RESEARCH ARTICLE

REVISED **Inhibition of CD34+ cell migration by matrix metalloproteinase-2 during acute myocardial ischemia, counteracted by ischemic preconditioning** [version 2; referees: 1 approved, 1 approved with reservations]

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Abstract

Background. Mobilization of bone marrow-origin CD34+ cells was investigated 3 days (3d) after acute myocardial infarction (AMI) with/without ischemic preconditioning (IP) in relation to stromal-derived factor-1 (SDF-1α)/ chemokine receptor type 4 (CXCR4) axis, to search for possible mechanisms behind insufficient cardiac repair in the first days post-AMI. **Methods.** Closed-chest reperfused AMI was performed by percutaneous balloon occlusion of the mid-left anterior descending (LAD) coronary artery for 90min, followed by reperfusion in pigs. Animals were randomized to receive either IP initiated by 3x5min cycles of re-occlusion/re-flow prior to AMI (n=6) or control AMI (n=12). Blood samples were collected at baseline, 3d post-AMI, and at 1-month follow-up to analyse chemokines and mobilized CD34+ cells. To investigate the effect of acute hypoxia, SDF-1α and matrix metalloproteinase (MMP)-2 *in vitro* were assessed, and a migration assay of CD34+ cells toward cardiomyocytes was performed. **Results.** Reperfused AMI induced significant mobilisation of CD34+ cells (baseline: 260±75 vs. 3d: 668±180; P<0.001) and secretion of MMP-2 (baseline: 291.83±53.40 vs. 3d: 369.64±72.89; P=0.011) into plasma, without affecting the SDF-1α concentration. IP led to the inhibition of MMP-2 (IP: 165.67±47.99 vs. AMI: 369.64±72.89; P=0.004) 3d post-AMI, accompanied by increased release of SDF-1α (baseline: 23.80±12.36 vs. 3d: 45.29±11.31; P=0.05) and CXCR4 (baseline: 0.59±0.16 vs. 3d: 2.06±1.42; P=0.034), with a parallel higher level of mobilisation of CD34+ cells (IP: 881±126 vs. AMI: 668±180; P=0.026), compared to non-conditioned AMI. *In vitro*, CD34+ cell migration toward cardiomyocytes was enhanced by SDF-1α, which was completely abolished by 90min hypoxia and co-incubation with MMP-2. **Conclusions.** Non-conditioned AMI induces MMP-2 release, hampering the ischemia-induced increase in SDF-1α and CXCR4 by cleaving the SDF-1α/CXCR4 axis, with diminished mobilization of the angiogenic CD34+ cells. IP might influence CD34+ cell mobilization via inhibition of MMP-2.

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Introduction

Heart regeneration after ischemic insult is still a matter of debate in spite of extensive research conducted in this field. One of the endogenous cardiac repair mechanisms is the mobilization of regenerative cells derived from bone marrow (BM), followed by migration and homing of the cells in the ischemic myocardial tissue[1]. Several factors have been identified that play a role in the mobilization of BM-origin stem and progenitor cells, and assist in migration and homing, such as chemotactic factors, complement fractions, cytokines, microRNAs or microvesicles. Among these substances, the axis of the stromal-derived factor-1 alpha [SDF-1α; chemokine receptor 12 (CXCL12)] and chemokine receptor type 4 (CXCR4) exerts the strongest chemoattractant stimulus for migration and homing of cells in the BM and tumors, but also in ischemic tissues, such in case of myocardial ischemia or ischemic stroke[2]. The local upregulation of SDF-1α attracts the cells covered with CXCR4 receptors towards the SDF-1α gradient, facilitating the migration of the cells into the target organ tissues.

Exploiting the beneficial effect of the SDF-1α/CXCR4 axis in cardiac repair has been performed by repeated injections of granulocyte-colony stimulating factor (G-CSF) applied in patients, which aims to release the stem and premature cells from BM and activate the cellular CXCR4 expression of the reparative cells by interrupting the BM-SDF-1α/CXCR4 axis[3]. However, despite the enhanced cell release and migration, and stimulation of the endogenous cardiac progenitor cells, the efficacy of clinical cardiac cell-based therapy in patients with recent acute myocardial infarction (AMI) led to ambiguous results[4], especially if the regenerative cell therapy was performed very early after ischemic injury[5].

Among several mechanisms explaining the cardiac regenerative processes, secretion of distinct chemokines, cytokines, and growth factors may play an important role; which factors are released after myocardial infarction[2]. Tang *et al* demonstrated upregulated SDF-1α expression in infarcted mouse myocardial tissue after implantation of mesenchymal stem cells induced with vascular endothelial growth factor (VEGF). This led to increased mobilisation of BM-derived stem cells[6]. Also, the upregulation of pro-inflammatory cytokines, such tumor necrosis factor (TNF)α[7] and interleukin (IL)-8[8], might initiate processes triggering increased cell trafficking, since myocardial infarction is associated with the inflammatory response[8]. In contrast, matrix metalloprotease (MMP)-2 cytokine is known to be the inhibitor of SDF-1α, implicating its inactivation[9]. Another mechanistic process is

cardioprotection, induced by either ischemic pre-, post- or remote conditioning, or by numerous cardioprotective substances, but their clinical importance is doubtful. Ischemic preconditioning (IP) has been shown to exhibit cardioprotective mechanisms, and stimulates the recruitment and homing of progenitor cells toward ischemic myocardium in early phases of cardioprotection in several animal models[7].

In our present experiment, we have investigated the mobilization of BM-origin CD34+ cells 3 days after reperfused acute myocardial infarction (AMI) in relation to the SDF-1α/CXCR4 axis. We measured the release of several cytokines, such as MMP-2, VEGF, fibroblast growth factor (FGF)-2, IL-8 and TNFα, to investigate and explain the possible mechanism behind insufficient cardiac repair in the first days post-AMI. In addition, the current study explored a possible counteracting effect of IP on cytokines, CD34+ cell release and MMP-2 expression.

Methods

Design of the porcine closed-chest reperfused acute myocardial infarction (AMI)

Randomly selected female domestic pigs (n=21; weight, 30–35kg) underwent percutaneous coronary intervention (PCI) under general anaesthesia in order to perform either ischemic preconditioning (group IP; n=6) or non-conditioned AMI (group control; n=12), with a block 1:2 randomisation, due to expected higher mortality in the control group. Animals in both groups underwent 90min percutaneous balloon occlusion of the left anterior descending (LAD) coronary artery at the origin of the first diagonal branch following reperfusion (balloon deflation). IP was initiated prior to 90min LAD occlusion by 3×5min repetitive cycles of artery re-occlusion and reperfusion. One pig in the IP and two animals in the control group died during the AMI intervention, all remaining animals (n=6 in IP and n=12 in control group) survived for 1 month after the experimental procedure (Figure 1).

Porcine closed-chest model of ischemia/reperfusion

All procedures were performed with the approval of the local Experimental Animal Care Committee (EK SOI/31/26-11/2014) of the University of Kaposvar, Hungary, conforming to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996). All animal experiments were conducted at the institute of diagnostic imaging and radiation oncology, University of Kaposvar.

