focus on the role of endothelial cells during IRI.

In addition, several studies indicate that ECs are more sensitive to I/R than cardiomyocytes and are critical mediators of cardiac IRI[32–36].

Vascular endothelial growth factor (VEGF), a sub-family of growth factors that induce angiogenesis and vasculogenesis, could play a role in left ventricular remodeling after myocardial infarction[37]. During angiogenesis, ECs migrate through the matrix, start to proliferate and then become quiescent to reorganize in a new vascular tube; these are modulated by intracellular Ca^{2+} signaling and extracellular molecules such as VEGFs, FGFs, IGFs, PDGF and EGF. In the adult body, endothelial cells retain their proliferative and mobile behavior allowing vascular damage repair (wound healing) or generation of new vessels, principally through angiogenesis at microcirculation level[38,39].

Vascular ECs restore blood perfusion in hypoxic tissues and organs through the process of sprouting angiogenesis, which is entirely sustained by local ECs[40,41].

Indeed, ECs injury and vascular function strongly correlates with cardiac function following IRI[42].

The process of angiogenesis, which is *de novo* formation of micro vessels, is essential in many physiological processes, from embryonic development[43,44] to the vascularization of many different tissues[45–47]. Other physiological conditions include the creation of new vessels for example to endure physical exercise in the skeletal muscle[48]. In pathological conditions such as heart

failure, this process is not just essential to prevent the transition to heart failure in the long-term, but it also has the potential to rescue the ischemic myocardium in the days following myocardial infarction[49,50].

Angiogenesis is crucial after myocardial infarction, in fact it has been showed that the impairment of myocardial angiogenesis causes a reduction in myocardial perfusion and fatal ischemic cardiomyopathy[51].

During IRI, ECs release many mediators such as endothelin, angiotensin, prostacyclin, nitric oxide, neuregulin, parathyroid hormone-related peptide (PTHrP), or natriuretic peptides. These factors modulate cardiomyocyte survival and contractile function in a positive fashion[31].

In endothelial cells, the uncontrolled depletion of Ca^{2+} in the endoplasmic reticulum can cause ER-stress. This leads to the cleavage of ER-bound caspase-12 and caspase-3, and cytochrome-c leads to the activation of the apoptotic process[52,53].

When ECs are activated, they first express P-selectin, this molecule mediates leukocytes rolling on the vessel wall surface[54]. E-selectin, ICAM-1 and RAGE are expressed *de novo* several hours after reperfusion (4-6 hours), they induce the firm adhesion of leukocytes via activated ß2-integrins leading to the transmigration of these inflammatory cells into the myocardium[55,56].

To prevent pathological remodeling in the structure of blood vessels, ECs produce and release two endogenous gasotransmitters, hydrogen sulfide (H_2S) and nitric oxide (NO). These two molecules physiologically act as vasorelaxants[57,58].

1.3 Hydrogen sulfide as a preconditioning agent.

Gasotransmitters are endogenous gaseous molecules that act as secondary messengers (e.g. calcium o cyclic AMP). Differently from other secondary messengers, they can all transmit the signal without a set of regulatory mechanisms, since they can diffuse through membranes[59].

Almost twenty years ago, Abe and Kimura were the first to propose the role of H_2S as the endogenous mediator in mammals[60].

There are three enzymes that endogenously generate H_2S: cystathionine-β-synthase (CBS), 3-mercaptopyruvate sulfurtransferase (3-MST) and cystathionine-γ-lyase (CSE). CBS and CSE catalyze the condensation reaction of homocysteine with cysteine, whereas 3-MST produce H_2S from 3-mercaptopyruvate (3MP)[61–63].

It is now recognized that H_2S is involved in the regulation of the inflammatory and immune response, cardiovascular system, kidney, nervous system and gastrointestinal tract function[64,65].

H_2S has been known for many years for being a toxic agent. In high concentrations, indeed, H_2S inhibits mitochondrial respiration in intact cells (30μM) or isolated mitochondria (10μM). In contrast, low concentrations (<20μM) of NaHS (an H_2S donor) enhance oxygen consumption and increase $\Delta\psi m$[66].

Exogenously given H_2S limits the extent of infarction; this effect is accompanied by a decrease in production of anti-angiogenic factors and a preservation of growth factors such as VEGF[37].

Studies on isolated rat liver mitochondria showed a biphasic effect of H_2S on mitochondrial electron transport. Concentrations from 100nM to 1μM stimulated electron transport, whereas 10μM and above produced inhibition[67]. The same bi-modal effect has been shown by Pupo et al where H_2S induced no effect at the lowest and highest concentrations (0.5 and 100 μM) and a significant cellular response at 1 μM[68].

Previous studies suggest that the heart is one of the major sources of H_2S[69]. H_2S can act as a cytoprotectant to protect cells from oxidative stress and as an anti-apoptosis agent by preserving mitochondrial function during ischemia-reperfusion[70,71].

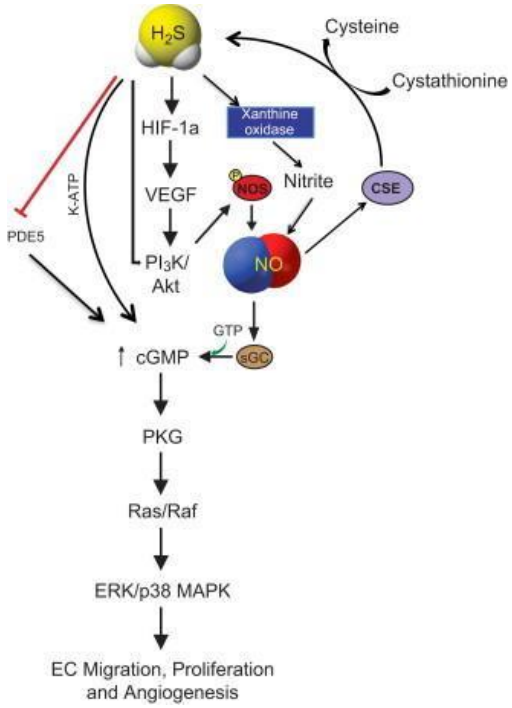


Fig. 2. H2S signaling pathway in endothelial cells.

ERK is an effector of H2S signaling cascade[72].

Despite HIF-1a is a direct effector of H_2S, since it is a soluble molecules, many other proteins could be targeted by it, making it worth investigating other pathways and effectors.

1.4 ERK 1/2 cascade and interaction with H_2S.

The RISK pathway is a group of kinases that confer cardioprotection during myocardial reperfusion[73]. They prevent the lethal reperfusion injury by mediating a programmed cell survival[14].

The RISK pathway is linked to the extracellular signal-regulated kinase (ERK) 1/2 cascade, which is one of the four mitogen-activated protein kinase (MAPK) cascades.

ERK1/2 cascade mainly regulates the initiation of proliferation, but it also takes part in several other fundamental processes such as cell migration, survival, differentiation, cellular metabolism, and apoptosis[74–77].

Moreover, in resting cells, ERK is present in the cytoplasm but it can translocate to other organelles including mitochondria[78]. ERK can phosphorylate mitochondrial membrane fusion protein MFN-1, located on the outer membrane of the organelle, initiating the fragmentation process, ultimately leading to cytochrome *c* release and resulting in cell apoptosis[79].