Female domestic pigs received 12mg/kg ketamine, 1mg/kg xylazine and 0.04mg/kg atropine as anaesthesia. The anaesthesia was deepened via mask maintaining 1.5–2.5 vol % isofluran, 1.6–1.8 vol % O_2 and 0.5 vol % N_2O. In total, 200IU/kg of heparin was administered via the right femoral artery, and selective angiography of LAD arteries was performed prior to induction of myocardial ischemia (MI). MI was induced by 90min balloon occlusion (3.0mm ø, 15mm length, 5atm; Maverick, Boston Scientific, MA, USA) at the mid-part of the LAD artery following balloon deflation. The % O_2 saturation, blood pressure and electrocardiogram were continuously measured during the intervention.
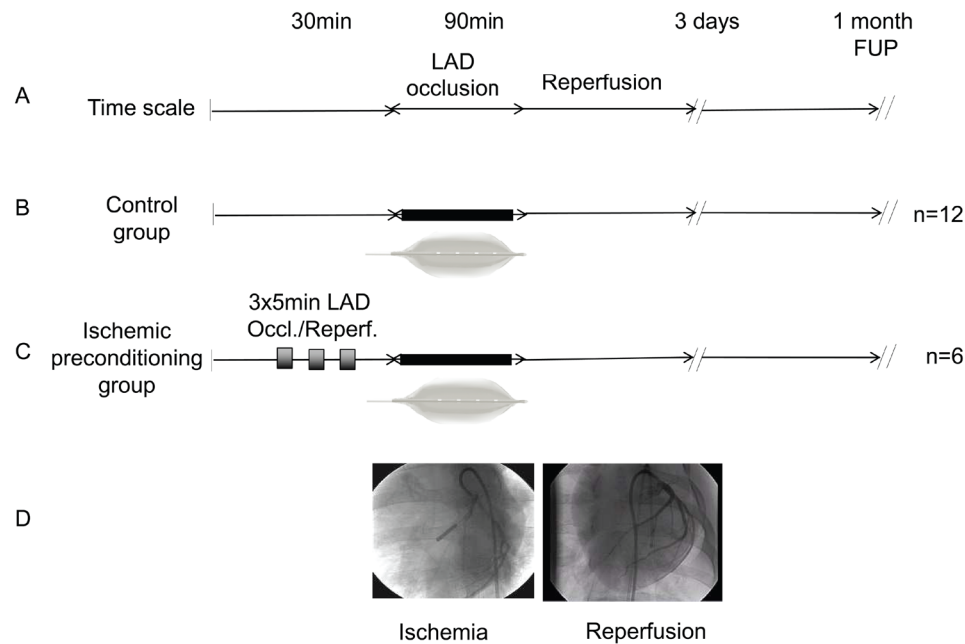
Figure 1. Design of the porcine closed-chest reperfused myocardial infarction experiments. (A) Time scale: 30min anaesthesia, followed by 90min occlusion of the mid left anterior descending coronary artery (LAD), followed by reperfusion. Follow-up (FUP) times 3 days and 1 month. **(B)** Control group (n=12). **(C)** Ischemic preconditioning group (n=6) was induced by 3x5min cycles of ischemia/reperfusion (balloon inflation/deflation) prior to 90min balloon occlusion of the LAD. **(D)** Angiographic pictures of the balloon occlusion of the LAD (left, left anterior oblique acquisition at 45°) and control angiography after restoration of the reperfusion (right, anteroposterior view).

Blood sampling

Blood samples were collected from the femoral vein for the detection of biological markers. Samples were centrifuged at 2000×g for 10min, and the plasma and serum samples were stored at -20°C until the analysis was performed. For fluorescent activated cell sorting (FACS) analysis, whole blood was collected into EDTA-treated tubes (BD Vacutainer®; Becton, Dickinson and Company, New Jersey, USA) at baseline, 3 days post MI and 1 month follow-up (FUP). All blood samples were processed within 6h.

Enzyme linked immunosorbent assay (ELISA)

Plasma level of stromal cell-derived factor-1 (porcine SDF-1α ELISA Kit; Neoscientific, Germany), chemokine (C-X-C motif) receptor 4 (pig CXCR4 ELISA Kit; Abbexa, UK), 72kDa isoform of matrix metalloproteinase-2 protein (porcine MMP-2 ELISA Kit; MyBioSource, CA, USA), fibroblast growth factor-2 (porcine FGF-2 ELISA Kit; Neoscientific, Germany), and vascular endothelial growth factor (porcine VEGF ELISA Kit; Neoscientific, Germany) were detected using commercial ELISA kits, according to the manufacturer's instructions. Tumor necrosis factor alpha (Porcine TNFα Quantikine ELISA Kit; R&D Systems, MN, USA), and interleukin-8 (pig IL-8 ELISA Kit; Abcam, UK) were detected from serum, according to the manufacturer's instructions.

Absorbance readings at wavelength 450nm were performed on the automated plate reader VIKTOR3 (Perkin Elmer, MA, USA),

and the resulting values were determined by interpolation from a standard curve. Measurements were performed in duplicates. Plasma or serum levels of markers were measured at baseline, 3d post MI and at 1 month FUP.

FACS analysis of CD34+ cells in peripheral blood

FACS analysis of whole blood samples was performed at baseline, 3d post MI and at 1 month FUP in order to address the kinetics of mobilized CD34+ cells *in vivo*. EDTA-treated venous blood samples (100μl) were labelled with PE-DY647-conjugated CD34+ antibody (monoclonal antibody; host/isotype: mouse/IgG1; cat# MA1-19770; Thermo Fisher Scientific, Waltham, MA, USA) or the corresponding isotype control (PE-conjugated mouse IgG1; cat# MA1-10415; Thermo Fisher Scientific, Waltham, MA) for 20min at room temperature. Anti-human CD34+ antibody was utilized due to lack of commercially available porcine-specific CD34+ marker (dilution: 5μl antibody/100μl whole porcine blood). Subsequently, erythrocyte cell lysis was performed, according to the manufacturer's protocol, using Dako-Uti Lyse™ (Dako, Agilent Technologies, Santa Clara, CA, USA) following fixation with PBS containing 1% paraformaldehyde. FACS analysis was performed on CyFlow® ML/space flow cytometer (Sysmex Partec, Görlitz, Germany) with acquired 100.000 events within the gated region of mononuclear cells of forward versus side scatter. Absolute counts of CD34+ cells were obtained by multiplying the ratio of the CD34+ cells obtained in the flow cytometry analysis and absolute count of leucocytes per 1μl of blood.

Isolation of human adult cardiac myocytes

Human adult cardiac myocytes (HACMs) were isolated from the left ventricular tissue obtained from the hearts of patients undergoing heart transplantation. Mechanical dissociation of the tissue and separation of the cardiomyocytes from fibroblasts detached to Petri-dish surface was performed, as described previously[10]. All tissue donors gave their informed written consent to the study. The study was approved by the local ethical committee (Medical University of Vienna, Austria; EK 151/2008) and complies with the Declaration of Helsinki.

Human cord blood CD34 positive cells (CD34+ cells) were purchased from StemCell Technologies Company (Grenoble, France).

Cell migration assay

Migration of CD34+ cells was monitored by commercially available Roche xCELLigence System (Acea Bioscience, CA, USA), according to the manufacturer's instructions. Briefly, 160µl suspension of HACM cells (conc. 10.000 cells/well) was resuspended in M199 cardiac cell culture media (Sigma-Aldrich, Vienna, Austria) containing 20% FBS and 1% Pen/Strep solution (Gibco™, Thermo Fischer Scientific, MA, USA). Cell suspension was transferred to the lower chamber of the CIM-Plate with integrated gold microelectrode sensors (Figure 2).

HACMs were incubated under normoxic conditions with increasing doses (0.005, 0.5 and 5.0ng/mL) of SDF-1α (Sigma-Aldrich,

Vienna, Austria) to evaluate the maximal SDF-1α chemoattractant effect on CD34+ cells towards HCAMs.

In order to analyse the effect of MMP-2 and hypoxia on the mobilisation of CD34+ cells toward HCAMs, HCAMs were incubated with SDF-1α (with the elaborated maximal effect of 5.0ng/mL) either with co-incubation with MMP-2 (5.0ng/mL; Sigma-Aldrich, Vienna, Austria) under normoxia, or under 90min hypoxic conditions.

Hypoxia (90min, 37°C, 1% O_2) was induced in HCAM cell culture on the CIM-Plate by sealing the cell culture plate in an airtight plastic bag (Microbiology Anaerocult® IS Bag; Merck Millipore, Vienna, Austria) containing a dry anaerobic indicator strip.