It is reported in literature that H_2S induces the phosphorylation of Heat Shock Protein-27 (HSP27)[80], leading first to actin polymerization and ultimately to cell migration[81–83].

Not only ERK1/2 but also activation of Akt in endothelial cells after H_2S stimulation exerts a stimulation of angiogenesis[84]. It is well known that Akt plays a central role in the intracellular signaling of angiogenesis. Its activation increases endothelial cell proliferation[85], migration[86] and formation of capillary-like structures *in vitro*[87]. It also promotes neovascularization *in*

vivo[88]. There is evidence that H_2S might upregulate the pro-angiogenic pathway triggered by Akt in normoxia by inducing Akt phosphorylation[84,89].

Hu et al[90] observed that preconditioning rat myocytes with NaHS (1–100 μM) not only increased cell viability, but if ERK1/2 or Akt were blocked during preconditioning or ischemia significantly decreased the cardioprotective effect of H_2S.

H_2S activates vascular endothelial growth factor receptor 2 (VEGFR2) by reducing a disulfide bond between Cys1045 and Cys1024, therefore inducing angiogenesis[91].

It has been reported for the first time in 2012 that H_2S promotes migration in human endothelial cells though Rac1-mediated actin cytoskeleton remodeling[89].

Moreover, there is evidence that low concentrations of H_2S (<10 μM) can decrease DNA damage, thus cell death, by S-Sulfhydration of MEK1, leading to the activation of the MEK/ERK pathway[92].

In literature, there is still debate as to whether hydrogen sulfide should be administered before the ischemic event (pre-conditioning) or after (post-conditioning)[17,34,90,93,94].

After a thorough study, we decided to give it as a pre-conditioning agent. Moreover, our main goal was to investigate if a 24 hours treatment could trigger a cascade that could enhance the endothelial response after an ischemic insult *in vitro*.

2. MATERIALS AND METHODS

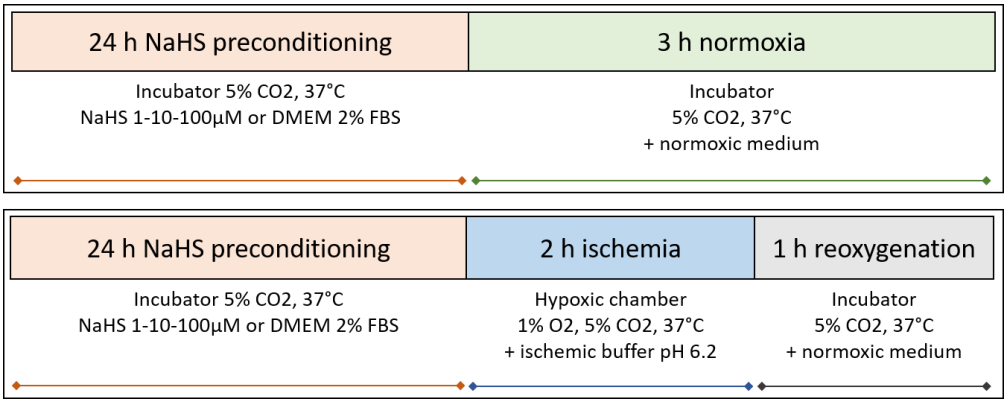
2.1 Cell culture

Human microvascular endothelial cells 1 (HMEC-1) (ATCC® CRL3243™) were cultured in EndoGRO™-MV Complete Culture Media Kit (Millipore), 1% penicillin/streptomycin (Pan Biotech) and passaged 80-90% confluence.
Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) 2% FBS (Microgem), 1% penicillin/streptomycin, 1% L-glutamine (Microgem) was used for preconditioning and re-oxygenation processes.

2.2 Hydrogen sulfide preconditioning and Ischemia/Reperfusion protocol

Sodium hydrosulfide hydrate (NaHS) (Sigma-Aldrich) was used as a saline hydrogen sulfide donor. NaHS was freshly prepared in DMEM 2% FBS on the day of the experiment at a concentration of 5mM, this stock solution was used for dilutions to reach our working conditions (1-10-100 μM).
In vitro simulation of ischemia/reperfusion injury was induced using an ischemic buffer (pH 6.2) as described in literature. [95] The buffer was equilibrated overnight in hypoxic chamber (1% O_2, 5% CO_2, 37°C) (Invivo$_2$ 200, Ruskinn, United Kingdom) in order to guarantee proper equilibration with the atmospheric oxygen levels. A minimum level of 1% O_2 can be achieved by this system and no complete anoxia is guaranteed from the manufacturer.

Moreover, to the best of our knowledge, 1% O_2 at atmospheric pressure, matches the oxygen partial pressure of ischemic tissues, including myocardium. Cells were put in the hypoxic chamber and the medium was changed with the equilibrated ischemic buffer. Ischemic protocol lasted for 2 hours in every experiment, and then cells were reoxygenated with fresh medium for 1 hour in incubator (37°C, 5% CO_2).



Schematic representation of the experimental design used. On the upper side is represented the "normoxia" setting, whereas on the lower panel is the setting used to simulate the *in vitro* "ischemia-reperfusion" condition.

2.3 Solutions and reagents

Ischemic buffer. The buffer was freshly prepared before each experiment. The following formulation was used: NaCl 750 mg, KCl 88 mg, $MgCl_2$ 9.9 mg, $CaCl_2$ 13.2 mg, HEPES 95 mg, Na L-lactate 224 mg (all purchased from Sigma Aldrich). The ingredients were dissolved into ddH2O

and pH was adjusted to reach 6.2 before bringing the solution to the required volume (100 mL).

AZD6244. Also called Selumetinib, is a selective MEK1/2 inhibitor. The inhibitor is dissolved in DMSO, as 10 mM stock solutions at -20°C. The stock was diluted to reach a working concentration of 1 μM.

SCH772984 (Aurogene). Selective ERK1/2 inhibitor was dissolved in DMSO, as 5 mM stock solutions at -80°C. The stock was diluted to reach a working concentration of 1 μM.

Antibodies. Primary antibodies anti- Heat-shock protein 90 (HSP90), p44/42 MAPK (ERK1/2) and phospho-p44/42 MAPK (p-ERK1/2) were all purchased from Cell Signaling Technology (The Netherlands). Secondary anti-rabbit antibody was purchased from ImmunoReagents, Inc. (North Carolina, USA). All antibodies were used according to their manufacturer's protocols.

2.4 Viability assay

In order to see how cell viability could be influenced by preconditioning, an MTT assay was performed at 24 hours from the end of reoxygenation.
Cells were plated in a 96-well plate at a density of $0.5x10^4$ cells/well in growth medium.
On the next day, cells were preconditioned as previously described. After 24 hours preconditioning, cells would undergo normoxic or I/R condition following the ischemia/reperfusion protocol mentioned earlier.

At the end of reperfusion, media was changed to all cells to DMEM 2% FBS, in order to keep cells alive for the next 24 hours, but avoiding cell proliferation. After one day, MTT solution was added to each well (10 µL/well), plates were kept in the dark in incubator for 3 hours. Then, media was removed and 100 µL DMSO were added to each well and absorbance was detected at 570 nm using a microplate reader. At least eight wells for each condition were analyzed.