In total, 25µl serum-free medium (M199 containing 0.1% FBS) was added to the upper chamber 30min after treatments with the various substances, and the chambers were combined for background measurements. Subsequently, CD34+ cells (100.000 cells/well) were transferred to the upper chamber of the CIM-Plate with polyethylene terephthalate membrane (PET) with 8µm pore diameter and measurements were repeated. Migrated cells translocated through the PET-membrane and changed the impedance signal captured by sensors in the lower chamber. The background was subtracted from all results and each experiment was repeated three times (Figure 2).
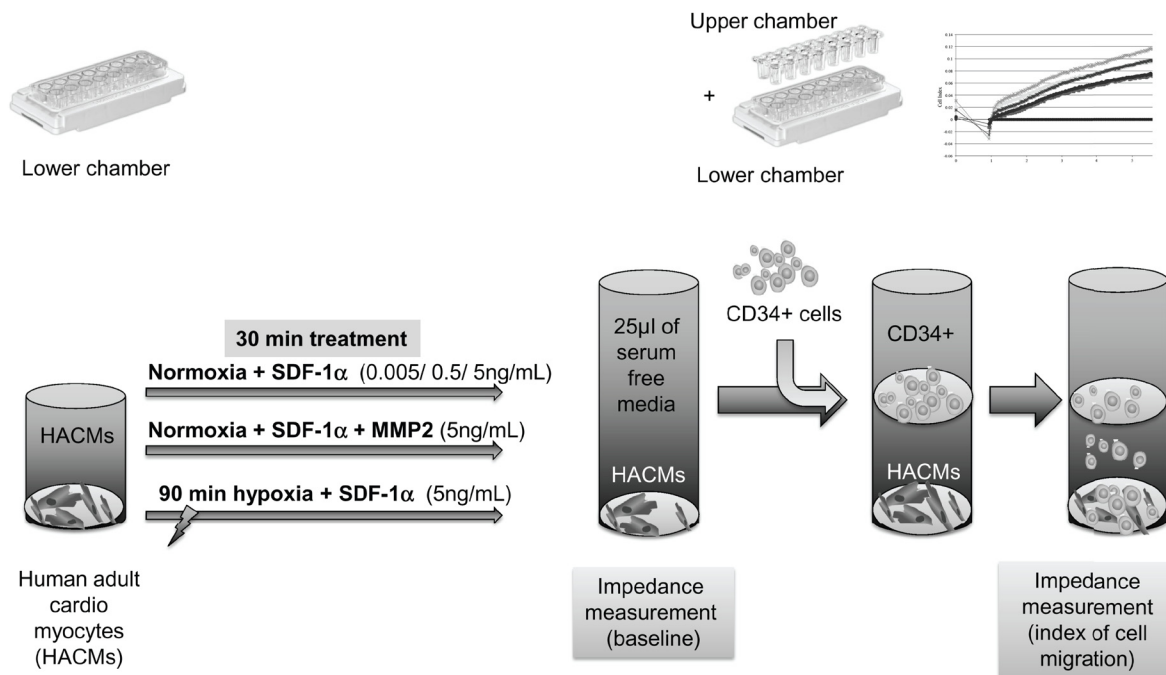
Figure 2. Design of the cell migration assay. Human adult cardiac myocytes (HACMs) were incubated in the lower chamber. After 30min incubation with different treatments: either adding stromal-derived factor-1-alpha (SDF-1α) in increasing concentrations, or adding the highest concentration of SDF-1α and matrix-metalloprotease-2 (MMP-2), or the cells were kept under hypoxia for 90min, followed by the addition of SDF-1α. Lower and upper chamber were then combined, and after adding serum-free media to the upper chamber, baseline impedance measurements were performed, followed by adding CD34+ cells. Impedance measurements were performed to quantify the CD34+ cell migration towards HAMCs.

Statistical analysis

Continuous parameters were expressed as means ± standard deviation. The effects between the groups and within the groups (baseline vs. 3d post-AMI) were analyzed by two-way analysis of variance (ANOVA) with repeated measures model with Bonferroni correction. The mean differences between the groups were detected by independent Student's t-test. Differences were considered statistically significant at $P<0.05$. Statistical analyses were performed with SPSS software (version 17.0; Macintosh; SPSS IBM).

Results

### AMI-induced cytokine release and CD34+ mobilization

As shown by the control group, reperfused AMI induced a significant increase in the circulating level of CXCR4 (baseline: 0.47 ± 0.22 vs. 3 days post-AMI: 1.15 ± 0.95ng/ml; $P=0.034$) with parallel significant mobilization of CD34+ cells (baseline: 260 ± 75 vs. 3 days post-AMI: 668 ± 180cells/µl; $P<0.001$), but without an increase in SDF-1α at 3 days post-AMI (baseline: 32.02 ± 24.35 vs. 3 days post-AMI: 26.97 ± 15.43pg/ml; $P=0.41$). The

circulating levels of the angiogenic cytokines (FGF-2, VEGF, IL-8 and TNFα) were not changed at 3-days post-AMI. However, the level of MMP-2 was increased significantly (baseline: 291.83 ± 53.40 vs. 3 days post-AMI: 369.64 ± 72.88pg/ml; $P=0.011$), which might explain the cleaved SDF-1α/CXCR4 axis (Figure 3).

IP induces CD34+ cell mobilisation via CXCR4/SDF-1α axis

IP led to the significantly higher stimulation of SDF-1α chemokine release with its putative receptor, CXCR4, into circulation, accompanied by downregulation of MMP-2. The number of CD34+ cells significantly increased as compared to the animals in the control group (non-conditioned AMI).

The plasma level of SDF-1α significantly increased 3 days post infarction in the IP group as compared to control AMI group (IP: 45.29 ± 11.31 vs. control: 27.00 ± 15.43pg/ml; $P=0.037$), with normalization at the 1-month FUP (IP: 28.87 ± 3.81 vs. control: 26.91 ± 20.24pg/ml; $P=0.85$) (Figure 3A). Enhanced SDF-1α secretion was accompanied by significant increase of its soluble CXCR4 receptor after 3 days post-AMI (baseline: 0.59 ± 0.16 vs. 3
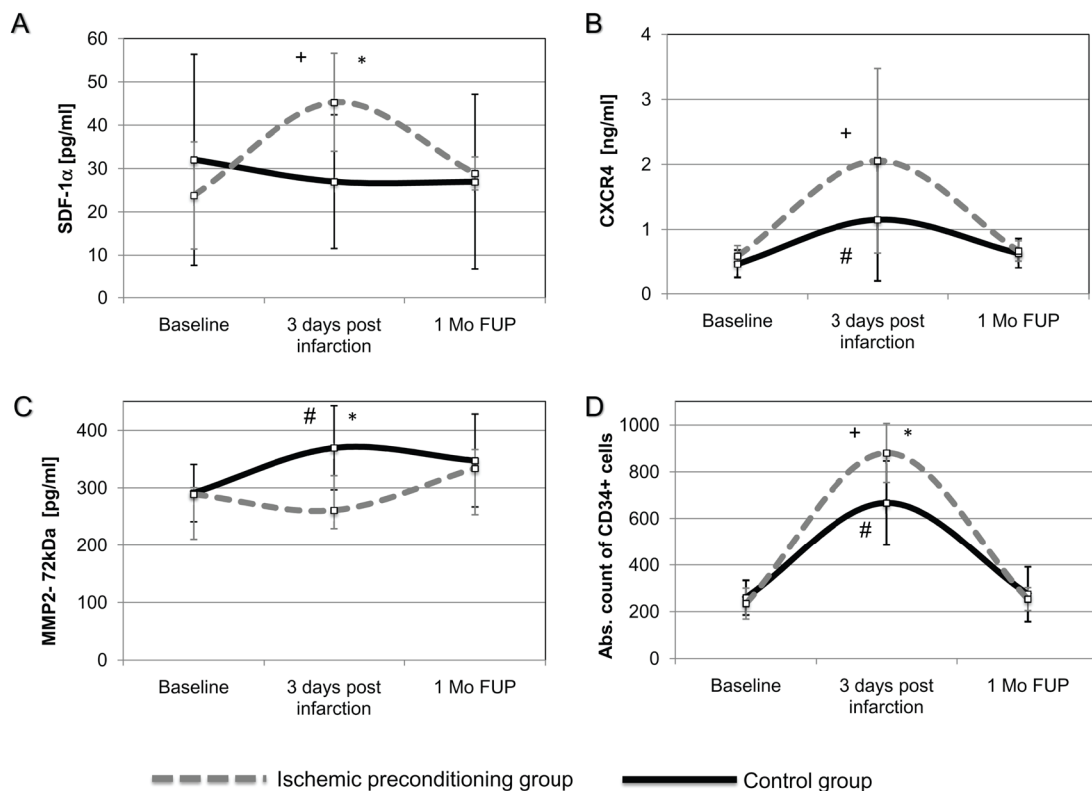
Figure 3. Time-dependent changes of plasma SDF-1α, CXCR4 and MMP-2 (72kDa), and CD34+ cell count in IPC and AMI group. Plasma concentrations of circulating (**A**) SDF-1α; (**B**) CXCR4; (**C**) MMP-2 (72kDa) protein isoform measured by porcine-specific ELISAs. (**D**) Absolute count of circulating CD34+ cells determined by FACS analysis. Concentrations are expressed as mean± standard deviation. *$P<0.05$ between the IP and control group; +$P<0.05$ between baseline and 3 day values within the IP group; #$P<0.05$ between baseline and 3 day values within the control (non-conditioned AMI) group. SDF-1α, stromal-derived factor-1 alpha; CXCR4, C-X-C motif chemokine receptor 4; MMP-2 (72kDa), matrix metalloproteinase-2, 72kDa isoform; IPC, ischemic preconditioning; AMI, acute myocardial infarction; 1 Mo FUP, 1 month follow-up.

days post-AMI: 2.06±1.42ng/ml; P=0.034); however, this did not reach statistical significance between the groups (IP: 2.06 ±1.42 vs. control: 1.15 ±0.95ng/ml; P=0.79) (Figure 3B).

IP significantly downregulated the secretion of MMP-2 into plasma at 3 days FUP as compared to the control AMI group (IP: 165.67±47.99 vs. control: 369.64±72.89pg/ml; P=0.004), which returned to the baseline level at the 1-month FUP control (IP: 334.00±93.10 vs. control: 347.58±80.47pg/ml; P=0.074) (Figure 3C).