2.5 Wound healing assay

To assess cell migration cells were plated into three-chamber silicone-culture inserts (Ibidi) in a 12-well plate at a density of $4x10^5$ cells/mL in growth medium. This density was chosen as the most appropriate to have cells at 90-100% confluence overnight.

On the next day, cells were treated with NaHS as described before, by removing the medium from each chamber and adding the desired treatment or just control medium, being careful not to scratch cells away.

After 24 hours, all culture inserts were removed and cells were gently washed with warm PBS with Ca/Mg. At this point, cells underwent I/R protocol for 2 hours.

At the end of the ischemia, cells were gently washed with warm PBS and growth medium was added to each well.

After 6 hours of migration, cells were fixed in 4% PAF. Images were then acquired using a Nikon Eclipse Ti-E microscope with a 10× lens.

The Metamorph software was used to both acquire and analyze all images. Cell motility was expressed as percentage of wound closure. At least three fields for each condition were analyzed.

2.6 In vitro angiogenesis assay

The formation of capillary-like structures *in vitro* was studied on growth-factor reduced Matrigel (Corning, USA). Matrigel was used according to the manufacturer's instructions.

ECs were plated into Petri dishes and treated according to our protocols on the following day.

After *in vitro*-simulated I/R injury, cells were seeded at $2.5x10^4$ cells per well onto Matrigel pre-coated 24-well plate in growth medium.

After 16 hours, cell organization was observed using a 5x lens. Images were acquired using the Infinity Analyze software (Lumenera Corporation). A minimum of three fields were analyzed for each condition.

2.7 Quantification of mitochondrial mass and membrane depolarization

To measure the depolarization of mitochondrial membrane in relationship with mitochondrial mass, endothelial cells were plated at a density of $2x10^4$ cells/well on 1.5 mm ø cover slips in growth medium. Cells were treated as previously described (see "Ischemia/reperfusion protocol") with 1uM NaHS.

After reperfusion, all specimens were incubated in DMEM 2% FBS with both MitoTracker™ Green FM (Thermo Fisher) 200nM and MitoTracker™ Red CMXROS (Thermo Fisher) 50nM in the dark for 30 minutes at 37°C. After staining, cells were washed with PBS and fixed with 4% PAF. After fixation, coverslips were mounted on microscope slides and observed at confocal microscope (Zeiss LSM800) with a 63x oil-immersion objective. MTG has an absorption/emission spectrum of 490 nm/516 nm, while the MTR one is 579 nm/599 nm. Images were acquired with ZEN System software with a resolution of 512x512 pixels.

2.8 Western Blot

Cells were seeded into petri dishes and were allowed to reach confluence before being treated with NaSH (1-10-100 μM) and/or AZD6244 1 μM, then underwent I/R simulation protocol. At the end of the protocol, cells were scraped and lysed with RIPA buffer (Sigma-Aldrich), containing protease and phosphatase inhibitors (Complete protease inhibitor tablets and PhosStop, Roche).

Whole cell lysates were separated by SDS-PAGE and wet-transferred on PVDF membranes, according to the manufacturer's protocol (Bio-Rad).

After transfer, membranes were blocked in 4% non-fat dried milk or 4% BSA (depending on the primary antibody), for 1 hour at room temperature.

Membranes were washed once in TBST and incubated with antibodies against HSP90 (1:1,000), ERK1/2 (1:1,000), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:1,000) overnight at 4°C.

Membranes were washed with TBST three times for 10 min. Anti-rabbit secondary antibody incubation (1:5,000) lasted for 1 hour. After another 30' of washing with TBST 1x, chemiluminescence was detected using the ECL system (Bio-Rad) using a Chemidoc touch instrument (Bio-rad).

2.9 Image analysis

Tubulogenesis assay. During the analysis, we kept into consideration the following parameters: number of master segments, number of master junctions, total master segments length and number of isolated segments.

A well-constructed network should have more master elements (segments and junctions), thus creating a more physiological and functional structure.

The images were analyzed with ImageJ's Angiogenesis Analyzer plugin, and only the aforementioned parameters were kept into consideration. All images were converted to RGB format if needed.

Mitochondrial staining. Multi-channel images were split to separate the two channels. Then, the corrected total cell fluorescence (CTCF) was calculated for each cell in both channels as described in literature[96].

For each cell, the ratio between mitochondrial depolarization and mass was calculated and normalized on the control condition.

Image lab quantification. Using Image Lab software, bands were detected automatically and then subtracting the background to obtain a better quantification. Density of bands was obtained keeping into consideration the value of the integrated density adjusted by subtracting the background. All proteins were quantified by normalizing their density on our loading control (HSP90).

2.10 Statistical and computer analysis.

Results are expressed as mean ± SEM. Differences between groups were analyzed by one-way ANOVA or Kruskal-wallis or Mann-Whitney t-test. Significance was established at p-value <0.05. All raw data was analyzed using Microsoft Excel and Prism 6.

3. RESULTS.

3.1 NaHS preconditioning effects on cell viability in both normoxia and I/R.

During acute myocardial infarction, endothelial cells can go towards death through apoptosis, necrosis or autophagy[97–99]. To assess endothelial cell response after I/R injury in terms of viability, an MTT assay was performed after 24 hours from the injury.

As shown in Fig. 1, there was a decrease in the IR condition compared to normoxia, but H2S did not significantly increase cell viability in the post-IR condition. There was an important decrease in the I/R condition compared to the normoxia.

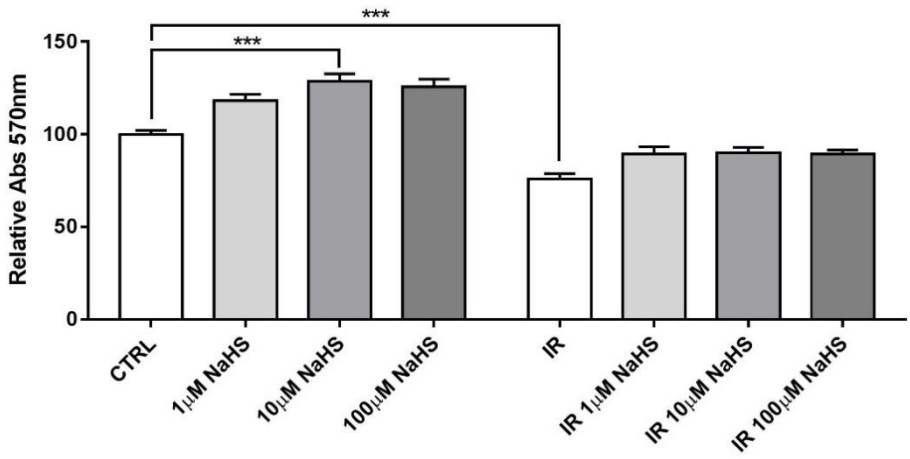


Fig. 1. Figure 1. Normalized viability on the normoxic control condition. Statistical significance was set at p-value <0.05 (*** = <0.001).

3.2 Cell migration is strongly enhanced after NaHS preconditioning.

Cell migration is one of the essential processes during angiogenesis. In fact, cells have to migrate to create new vessels. The creation of new vessels is important after I/R injury in order to allow a right blood supply to the myocardium and to eventually replace arteries that might have been disrupted by the ischemic event and subsequent reperfusion. To study this process, cells would migrate following I/R-simulation. After 6 hours, the percentage of wound closure was calculated and compared to the normoxic control condition (Fig. 2B).

The strongest effect was observed at 1 μM NaHS both in normoxia and post-I/R.

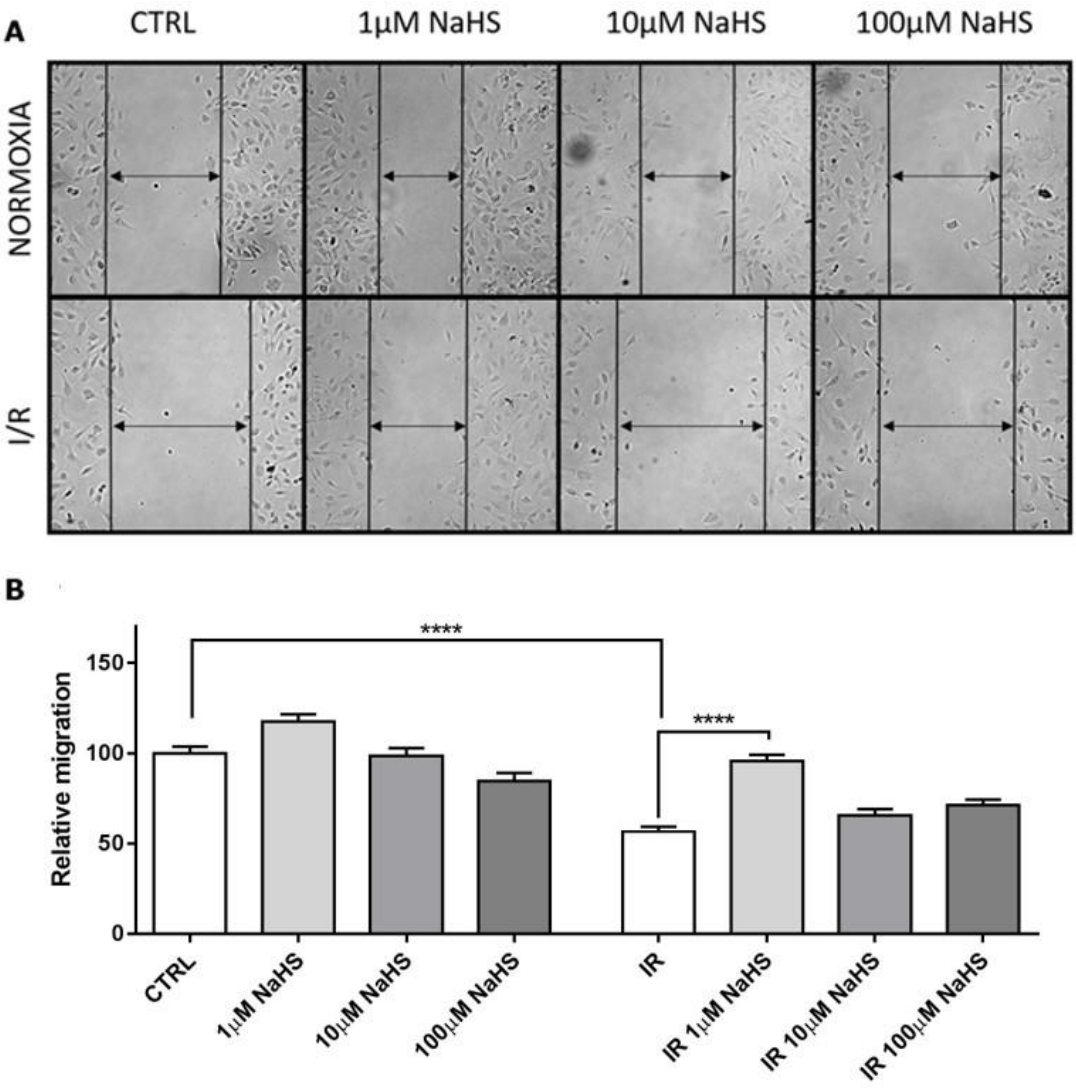


Fig 2A. Representative images of cells after 6 hours in the different experimental conditions; **Fig 2B.** Relative migration, all data sets were normalized on the normoxic control condition (CTRL).

Statistical significance: **** = p-value <0.0001.

3.3 Treatment evoked a pro-angiogenic effect after I/R injury.

To study if the NaHS treatment had a pro-angiogenic effect on cells that underwent I/R injury simulation, we studied their ability to form capillary-like structures *in vitro* focusing on the most promising concentration (1 μM NaHS).

After I/R, cells were detached and plated in Matrigel precoated 24-well multiwell in EndoGRO 10% FBS for 16 hours. As Fig 3 shows, there is a positive trend in both normoxia and I/R when cells are treated with 1 μM NaHS.