FACS analysis was performed to reflect the impact of chemoattractant release on cell migration. We observed a significant parallel increase of mobilized CD34+ cells in both non-conditioned AMI and IP groups 3 days post infarction (IP: 881±126 vs. control: 668±180cells/µl; P=0.026, returning to the baseline level after 1 month FUP (IP: 255±50 vs. control: 275±118cells/µl; P=0.85) (Figure 3D).

IP-induced changes in the levels of circulating cytokines

A trend towards increase of IL-8 was observed in the IP group at 3 days and 1 month post infarction (IP: 100.18±60.42 vs. control:

49.52±16.68pg/ml; P=0.055) at day 3 and (IP: 59.32±32.88 vs. control: 25.19±5.76pg/ml; P=0.059) at 1 month (Figure 4A).

The concentration of VEGF in plasma significantly increased in IP group at day 3 post infarction as compared with controls (IP: 41.35±5.12 vs. control: 29.01± 10.18pg/ml; P=0.021) (Figure 4B).

IP did not affect the changes in serum concentrations of TNFα as compared to the control group (Figure 4C).

The plasma level of FGF-2 was not significantly changed at day 3 by IP as compared to the control group (IP: 1.90±0.41 vs. control: 2.22±0.88ng/ml; P=0.45) (Figure 4D).

Inhibition of SDF-1α-induced CD34+ cell migration by MMP-2 *in vitro*

In order to prove the oppositional effect of MMP-2 on CD34+ cell mobilisation, we added MMP-2 to cultured HACMs, stimulated with SDF-1α, and quantified the CD34+ cell migration towards the HACMs (Figure 5).

SDF-1α treatment stimulated the migration of CD34+ cells toward HACMs under normoxic conditions in a dose-dependent manner.
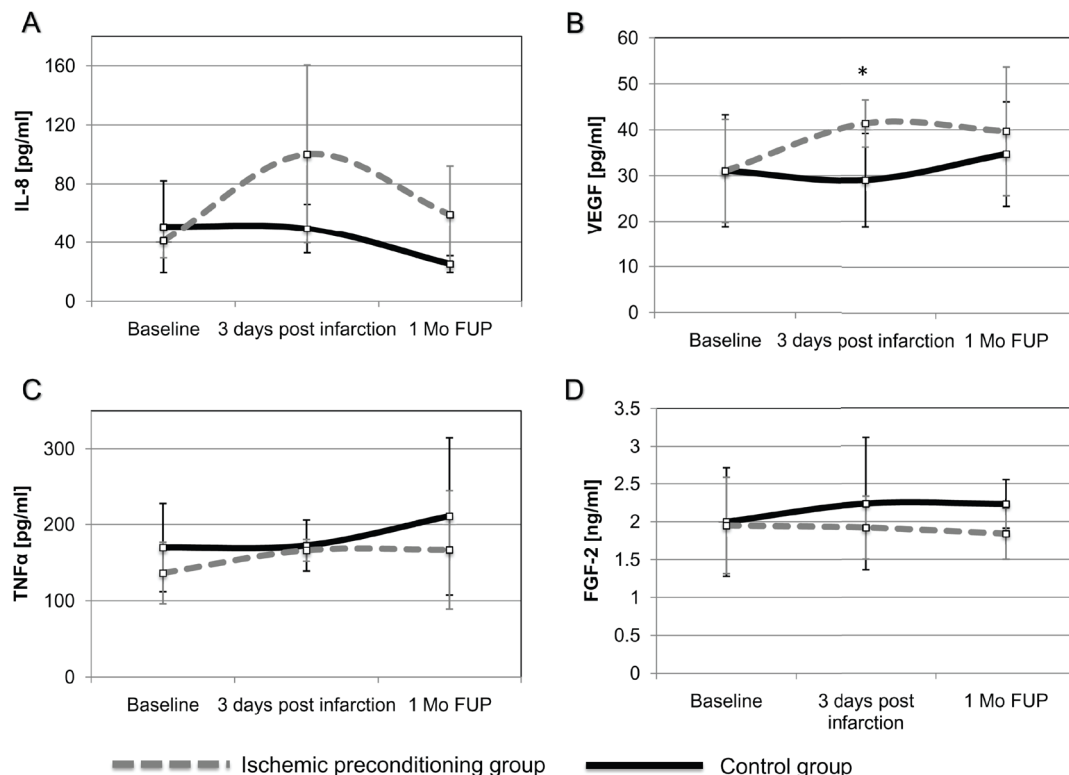
Figure 4. Time-dependent changes of plasma FGF-2, VEGF, serum IL-8 and TNFα analysed in IPC and AMI group. Plasma concentrations of circulating (**A**) IL-8, (**B**) VEGF, (**C**) TNFα, (**D**) FGF-2 Concentrations are expressed as mean± standard deviation. *P<0.05 between the IP and control group. FGF-2, fibroblast growth factor-2; VEGF, vascular endothelial growth factor; IL-8, interleukin-8; TNFα, tumor necrosis factor alpha; IPC, ischemic preconditioning; AMI, acute myocardial infarction; 1 Mo FUP, 1 month follow-up.

CD34+ cell migration (6h)

Upper chamber	CD34+ cells	-	+	+	+	+	+	+
	Medium 0.1% serum	+	+	+	+	+	+	+
Lower chamber	Cardiac myocytes	-	+	+	+	+	+	+
	Hypoxy 90min	-	-	-	-	-	+	-
	SDF-1α	-	-	5 ng/ml	0.5 ng/ml	0.005 ng/ml	5 ng/ml	5 ng/ml
	MMP-2	-	-	-	-	-	-	5 ng/ml
	Medium 20% serum	+	+	+	+	+	+	+
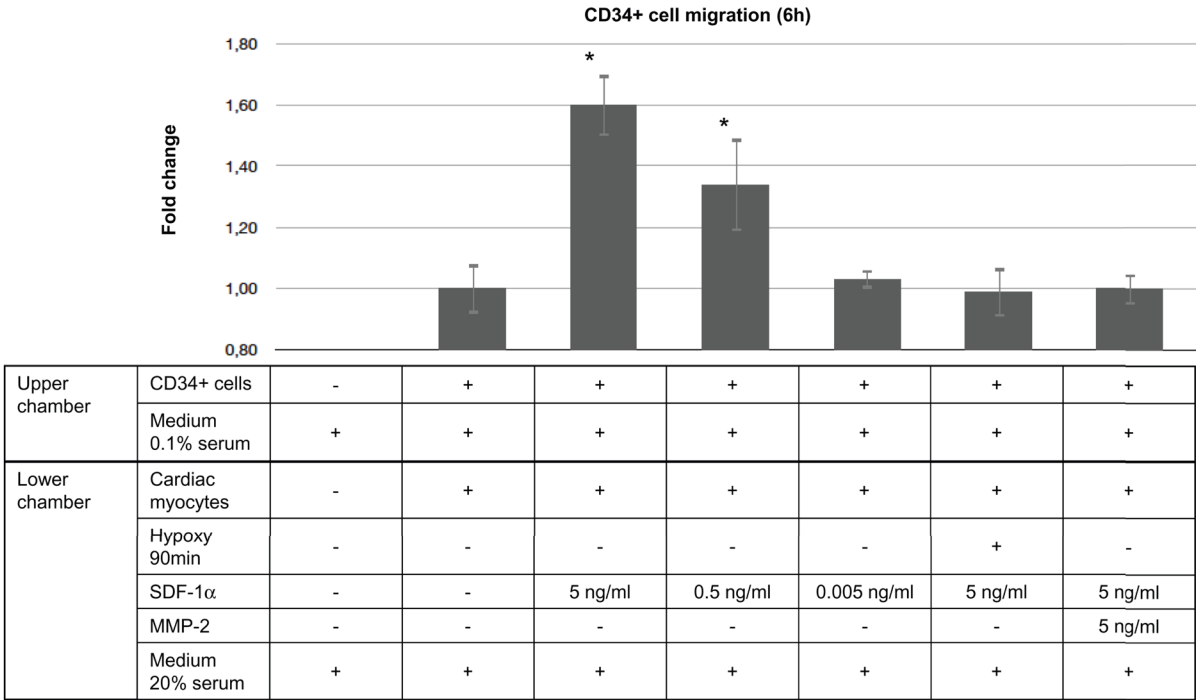
Figure 5. CD34+ cell migration toward human adult cardiomyocytes culture under different conditions. Migration was quantified as fold change impedance compared to the baseline conditions. Adding of SDF-1α in different concentrations induced chemotaxis of the CD34+ cells in a dose-dependent manner. A total of 90min hypoxia followed by a change of the medium eliminated the chemotactic effect of SDF-1α, and blocked CD34+ cell migration. This migration effect of SDF-1α was similarly eliminated if MMP-2 was added to the normoxic cell culture. Depicted results express impedance values measured at 6h post-treatment. Background results were subtracted from each impedance measurement. Parameters are expressed as mean ± standard deviation. Each experiment was repeated three times. *P<0.001 compared to the baseline normalized value. SDF-1α, stromal derived factor-1α; MMP-2, matrix metalloproteinase-2.