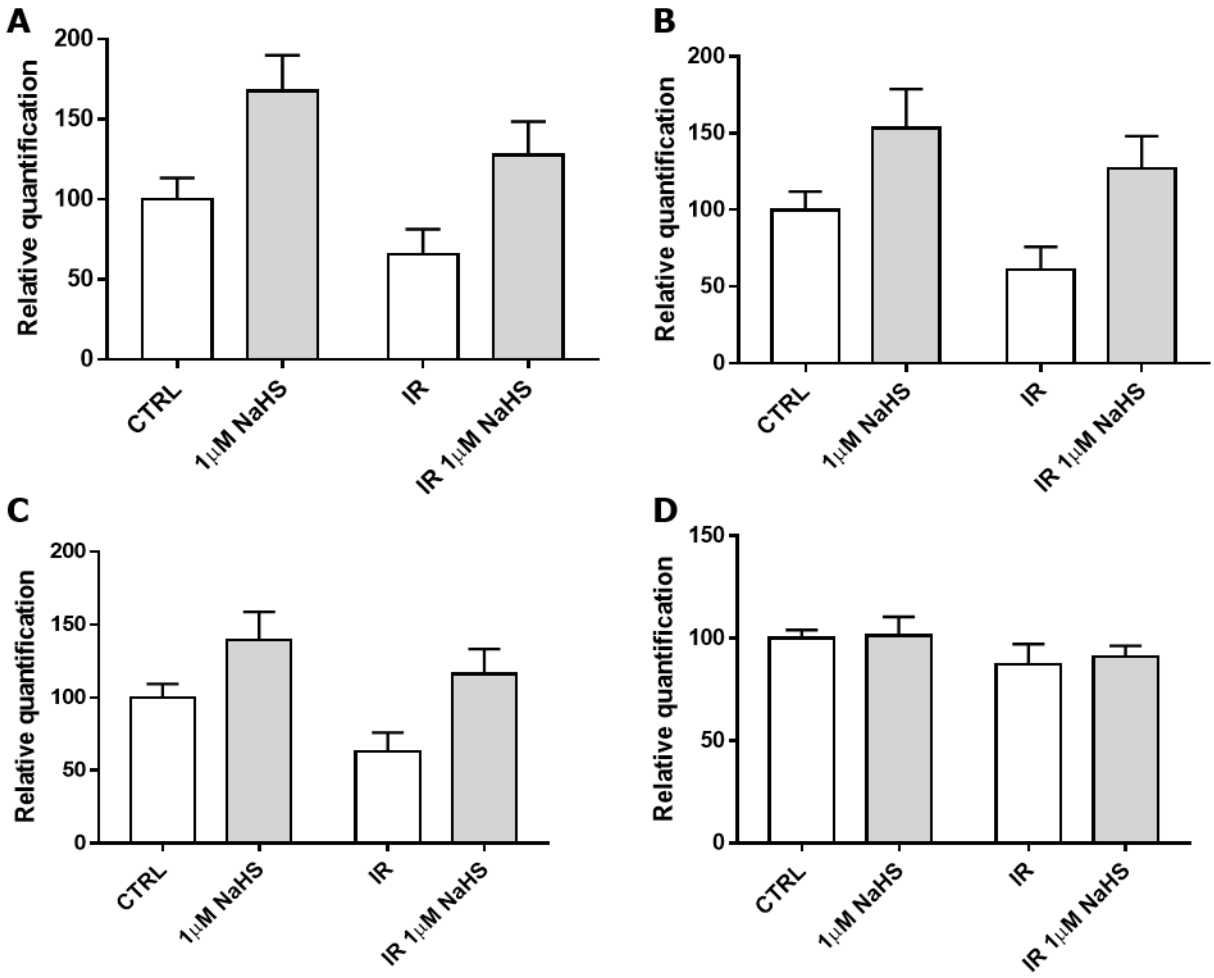


Fig 3. Quantification of main elements of the *in vitro*-capillary network.

3A. Total master segments length. **3B.** Number of master segments. **3C.** Number of Junctions. **3D.** Number of Isolated Elements.

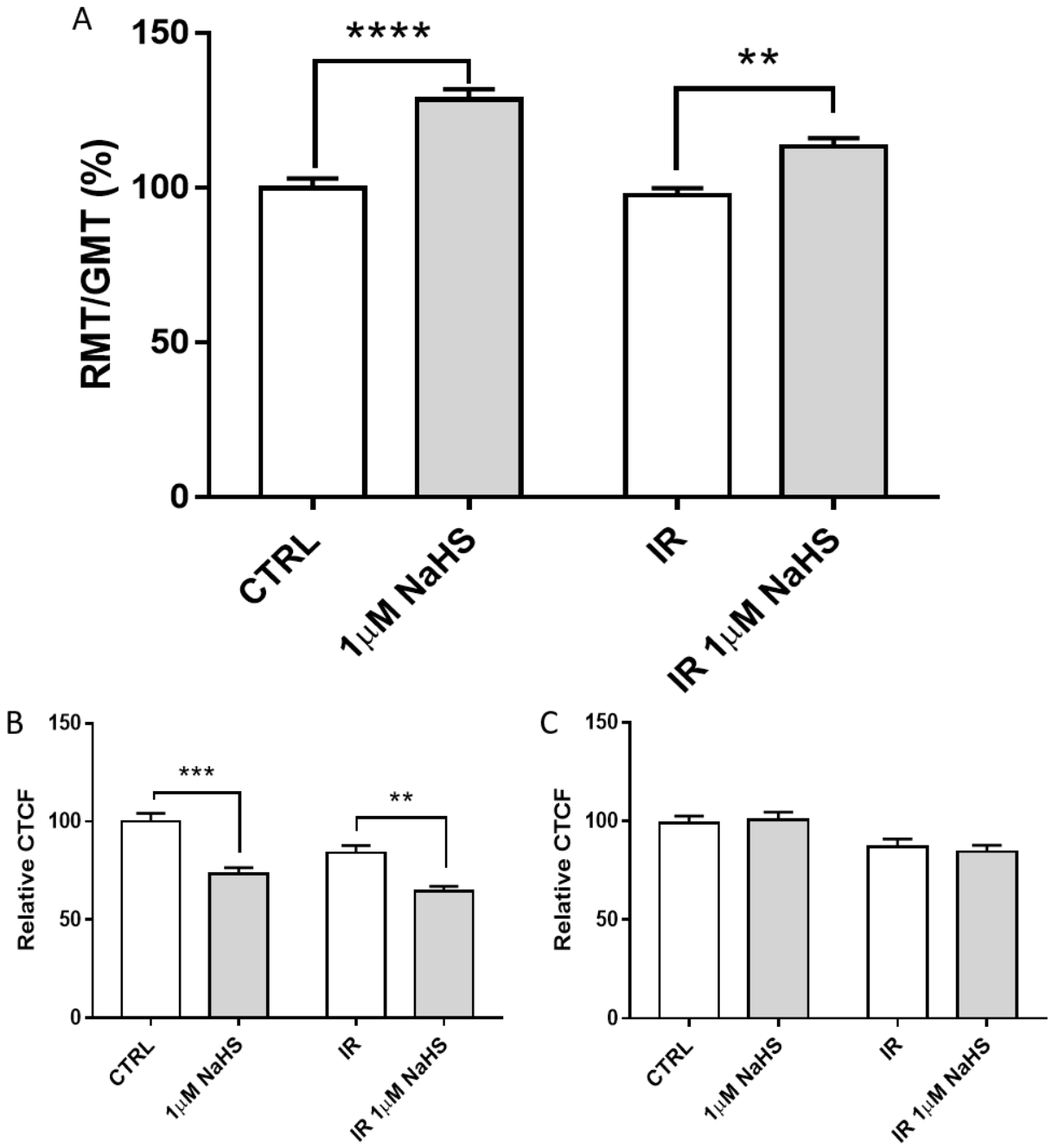
3.4 NaHS preconditioning prevents mitochondrial dysfunction by preserving mitochondrial membrane energy.

We decided to perform a double MitoTracker™ staining on all samples to investigate whether mitochondria were affected by NaHS preconditioning.

MitoTracker™ Green FM stains all mitochondria, whereas MitoTracker™ Red CMXROS stains only viable mitochondria. Red fluorescence is directly proportional to membrane depolarization.

By calculating the ratio between red and green fluorescence, we tried to determine the influence of NaHS and I/R on mitochondria in endothelial cells.

NaHS treatment decreased the mitochondrial mass with a similar fashion in normoxia and I/R (Fig 4B). However, the mitochondrial function did not change much between the two I/R experimental conditions. Since the mitochondrial mass is less in the NaHS treated I/R condition when compared to untreated cells exposed to I/R, the outcome ratio is higher (Fig 4A). The same trend can be observed also in normoxia, where the 1 μM NaHS produces a higher ratio than the normoxic control.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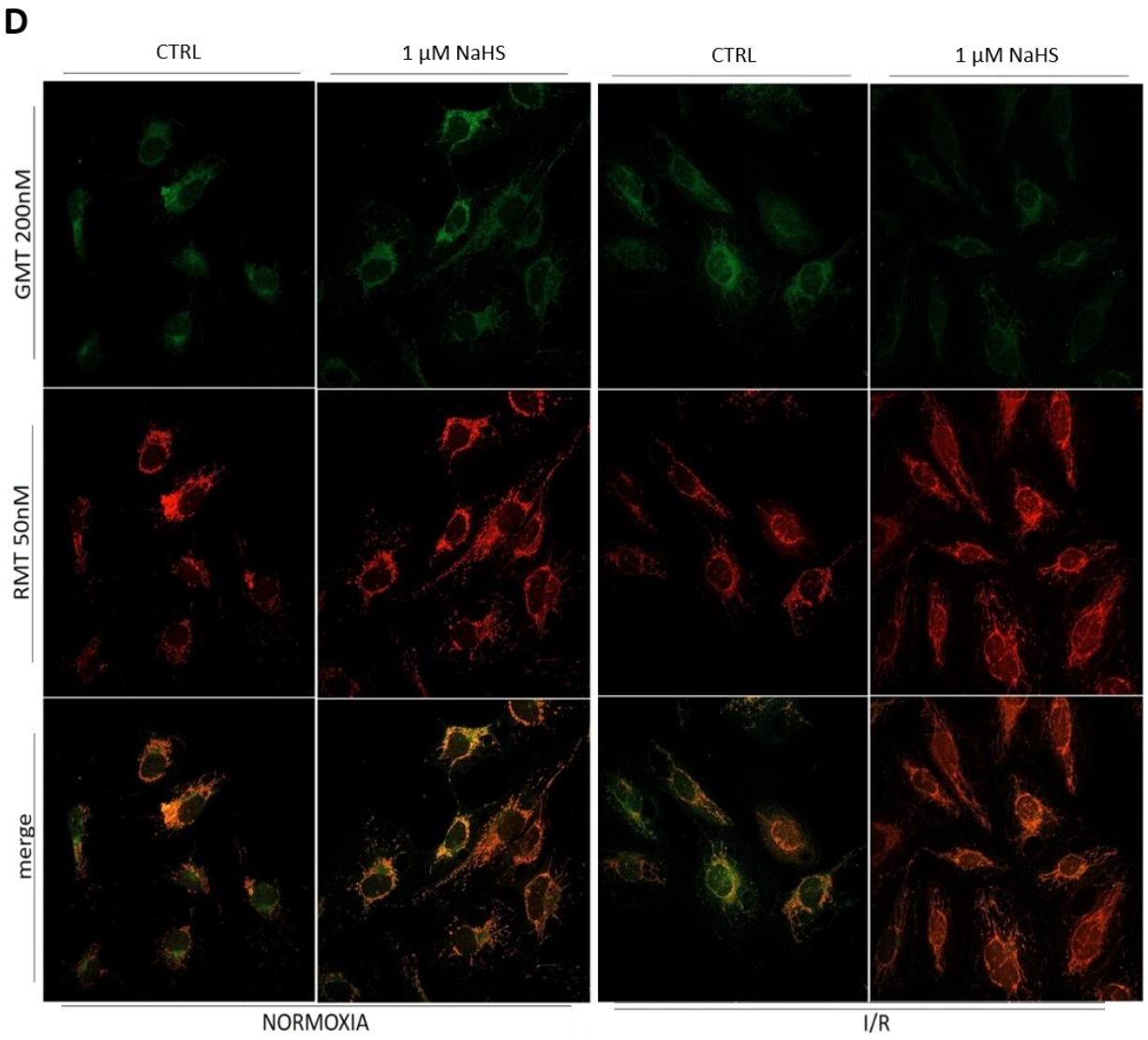


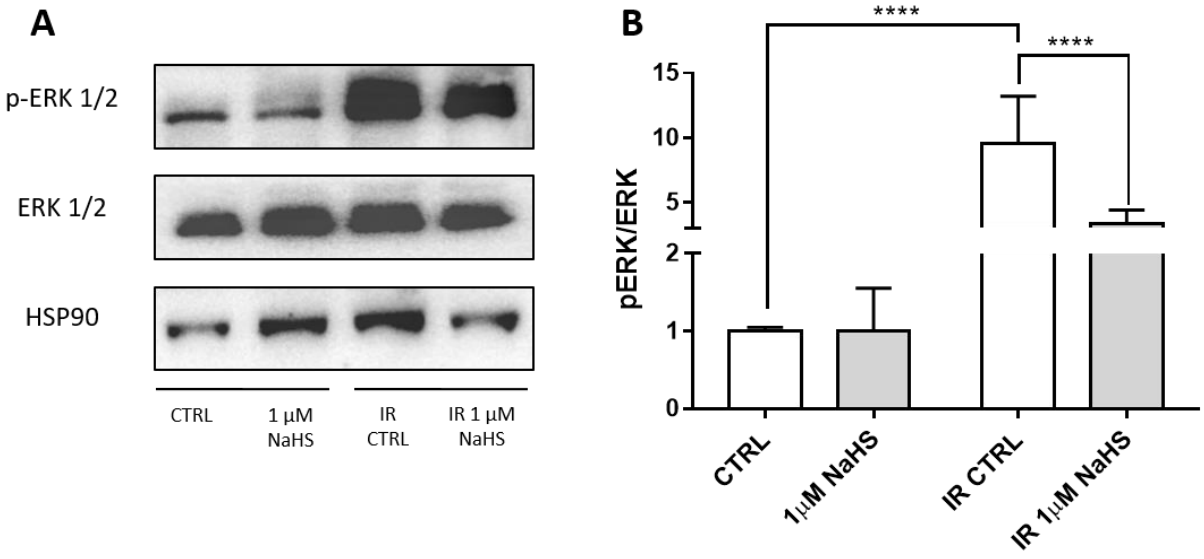
Fig 4A. Ratio between red (RMT) and green (GMT) MitoTracker™. **4B-C.** GMT and RMT fluorescence respectively, calculated for each experimental condition. **4D.** Representative fields of stained cells. All data sets were normalized on the normoxic control condition (CTRL). Statistical significance: ** = p-value <0.01, **** = p-value <0.0001.

3.5 NaHS preconditioning and I/R injury influence ERK1/2 phosphorylation.

Since ERK1/2 is deeply implicated in both cell migration and angiogenesis, but also in inducing apoptosis, we evaluated whether I/R and/or NaHS treatment could influence its level of phosphorylation.

Protein expression and phosphorylation levels were assessed through western blot.

ERK appeared to be strongly phosphorylated in I/R condition, whereas NaHS treatment seemed to attenuate the ratio p-ERK/ERK, bringing it towards a more physiological condition.



5A. Representative experiment of ERK 1/2 and p-ERK 1/2. **5B.** Ratio of p-ERK on total ERK (n=4). Both total and phosphorylated proteins were first

normalized on the housekeeping gene (HSP90) and then the ratio was calculated. All data sets were normalized on the normoxic control condition (CTRL). Statistical significance: **** = p-value <0.0001.