The maximal chemotactic effect (1.6± 0.11 fold change; P<0.001) was achieved by adding 5ng/ml concentration of SDF-1α, while 0.5ng/mL and 0.05ng/mL SDF-1α resulted in a migration rate of 1.35± 0.18 fold (P<0.001) and 1.08±0.02 fold change (P=0.43), respectively, compared to the control HACMs and CD34+ cells culture without SDF-1α.

Co-incubation of HACMs with MMP-2 under normoxic conditions completely eliminated the SDF-1α chemotactic effect to CD34+ cell migration towards the cardiomyocytes (0.98±0.1 fold change; P=0.71).

Interestingly, incubation of the cardiomyocytes under 90min hypoxic conditions inhibited migration of CD34+ cells, even if the highest effective dose of SDF-1α (5ng/ml) was added to the cell culture as a chemoattractant (1.00±0.04 fold change; P=0.75).

Discussion

Here, we demonstrate that 1) myocardial ischemia triggers the release of circulating MMP-2, which inhibits SDF-1α and CXCR4 release; 2) SDF-1-induced migration of CD34+ cells towards cardiomyocytes was inhibited by MMP-2 *in vitro*; 3) IP inhibited MMP-2 release, thereby increasing both SDF-1α and CXCR4 levels, resulting in a higher level of CD34+ cell mobilization 3 days post ischemic injury in *in vivo* condition; 4) IP induced VEGF secretion in the second window of cardioprotection.

AMI leads to MMP-2 release

Reperfused AMI led to an increase in CXCR4, but not SDF-1α, at 3 days post-infarction, with moderate enhancement of circulating CD34+ counts. Similarly, AMI caused significant elevation of MMP-2, produced by macrophages in case of acute tissue injury. MMP-2 disrupts the SDF-1α/CXCR4 axis, by cleaving SDF-1α to N-terminally truncated SDF-1[11]. This form of SDF-1 is unable to

trigger CXCR4 signalling and prevent the chemoattractant function of SDF-1α/CXCR4 in human progenitor cells[12]. Since the increased upregulation of MMP-2 post-AMI may inhibit retention of hematopoietic stem cells in the ischemic injury site, targeted modulation of MMP-2 expression has potential to improve outcome of regenerative therapies[9].

IP induces CD34+ mobilisation by SDF-1α/CXCR4 axis *in vivo*

Our previous study demonstrated that IP in early phase post-infarction (early window of protection, 2h after reperfusion start) induced mobilization of BM-derived haematopoietic (HSCs) and mesenchymal stem cells (MSCs) involving the release of distinct cytokines[7]. In our present work, we analyzed the effect of IP on the mobilization of CD34+ regenerative cells and measured the cytokine release (MMP-2, VEGF, FGF-2, IL-8 and TNFα) in the late (second) window of protection.

In contrast with the non-conditioned AMI group, we observed significantly elevated SDF-1α plasma level in the IP group at 3 days post infarction, as compared to the AMI group. This confirmed our earlier assumptions that SDF-1α is released in a later time window after IP[7]. Previous *in vitro* and *in vivo* experiments have shown an increased cell migration ability responding to treatment with SDF-1α[13] or increased mobilisation of BM-derived cells toward injured tissue after SDF-1α overexpression[6,14]. The putative receptor for SDF-1α chemokine is CXCR4, which is expressed also in mouse cardiomyocytes[14] and mobilises mesenchymal stem cells in the ST-segment elevation of myocardial infarction patients[2]. The elevated level of SDF-1α was paralleled by an increased number of circulating CD34+ cells. This suggests that IP stimulates CD34+ cell migration by SDF-1α/CXCR4 upregulation within the first days after AMI.

The increased concentration of MMP-2 (72kDa) at 3 days post-infarction was completely abolished by IP, which might be an additional beneficial effect of IP in a translational large animal model, and is similar to mice experiments[15]. IP has shown cardioprotective effects against ischemia/reperfusion injury in accepted experimental models. Induction of IP in a mouse model led to improvement of cardiac function and increasing cell survival, accompanied by release of BM- derived cells[16]. Accordingly, our previous[4] and present study suggest that IP stimulates endogenous mechanisms, promoting the recruitment of CD34+ cells in both early and late windows of cardioprotection.

MMP-2 and hypoxia abolish SDF-1α-induced CD34+ cell mobilisation *in vitro*

In order to prove the direct confounding effects of MMP-2 on SDF-1α/CXCR4, we have performed *in vitro* experiments, and observed that MMP-2 completely inhibited SDF-1α -induced CD34+ cell mobilization.

Interestingly, our experiments also revealed that 90min hypoxia abolishes the SDF-1α chemotactic effect *in vitro*. By contrast, it has been reported that hypoxia inducible factor 2, which is released in hypoxia, binds to the promotor sequence of CXCR4, the SDF-1α putative receptor, and activates the migratory activity of

the endothelial progenitor cells[17]. We cannot completely explain our findings, but we assume that the release of hypoxia-triggered factors, such as MMP-2, may locally inhibit the migratory capacity of the regenerative cells. This is also in concordance with the findings in humans; early administration of regenerative cells has debatable effects on myocardial regeneration[18].

Effect of AMI and IP on FGF-2, VEGF, IL-8 and TNFα release

Non-conditioned AMI did not influence the release of circulating cytokine FGF-2, VEGF, IL-8 and TNFα. In contrast, IP induced a marked release of circulating VEGF and a trend towards increase in IL-8 3 days post-AMI, indicating the stimulation of additional pro-migratory cytokines by IP for enhanced cardioprotection. IL-8 is a pro-inflammatory C-X-C chemokine that is also involved in activation of pro-angiogenic processes and re-introduction of progenitor cells into the circulation. The study of Schomig *et al.* demonstrated significantly increased IL-8 level in AMI patients as compared to patients diagnosed with stable angina[8]. In our study, we observed a trend toward increased release of IL-8 in the clinically relevant porcine reperfused "STEMI" model. The levels of CXCR4 increased both in controls (with AMI) and IP groups, with a trend towards higher increase in the IP group 3-day post AMI. The differences between our and other studies might be explained by the pre- and peri-AMI medication of patients with standard care that may contribute to changes in plasma levels of cytokines in AMI patients[8]. Results of plasma cytokine levels would be more informative if it measured more often, in an extended time window. The area under the curve (AUC) calculation of the cytokine release data might have delivered additional results. However, for a simple blood sampling, the animals must have been fully anaesthesized, which procedure signifies an additional stress for the animals with recent AMI with predicted higher mortality.

In our previous experiments, IP induced the release of VEGF plasma levels immediately after myocardial infarction (first window of protection)[7]. In the present experiment, VEGF was still increased 3 days post AMI in the IP group (second window of protection) as compared to the control AMI group. Similarly to our study, Kamota *et al.* showed an amplified secretion of VEGF and SDF-1α up until 6 hours post infarction in a mouse model of IP[16]. Tang *et al.* also reported induced mobilisation of stem cells by VEGF/ SDF-1α trafficking in a rat model[6].

FGF-2 is an important chemotactic factor and it is also a prominent cardioprotective and angiogenic agent[19]. Since FGF-2 was not significantly induced by IP in our experiment, we assume that this protein did not participate in mechanisms of IP-elicited late window of cardioprotection.

Acute phase of AMI after IP is characterized by an increased level of TNFα triggering a release of additional cytokines, such as IL-6, IL-8, and cell adhesion molecules. Our previous data demonstrated that IP resulted in elevated levels of TNFα in serum with concomitant IL-8 increase immediately after reperfusion induction[7]. A later time window after AMI revealed heterogeneous results. TNFα remained moderately increased 3 days post infarction with continuous moderate increase after 1 month FUP

in both groups, most probably due to developing chronic phase of myocardial infarction. Interestingly, IP induced a trend towards enhanced IL-8 release, which is a potent progenitor cell mobilisation enhancer responding to ischemia, although it is also associated with pro-inflammatory processes[8,9].

In conclusion, the present study revealed that AMI induces MMP-2 release, which hampered the ischemia-induced increase in SDF-1α and CXCR4 by cleaving the SDF-1α/CXCR4 axis. This led to diminished mobilization of the angiogenic CD34+ cells. IP induced CD34+ cell mobilization in the late phase (second window), thereby also increasing circulating SDF-1α and CXCR4, parallel with enhanced VEGF secretion. One mechanism of this beneficial effect of IP might be the inhibition of AMI-induced MMP2-release. *In vitro* migration assay confirmed the anti-migratory effect of MMP-2 and the direct negative association of MMP-2 and SDF-1α-induced cell migration. Accordingly, our experiment might explain the inhibited homing of mobilized or transplanted cells in the ischemic myocardium resulting in decreased efficacy of cell-based therapies early after AMI.

Limitations
Even though we demonstrate IP-induced mobilisation of CD34+ cells in a large animal model of reperfused AMI, the clinical relevance of IP remains uncertain. We have concentrated on mechanisms involved in cell mobilisation in terms of chemokine and cytokine secretion.

We revealed one additional possible beneficial mechanism of IP, namely the inhibition of MMP-2 release with consequent higher mobilization of CD34+ cells, which was confirmed in our *in vitro* experiment. However, a direct association between IP - MMP-2 - CD34+ axis had to be confirmed *in vivo*, by blocking MMP2 in animals subjected to AMI and IP. We have not measured myocardial MMP-2 level, which analysis would require harvesting of the animals maximal 72h post IP-AMI (second window of protection), and our animals survived 1-month follow-up.

An important limitation is the utilisation of human CD34+ FACS antibody due to lack of commercially available porcine products. However, the number of mobilized CD34+ cells correspond with the available mobilized cell numbers published several times[2,16,20];

bearing in mind, that the normal count of white blood cells of pigs is 12–20 thousand cells/μl blood.

We have chosen female pigs for the experiments because of a clear gender differences observed in female and male rodents, rabbits, dogs and pigs[21]; the incidence of cardiogenic shock and life-threatening arrhythmias were more frequent in male than female pigs by using the closed-chest reperfused AMI model[22].

We are aware, that serial blood sampling would have given more information, e.g. the evident changes in mobilisation of bone marrow-derived cells following myocardial infarction occur at day 3, 7 and 14 post-ischemia[23]. However, we have focused on the second window of protection, which ends at day 3.

Data availability
Dataset 1. Raw data for XCelligence measurements of cell migration assay (DOI: 10.5256/f1000research.9957.d142079[24]).

Dataset 2. Raw data obtained from ELISA and FACS analyses (DOI: 10.5256/f1000research.9957.d142080[25]).

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Data Source

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Data Source

Open Peer Review

Current Referee Status: ✔ ?

? **Amadou K.S. Camara**

Department of Anesthesiology, Medical College of Wisconsin (MCW), Milwaukee, WI, USA

General comments

The manuscript "Inhibition of CD34+ cell migration by matrix metalloproteinase-2 during acute myocardial ischemia, counteracted by ischemic preconditioning" describes a phenomenon, preconditioning, that has been demonstrated to be cardioprotective against ischemic insult. The authors elegantly showed that the preconditioning that leads to protection involves the activation of CD34+ cell migration to the injured site for tissue remodeling, and that inhibition of MMP-2 is a potential target of the preconditioning protection. The details of the experimental protocols and procedures are adequate and the number of animals per treatment are statistically appropriate. The manuscript and the study design have some concerns that warrant the attention of the authors.

Specific Comments

Introduction
The Introduction lacks a compelling rationale for why the in vivo studies were conducted in female pigs.

The Introduction section does not make a distinction between early and delayed or second window cytoprotection conferred by preconditioning, and which of the two is being targeted in the current study. The concept of second window protection was discussed later in the manuscript; however, the reader should be provided this information earlier to orient and set the stage for an overview of the work.

The last sentence in the Introduction "In addition, the current study explored a possible counteracting effect of IP on cytokines, CD34+ cell release and MMP-2 expression." What does the "counteracting effect" mean in this case? Doesn't this imply that IP diminishes the release of CD34+ cells, which are the necessary responses to protect against AMI during IP? Clarify or rephrase this sentence.

Materials
Design of the porcine closed-chest reperfused acute myocardial infarction (AMI)
The in vivo model lacks a sham group. This group should provide the proper basal level of the variables being monitored for the entire duration of the experimental protocol. The results shown in Figure 3 (3D) clearly attests and supports the inclusion of a sham group in this study.

Isolation of human adult cardiomyocytes

Were the cells obtained from male or female human subjects? If they are from male subjects, would the rationale for focusing on female pigs hold in this case? Or, does it not matter in this case?

The purpose of using human cord blood CD34+ cells is not clear.

Cell migration assay
Figure 2, hypoxia treatment should have been present in all the various concentrations of SDF-1a and in its absence? This information would strengthen the connection between SDF-1a and the migratory response to CD34+ cells during hypoxia. How would one conduct an IP experiment in this case to demonstrate the restitution of the CD34+ migratory response during hypoxia in the in vitro model?

The legend for Figure 2 (minor) "Lower and upper case chamber were then combined…" This should read, "Lower and upper case chambers were then combined…"

Results
AMI-induced cytokine release and CD34+ mobilization
"The circulating levels of the cytokines (FGF-2, VEGF, IL-8 and TNFa) were not changed at 3 days…" Where is the Figure/result that refer to this information? If there is none, please note in parenthesis (data not shown or displayed). What was the condition/treatment in this case?

Based on the initial discussion of the results, the appearance of Figure 3B and Figure 3A should be switched.

Cell migration
Why was 90 min hypoxia used?

Discussion
There seems to be a contradiction in the findings regarding the secretion of VEGF. As noted above in Results, there was no change in the levels of the cytokines (FGF-2, VEGF, IL-8 and TNFa) at day 3. In the Discussion, item 4, it notes "IP induced VEGF secretion in in the second window of cardioprotection." Please clarify the discrepancy.

Effect of AMI and IP PGF-2, VEGF, IL-8 and TNFa release
The comparison being made in this section does not seem to account for the fact that the data were obtained from two experimental models and that the IL-8 was from pigs and not human cardiomyocytes. The conclusion could be bolstered by some of the control studies suggested.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 25 Jan 2017
Dominika Lukovic, Medical University of Vienna, Austria

Dear Prof. Amadou K.S. Camara,

We greatly appreciate that you spent a substantial amount of time looking at our paper entitled "

Inhibition of CD34+ cell migration by matrix metalloproteinase-2 during acute myocardial ischemia, counteracted by ischemic preconditioning ".

General comments: The manuscript "Inhibition of CD34+ cell migration by matrix metalloproteinase-2 during acute myocardial ischemia, counteracted by ischemic preconditioning" describes a phenomenon, preconditioning, that has been demonstrated to be cardioprotective against ischemic insult. The authors elegantly showed that the preconditioning that leads to protection involves the activation of CD34+ cell migration to the injured site for tissue remodeling, and that inhibition of MMP-2 is a potential target of the preconditioning protection. The details of the experimental protocols and procedures are adequate and the number of animals per treatment are statistically appropriate. The manuscript and the study design have some concerns that warrant the attention of the authors.

Specific comments:
Introduction
1A. Comment: The Introduction lacks a compelling rationale for why the in vivo studies were conducted in female pigs.

Answer: Thank you for the comment. We agree with the reviewer in this aspect. Accordingly, we have corrected the Introduction section as follows (page 2):

In our present experiment, we have investigated the mobilization of BM-origin CD34+ cells 3 days after reperfused AMI in relation to the SDF-1α/CXCR4 axis in a clinically relevant in vivo pig model. We conducted the experiments in domestic female pigs, since the female pigs tolerate the ischemic burden better regarding mortality as compared to male pigs[22] (page 2).

1B. Comment: The Introduction section does not make a distinction between early and delayed or second window cytoprotection conferred by preconditioning, and which of the two is being targeted in the current study. The concept of second window protection was discussed later in the manuscript; however, the reader should be provided this information earlier to orient and set the stage for an overview of the work.

Answer: We fully agree with this comment of the reviewer. We added the relevant information in the Introduction section as follows (page 2):

Ischemic preconditioning-induced cardioprotection possesses bi-phasic effect by initiation of protective mechanisms in the early phase appearing within the first 3 hours following myocardial insult[10] and in the late phase, which re-appear 24 hours and lasts for 72 hours[16] (Kamota et.al). In our study, we focused on the late window of cardioprotection in relation with stem cell mobilization and cytokine releases.

1C. Comment: The last sentence in the Introduction "In addition, the current study explored a possible counteracting effect of IP on cytokines, CD34+ cell release and MMP-2 expression." What does the "counteracting effect" mean in this case? Doesn't this imply that IP diminishes the release of CD34+ cells, which are the necessary responses to

protect against AMI during IP? Clarify or rephrase this sentence.

Answer: Thank you for the comment. Indeed, we have rephrased the statement in the section Introduction (page 2):

In addition, the current study explored a possible additional benefits elicited by IP that involve release of cytokines and CD34+ cells, and MMP-2 expression.