3.6 Effects of upstream ERK inhibition.

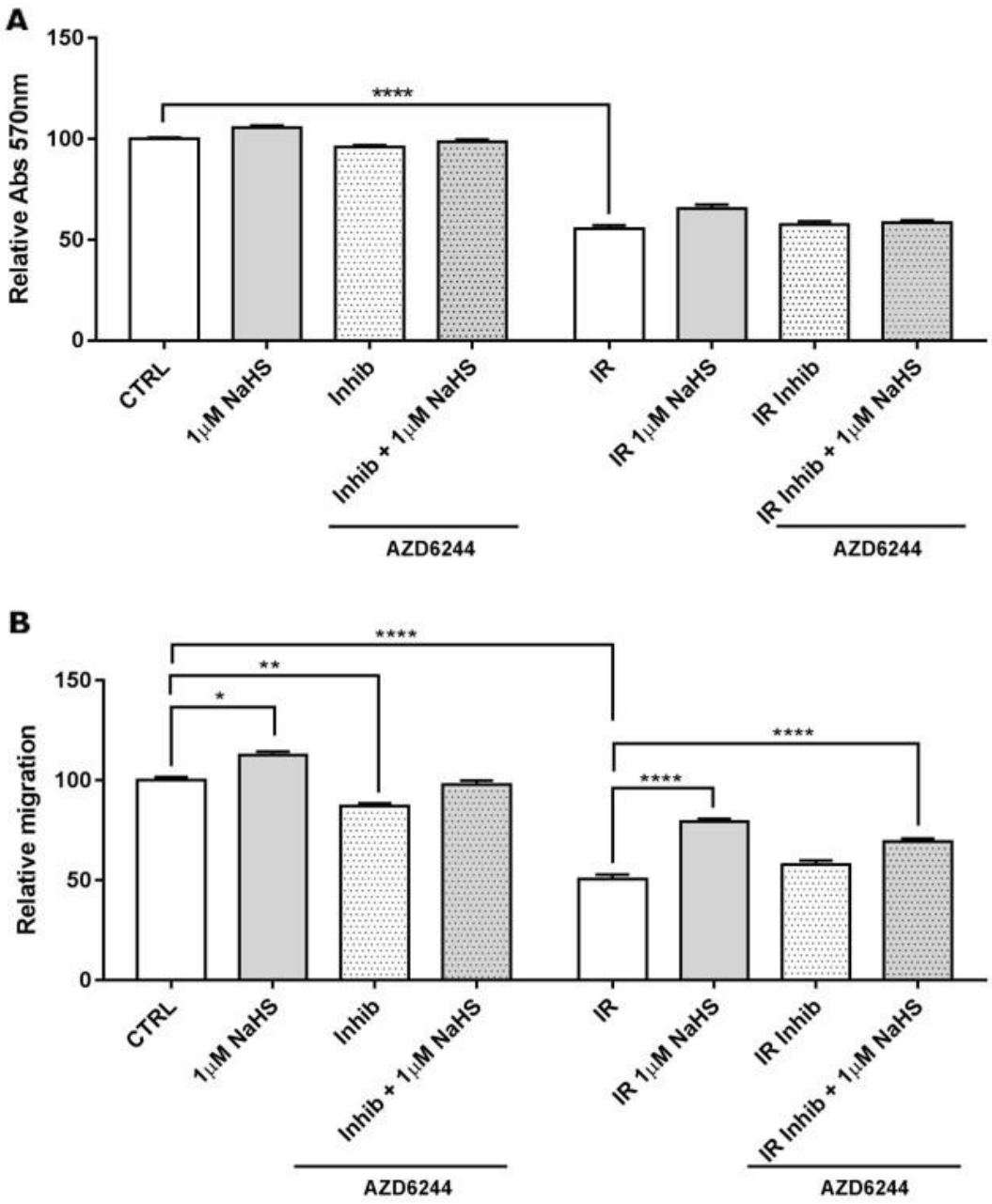
After assessing the levels of ERK phosphorylation, we decided to investigate if NaHS directly modulated ERK pathway.

At first, we targeted its upstream modulator, MEK, using its specific inhibitor AZD6244 before preconditioning.

Both viability and cell migration were tested.

Considering cell viability there was no relevant difference with or without inhibitor and/or 1μM NaHS, confirming what we had already observed (see Result 1).

When we tested the effects on cell migration, we still observed the same trend when cells were preconditioned with 1μM NaHS in both normoxia and I/R. MEK inhibitor slightly decreased the rate of cell migration, especially in normoxia. When we used both the inhibitor and NaHS, migration was still enhanced, but less than the 1μM NaHS condition alone (Fig. 6B).



6A. Cell viability after MEK inhibition (1 μM AZD6244) and 1 μM NaHS preconditioning. **6B.** Cell migration after MEK inhibition (1 μM AZD6244) and 1 μM NaHS preconditioning. Statistical significance: * = p-value <0.05, ** = p-value <0.01, **** = p-value <0.0001.

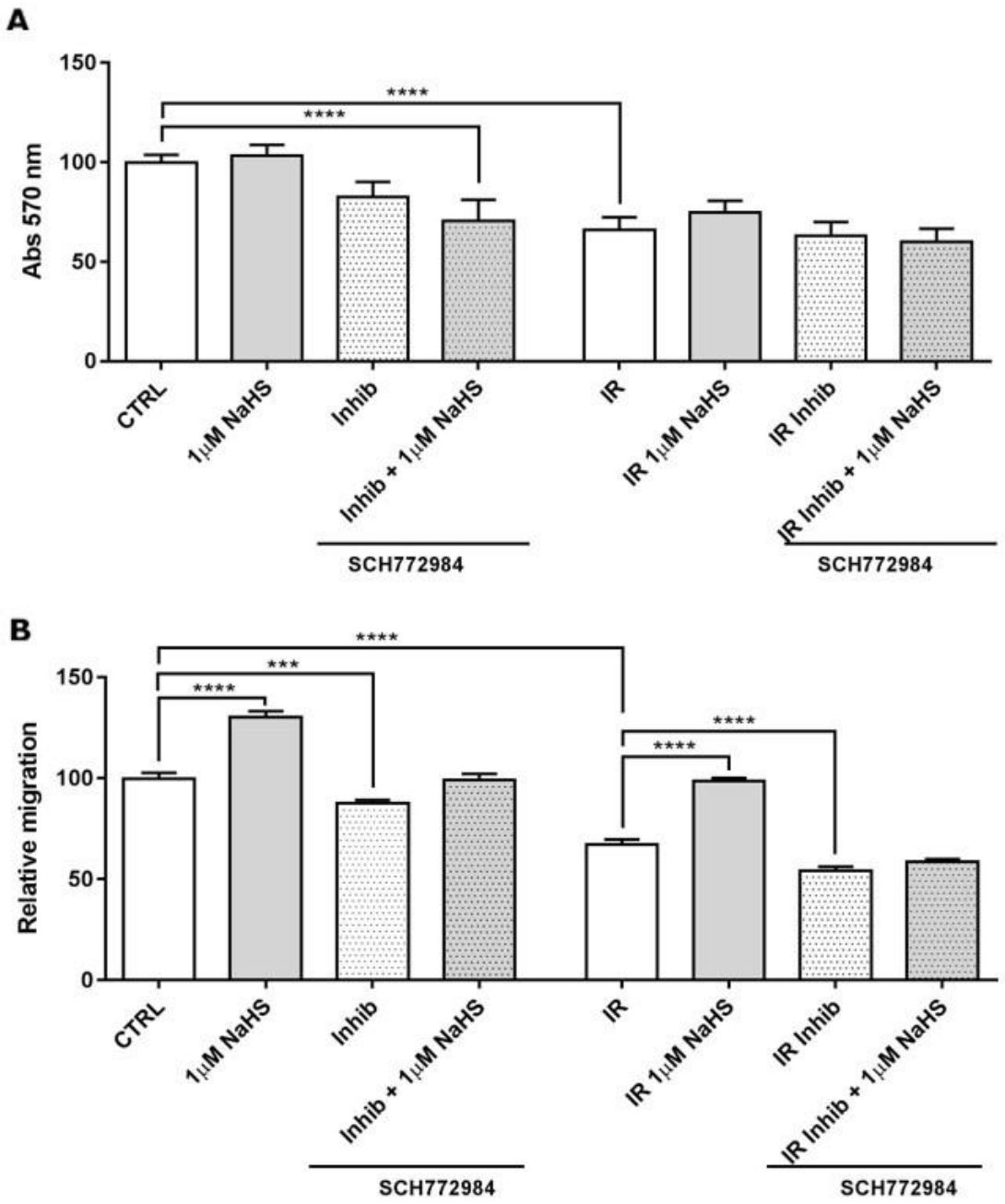
3.7 Effects of direct ERK inhibition.