Materials
2A. Comment: Design of the porcine closed-chest reperfused acute myocardial infarction (AMI). The in vivo model lacks a sham group. This group should provide the proper basal level of the variables being monitored for the entire duration of the experimental protocol. The results shown in Figure 3 (3D) clearly attests and supports the inclusion of a sham group in this study.

Answer: Thank you for the important comment. We agree, that inclusion of a sham-operated control group would promote better comparison of the results. However, we decided to not supplement the study groups with control group of animals without infarction with expected constant baseline level of biomarkers. Indeed, we intended to apply the "reduction" principle of the 3R (reduction, refinement and replacement) concept regarding the in vivo experiments.
We have added this comment in the Limitation (page 13).

2B. Comment: Isolation of human adult cardiomyocytes. Were the cells obtained from male or female human subjects? If they are from male subjects, would the rationale for focusing on female pigs hold in this case? Or, does it not matter in this case?

Answer: We thank the reviewer to highlight this issue. Isolation of human cardiomyocytes was performed from samples obtained from both female and male patients. Since the cells were utilized for *in vitro* experimental evaluation, the "gender" of the cells in the cell culture was not relevant for the in vivo experiments.
We have added this comment in the Limitation (page 10) as follows:

Isolation of human cardiomyocytes was performed from samples obtained from both female and male patients. Since the cells were utilized for in vitro experimental evaluation, the "gender" of the cells in the cell culture was not relevant for the in vivo experiments.

2C. Comment: The purpose of using human cord blood CD34+ cells is not clear.

Answer: We appreciate the reviewer's opinion. We clearly noted the purpose of using human cord blood CD34+ cell in Method section (page 4):

Human cord blood CD34 positive cells (CD34+ cells) were purchased from StemCell Technologies Company (Grenoble, France). The cells were used in *in vitro* cell migration assay to assess their migratory capacity toward HACMs. Since porcine CD34+ cells are not commercially available, we used human cardiomyocytes and human cord blood CD34+ cells.

2.D Comment: Cell migration assay
Figure 2, hypoxia treatment should have been present in all the various concentrations of SDF-1a and in its absence? This information would strengthen the connection between SDF-1a and the migratory response to CD34+ cells during hypoxia. How would one conduct an IP experiment in this case to demonstrate the restitution of the CD34+ migratory response during hypoxia in the in vitro model?

Answer: Thank you for the valuable comment. SDF-1a treatment of the HACMs was conducted under normoxic conditions, since we wanted to assess the maximal mobilization effect of SDF-1a. We agree with the Reviewer that parallel measurements under hypoxic conditions might better explain the connection between SDF-1a and the migratory response.
Ischemia and reperfusion stimuli of IP can be simulated *in vitro* by incubation of HACMs with alternation of normoxic and hypoxic conditioning cycles. This method was developed by Martou *et al.*

Martou G, O'Blenes CA, Huang N, McAllister SE, Neligan PC, Ashrafpour H, Pang CY, Lipa JE (2006) Development of an in vitro model for study of the efficacy of ischemic preconditioning in human skeletal muscle against ischemia-reperfusion injury. Journal of applied physiology 101: 1335-42

Results
3A. Comment: AMI-induced cytokine release and CD34+ mobilization
"The circulating levels of the cytokines (FGF-2, VEGF, IL-8 and TNFa) were not changed at 3 days…" Where is the Figure/result that refer to this information? If there is none, please note in parenthesis (data not shown or displayed). What was the condition/treatment in this case?

Answer: Thank you for the remark. We have added this comment, and corrected the Result with the following text (page 5):

The circulating levels of the angiogenic cytokines (FGF-2, VEGF, IL-8 and TNFα) were not changed significantly at 3-days post-AMI in the AMI group (Figure 4A-D).

3B. Comment: Based on the initial discussion of the results, the appearance of Figure 3B and Figure 3A should be switched.

Answer: Thank you for the remark. Accordingly, we have corrected the Result section (page 5):

Reperfused AMI did not enhance the release of SDF-1α at 3 days post-AMI (baseline: 32.02±24.35 vs. 3 days post-AMI: 26.97±15.43pg/ml; P=0.41) (Figure 3). In contrast, the circulating level of CXCR4 (baseline: 0.47±0.22 vs. 3 days post-AMI: 1.15±0.95ng/ml; P=0.034) increased significantly at 3 days post-AMI with concomitant induced mobilization of CD34+ cells (baseline: 260±75 vs. 3 days post-AMI: 668±180cells/µl; P<0.001).

3C. Comment: Cell migration

Why was 90 min hypoxia used?

Answer: The cells in *in vitro* cell migration assays were treated for 90 min, since 90 min LAD-occlusion was applied in the *in vivo* experimental protocol. *In vitro* experiment supposed to mimic the conditions applied in large animal experiment with already validated time for sufficient myocardial necrosis induced by 90 min coronary occlusion *(Gyöngyösi et. al, 2010).*

Gyöngyösi M, Posa A, Pavo N, et al.: Differential effect of ischaemic preconditioning on mobilisation and recruitment of haematopoietic and mesenchymal stem cells in porcine myocardial ischaemia-reperfusion. *Thromb Haemost.* 2010;104(2):376–384. 20352158 10.1160/TH09-08-0558

Discussion:
4A Comment: There seems to be a contradiction in the findings regarding the secretion of VEGF. As noted above in Results, there was no change in the levels of the cytokines (FGF-2, VEGF, IL-8 and TNFa) at day 3. In the Discussion, item 4, it notes "IP induced VEGF secretion in in the second window of cardioprotection." Please clarify the discrepancy.

Answer: Unchanged level of the FGF-3, VEGF and IL-8 and TNFa are related to the AMI group. We have corrected this in section Results (page 5). (Also Comment 3A).

4B Comment: Effect of AMI and IP FGF-2, VEGF, IL-8 and TNFa release
The comparison being made in this section does not seem to account for the fact that the data were obtained from two experimental models and that the IL-8 was from pigs and not human cardiomyocytes.
The conclusion could be bolstered by some of the control studies suggested.

Thank you for the comment. We agree, the inclusion of the control group would give additional comparison to the group of the present experiments. However, our findings are similar to the study of Husebye *et al*., reporting no increase in TNF-alpha and IL-8 levels in patients with STEMI and randomized to placebo group.

Husebye T1, Eritsland J2, Arnesen H3, Bjørnerheim R2, Mangschau A2, Seljeflot I4, Andersen GØ5. Association of interleukin 8 and myocardial recovery in patients with ST-elevation myocardial infarction complicated by acute heart failure. Plos One. 2014 Nov 12;9(11): e112359. doi: 10.1371/journal.pone.0112359. eCollection 2014

We have added this comment in the Discussion (page 9).
Additionally, we have expanded the Limitation section with the lack of sham-operated control group (page 10) as follows:

Inclusion of a sham-operated control group would promote better comparison of the results. However, we decided to not supplement the study groups with control group of animals without infarction with expected constant baseline level of biomarkers. Indeed, we intended to apply the "reduction" principle of the 3R (reduction, refinement and replacement) concept regarding the in vivo experiments.

Competing Interests: No competing interests disclosed.

Author Response 30 Jan 2017

Dominika Lukovic, Medical University of Vienna, Austria

Comment: Cell migration assay
Figure 2, hypoxia treatment should have been present in all the various concentrations of SDF-1a and in its absence? This information would strengthen the connection between SDF-1a and the migratory response to CD34+ cells during hypoxia. How would one conduct an IP experiment in this case to demonstrate the restitution of the CD34+ migratory response during hypoxia in the in vitro model?

Answer: Thank you for the valuable comment. SDF-1a treatment of the HACMs was conducted under normoxic conditions, since we wanted to assess the maximal effect of SDF-1a. We agree with the Reviewer that parallel measurement under hypoxic conditions might better explain the connection between SDF-1a and the migratory response.

Ischemia and reperfusion stimuli of IP can be simulated *in vitro* by incubation of HACMS under normoxic and hypoxic conditioning cycles. This method was developed by Martou *et al*.

Martou G, O'Blenes CA, Huang N, McAllister SE, Neligan PC, Ashrafpour H, Pang CY, Lipa JE (2006) Development of an in vitro model for study of the efficacy of ischemic preconditioning in human skeletal muscle against ischemia-reperfusion injury. Journal of applied physiology 101: 1335-42

Competing Interests: No competing interests were disclosed.

Referee Report 20 December 2016

✓   **Zhengyuan Xia**[1], **Michael Irwin**[2]
[1] Anesthesiology Research Laboratory, Department of Anaesthesiology, The University of Hong Kong (HKU), Hong Kong, China
[2] Department of Anesthesiology, University of Hong Kong, Pokfulam, K424, Hong Kong

Authors have adequately addressed reviewer concerns and improved the paper. No further comments.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 24 November 2016

Zhengyuan Xia[1], **Michael Irwin**[2]

[1] Anesthesiology Research Laboratory, Department of Anaesthesiology, The University of Hong Kong (HKU), Hong Kong, China

[2] Department of Anesthesiology, University of Hong Kong, Pokfulam, K424, Hong Kong

General comments: This is an interesting study with novel findings showing that myocardial ischemia triggers the release of circulating MMP-2, which inhibits SDF-1α and CXCR4 release, and that ischemic preconditioning (IP) inhibits MMP-2 release, thereby increasing both SDF-1α and CXCR4 levels, resulting in a higher level of CD34+ cell mobilization 3 days post ischemic injury in in vivo condition in large animals. In addition to the limitations mentioned by the authors, we have the following comments that the authors may need to consider to improve the manuscript.

Specific comments:

1. Is there any particular consideration why female but not male pigs were chosen as the study subjects? This needs to be clarified. Similarly, the reason why n=6 in the IP group but n=12 in the non-conditioned AMI group is not clear.

2. IP has been shown to reduce post-ischemic IL-8 release in most, if not all, models of myocardial ischemia and reperfusion or in some clinical trial studies. While in your model, IP almost significantly increased, and could actually significantly (see below), post-ischemic IL-8. This should be discussed in comparison of other studies in more detail.

3. One may expect that IP may also could have significantly increased post-ischemic CXCR4, if the authors have 1) observed more time points; or 2) the sample size of the two groups were 9 per group rather than 6 vs. 12; or 3) the area under curve (AUC) data are compared. This applies to the above IL-8 story.

4. Have the authors considered to perform repeated measures ANOVA with post hoc comparison to assess time effects or time-treatment interaction?

5. The conclusion that IP enforces CD34+ cell mobilization via inhibition of MMP-2 does not seem to have well supported by data, in particular you did not have intervention of MMP-2 in the presence or absence of IP in the in vivo model, and you did not measure cardiac MMP-2 proteins. Therefore, it needs to be toned down.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 15 Dec 2016
Dominika Lukovic, Medical University of Vienna, Austria

Dear Dr. Zhengyuan Xia,

We greatly appreciate the efforts to carefully review our paper and the valuable comments. We have modified the manuscript accordingly, as follows:

General comments: This is an interesting study with novel findings showing that myocardial ischemia triggers the release of circulating MMP-2, which inhibits SDF-1α and CXCR4 release, and that ischemic preconditioning (IP) inhibits MMP-2 release, thereby increasing both SDF-1α and CXCR4 levels, resulting in a higher level of CD34+ cell mobilization 3 days post ischemic injury in in vivo condition in large animals. In addition to the limitations mentioned by the authors, we have the following comments that the authors may need to consider to improve the manuscript.

Specific comments:
1. **A. Is there any particular consideration why female but not male pigs were chosen as the study subjects? This needs to be clarified.**

 Answer:
Thank you for the comment. Indeed, we have chosen female pigs, because a clear gender differences have been observed in female and male rodents, rabbits, dogs and pigs (Murphy CVRes); the incidence of cardiogenic shock and life-threatening arrhythmias were more frequent in male than female pigs by using the closed-chest reperfused AMI model.
We have added this comment into the Limitation in our revised paper (page…)

1. **B. Similarly, the reason why n=6 in the IP group but n=12 in the non-conditioned AMI group is not clear.**

 Answer:
Due to expected higher mortality in the control group, the block randomisation of 1:2 was chosen.
We have added this comment, and corrected the Method with the following text:

Randomly selected female domestic pigs (n=21; weight, 30–35kg) underwent percutaneous coronary intervention (PCI) under general anaesthesia in order to perform either ischemic preconditioning (group IP) or non-conditioned AMI (group control), with a 1:2 block randomization, due to expected higher mortality in the control group. Animals in both groups underwent 90min percutaneous balloon occlusion of the left anterior descending (LAD) coronary artery at the origin of the first diagonal branch following reperfusion (balloon deflation). IP was initiated prior to 90min LAD occlusion by 3×5min repetitive cycles of artery re-occlusion and reperfusion. One pig in the IP and 2 animals in the control group died during the AMI intervention, all remaining animals (n=6 in IP and n=12 in control groups) survived for 1 month after the experimental procedure.

(page 2)

2. And 3. IP has been shown to reduce post-ischemic IL-8 release in most, if not all, models of myocardial ischemia and reperfusion or in some clinical trial studies. While in your model, IP almost significantly increased, and could actually significantly (see below), post-ischemic IL-8. This should be discussed in comparison of other studies in more detail.One may expect that IP may also could have significantly increased post-ischemic CXCR4, if the authors have 1) observed more time points; or 2) the sample size of the two groups were 9 per group rather than 6 vs. 12; or 3) the area under curve (AUC) data are

compared. This applies to the above IL-8 story.

Answer:
Thank you for the remark. We agree that results of plasma cytokine levels would be more informative if it measured more often. However, an additional blood sampling would require a full anaesthesia in the pigs with recent AMI with expected higher mortality. Accordingly, we have added the following text in the revised manuscript:

IL-8 is a pro-inflammatory C-X-X chemokine that is also involved in activation of pro-angiogenic processes and re-introduction of progenitor cells into the circulation. The study of Schomig et al. demonstrated significantly increased IL-8 level in AMI patients as compared to patients diagnosed with stable angina[8]. In our study, we observed a trend toward increased release of IL-8 in the clinically relevant porcine reperfused "STEMI" model. The levels of CXCR4 increased both in controls (with AMI) and IP groups, with a trend towards higher increase in the IP group 3-day post AMI. The differences between our and other studies might be explained by the pre- and peri-AMI medication of patients with standard care that may contribute to changes in plasma levels of cytokines in AMI patients[8]. Results of plasma cytokine levels would be more informative if it measured more often, in an extended time window. The area under the curve (AUC) calculation of the cytokine release data might have delivered additional results. However, for a simple blood sampling, the animals must have been fully anaesthesized, which procedure signifies an additional stress for the animals with recent AMI with predicted higher mortality.

(page 9)

4 Have the authors considered to perform repeated measures ANOVA with post hoc comparison to assess time effects or time-treatment interaction?

Answer:
Thank you for the comment. Indeed, we have performed two-way ANOVA with repeated measurements, supplemented with Bonferroni correction, as described in the originally submitted paper (Statistical analysis section).
According to the Reviewer`s suggestion, we have also tested the intra-group differences according to the time-factor (statistical comparison between baseline and 3-day and 1 month), and also the "between-groups" differences by using independent t-test. The results are added into the Figure legends as follows:
+$P<0.05$ between baseline and 3 day values within the IP group;
 #$P<0.05$ between baseline and 3 day values within the control (non-conditioned AMI) group.
*$P<0.05$ between the IP and control group

The non-significant changes were not labeled.

We are aware, that serial blood sampling would have given more information, eg. the evident changes in mobilisation of bone marrow-derived cells following myocardial infarction occur at day 3, 7 and 14 post-ischemia[23] . However, we have focused on the second window of protection, which ends at day 3.

We have added this comment in the Limitation (page 10).

5. The conclusion that IP enforces CD34+ cell mobilization via inhibition of MMP-2 does not seem to have well supported by data, in particular you did not have intervention of MMP-2 in the presence or absence of IP in the in vivo model, and you did not measure cardiac MMP-2 proteins. Therefore, it needs to be toned down.

Answer:
We agree with the reviewer in this aspect. Accordingly, we have corrected the Conclusion in the Abstract and Discussion and supplemented the Limitation section as follows:

Abstract:
Conclusions. Non-conditioned AMI induces MMP-2 release, hampering the ischemia-induced increase in SDF-1α and CXCR4 by cleaving the SDF-1α/CXCR4 axis, with diminished mobilization of the angiogenic CD34+ cells. IP might influence CD34+ cell mobilization via inhibition of MMP-2. (page 1)

Discussion:
IP induced CD34+ cell mobilization in the late phase (second window) of cardioprotection, thereby also increasing circulating SDF-1α and CXCR4, parallel with enhanced VEGF secretion. One mechanism of this beneficial effect of IP might be the inhibition of AMI-induced MMP2-release. (page 10)

Limitation
We revealed one additional possible beneficial mechanism of IP, namely the inhibition of MMP-2 release with consequent higher mobilization of CD34+ cells, which was confirmed in our in vitro experiment. However, a direct association between IP - MMP-2 - CD34+ axis had to be confirmed in vivo, by blocking MMP2 in animals subjected to AMI and IP. We have not measured myocardial MMP-2 level, which analysis would require harvesting of the animals max. 72h post IP-AMI (second window of protection), and our animals survived 1-month follow-up. (page 10)

Competing Interests: No competing interests were disclosed.