After evaluating the effects on upstream inhibition, we decided to investigate whether NaHS preconditioning could rescue the biological effects after direct ERK1/2 inhibition.

Again, we assessed the inhibition on both cell viability and cell migration, which are two key pathways in which ERK1/2 is strongly involved.

No relevant changes were observed in I/R condition, but a significant decrease in cell viability was observed in normoxia when cells were treated with both ERK inhibitor and NaHS.

Focusing on cell migration, in normoxia a significant decreased was observed when cells had ERK inhibited but NaHS restored the migration, rescuing the inhibition. However, in I/R, the direct inhibition of ERK actually caused a decrease in cell migration when compared to the I/R untreated cells. NaHS in this case could not rescue the pathway, resulting in a cell migration rate very similar to the one observed with only ERK inhibitor.



7A. Normalized viability after direct ERK1/2 inhibition (1 μM SCH772984) and 1 μM NaHS preconditioning. **7B.** Cell migration on 1 μM NaHS preconditioned cells after ERK1/2 inhibition (1 μM SCH772984). Statistical significance: ** = p-value <0.05, *** = p-value<0.01, **** = p-value <0.0001.

4. DISCUSSION.

Cardiovascular diseases, and in particular coronary heart disease, are the leading cause of death worldwide (WHO, 2017). In 2016, approximately 17.9 million people died from CVDs, consisting of 31% of all global deaths. Moreover, 85% of these deaths are due to heart attack and stroke.

Many complications associated to these diseases are caused by ischemia-reperfusion injury[100–102]. This phenomenon can differently affect the organism depending on where it happens, the duration and the severity of the event itself. After the ischemic event, most interestingly, the worst effect is caused by the blood flow restoration. This leads at first to an increased inflammatory response and later on also to a profound tissue injury[3].

Along with the inflammatory response, there are several biochemical and cellular changes caused by this rapid oxygen and nutrients supply, that cells cannot bear altogether[8,9,11].

One of the issues of acute myocardial infarction is that there are more complications for patients undergoing revascularization, since a proper vascularization is a key step into restoring the physiological state[103,104].

Indeed, there is evidence that not only cardiomyocytes suffer from ischemia-reperfusion injury but endothelial cells suffer as well and are even more sensitive to this kind of injury[35]. Moreover, they are also critical mediators for the onset of the inflammatory response, the generation of ROS, and the rapid restoration of the physiological pH. Indeed, this all negatively affects the

surrounding myocardial tissue, leading in worst case to lethal reperfusion injury[3].

Since endothelial cells are such critical mediators of I/R injury, especially in the context of myocardial infarction, we decided to focus our attention on how to restore their physiological function. To do so, we tried to find a way to enhance their angiogenic activity and decrease the injury on the endothelium, based on the evidence that a functional endothelium is pivotal to avoid lethal consequences[51].

To this aim, we chose to use hydrogen sulfide released from NaHS as a protective agent on endothelial cells. H_2S is a gasotransmitter, known to act as a secondary messenger in mammals[59]. There is evidence that it can play a positive role in ischemia-reperfusion injury and could indeed be used as a therapeutic agent[105]. As we aimed at study the effects as a potential protective agent, we decided to settle on a 24 hours treatment and not right before the ischemic event that is usually unexpected an unpredictable.

In order to study the effects on the endothelium alone, we decided to use an *in vitro* model on which we could test the direct effects of hydrogen sulfide as a preconditioning agent with the aim to restore the physiological state after the ischemic event. Using it this way, we aimed at triggering a response in endothelial cells that would last since after the ischemic event, since it is know that NaHS rapidly hydrolyzes in water, establishing the equilibrium among H_2S, S^{2-} and HS^- species[106].

We are aware that testing the effects on other endothelial cell types, such as HUVEC or HAEC would be interesting and is definitely worth investigating in the future.

The first thing we decided to evaluate is whether the NaHS pretreatment could induce some changes in cell viability. There is debate in literature on what is the most efficient dose to induce a positive effect on cell viability[107]. What we observed was that in our I/R model there was a slight increase but nothing particularly relevant. On the other side, when cells were treated with NaHS alone and kept in normoxia, we detected an effect only in the 10 μM condition. It is true that usually the effects of hydrogen sulfide are not observed in our time-range, so this could be a new result that could be further explored in the future.

The next focus was on how the preconditioning could change the angiogenic activity of ECs. We tested the effects of cell migration and the ability to create capillary-like structures *in vitro*. The strongest effect we observed was that cells treated with 1 μM NaHS and then underwent I/R injury, showed a migration rate that was almost as much as the control condition in normoxia.

The same treatment showed a trend in *in vitro* angiogenesis but not statistically significant.

Then we decided to focus our attention on how the mitochondrial activity could be modulated by the preconditioning and the I/R injury. We observed that although the overall mitochondrial activity appeared to remain stable in all conditions, there was always a decrease in the mitochondrial mass. Considering this, the ratio between the number of mitochondria and their

activity was always enhanced in cells pretreated with NaHS. However, there appeared to be no big changes in mitochondrial mass and/or activity between the control and the IR condition. This could mean that H_2S could enhance mitochondrial activity. Moreover, we need to keep into consideration that despite having a higher mitochondrial mass, they could have been not viable or working in the wrong way, decreasing the overall ratio in the control (CTRL) and ischemic-induced condition (IR).

The next step was to try to understand whether the 24 hours preconditioning before the ischemic event could trigger a change in ERK phosphorylation state, due to the role that the MEK/ERK pathway has in both apoptosis and cell migration.

What we saw was that I/R alone significantly enhanced ERK1/2 phosphorylation and that the 1 μM NaHS treatment managed to drastically reduce this effect, going towards a more physiological phosphorylation state. On the other hand, we saw no change in p-ERK1/2 when cells were treated and kept in normoxia.

Lastly, since it appeared that ERK was triggered by the NaHS treatment, we inhibited both ERK1/2 and its upstream activator, MEK with selective inhibitors (AZD6244 and SCH772984).

After testing the effects on cell viability and cell migration, we observed that the direct inhibition of ERK decreased cell migration and could not be rescued by H_2S. The use of these inhibitors gave us the information that, apparently, the inhibition of ERK cannot be rescued by H_2S. This suggests that our pathway is triggered upstream, after MEK, although the final effector seems

to be ERK1/2. Obviously, further experiments are needed in order to elucidate how this pathway is activated 24 hours ahead of the ischemic insult and how it is prolonged in time.

In our study, we showed that H_2S could be used as a long-term preconditioning agent and not just in the exact moment of the ischemic event, despite its rapid metabolization. This opens the discussion that an ERK1/2-dependent pathway is activated helping microvascular endothelial cells to better respond to ischemia-reperfusion injury, thus helping the overall response of the tissue and avoiding lethal consequences.

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