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# Isoflurane post-conditioning stimulates the proliferative phase of myocardial recovery in an ischemia-reperfusion model of heart injury in rats

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**Summary.** The application of isoflurane in a post-conditioning manner, during early reperfusion following a period of coronary occlusion, has numerous beneficial effects on the ischemic myocardium, including reduction of infarct size. It does so by stimulating a sequence of well studied anti-apoptotic pro-survival mechanisms in a similar manner to various 'ischemic' pre-/post-conditioning approaches which achieve their cardio protective effects in both laboratory and clinical situations. Proliferation of newly formed blood vessels, resulting in formation of highly vascularized granulation tissue, is an essential stage of infarct healing. It can be evaluated by detecting various angiogenic factors, including vascular endothelial growth factor (VEGF) and platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) or by quantification of expression of vascular smooth muscle progenitors, such as Nestin. Expression of these three markers was used to evaluate the effect of early isoflurane post-conditioning in ischemia-reperfusion type cardiac injury. A large reduction in infarct size (59.3% of control), and marked increase of expression of VEGF (43.4%), PECAM-1/CD31 (136%) and Nestin (77.9%) was found in experimental animals when compared to control animals that did not receive isoflurane treatment. Hence, based on our results, we can emphasize two morphologically detectable benefits of isoflurane post-conditioning: a marked reduction in infarct size and much better organization/vascularization of necrotic tissue.

**Key words:** Myocardial infarction, Ischemia-reperfusion model, VEGF, PECAM/CD31, Nestin

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## Introduction

The size of myocardial infarction (MI) is directly related to both severity and duration of coronary occlusion. However, the process of restoration of coronary blood flow, known as reperfusion, paradoxically exacerbates the injury caused by ischemia itself, mainly through activation of apoptotic events (Freude et al., 2000; Eefting et al., 2004), microvascular dysfunction and/or overzealous inflammatory response (Gross and Auchampach, 2007). There are several well studied approaches to reducing this 'reperfusion injury': from the introduction of various pre- or post-conditioning scenarios, including either short, repetitive ischemic episodes (known as 'ischemic' pre-/post-conditioning) to pharmacological interventions using volatile anesthetics (termed 'anesthetic' pre-/post-conditioning, see a review article by Pagel) (Pagel, 2008). Since coronary artery occlusion cannot be predicted in most patients, therapeutic interventions should be applicable during myocardial ischemia and/or at the onset of reperfusion, the latter commonly being a result of a clinical intervention (during coronary angioplasty) (Staat et al., 2005). Experimental and clinical investigations state that a particularly important period for interventions into coronary hemodynamics, appears to be during early reperfusion (Kin et al., 2004; Piper et al., 2004), highlighting the clinical importance of post-conditioning. In that period, instead of performing repetitive coronary occlusion-reperfusion approaches (which might be clinically challenging), brief application of volatile anesthetics might be attempted to achieve a significant reduction of the infarcted area. This is the reason why anesthetic post-conditioning, as an experimental model, has gained more attention in the last decade (Frassdorf et al., 2009). Not

only do volatile anesthetics have a proven inhibitory effect on adhesion of neutrophils in coronary arteries after ischemia (Heindl et al., 1999), they also activate the so called reperfusion injury salvage kinases (RISK): pro-survival antiapoptotic kinases, including Akt and ERK1/2 (Hausenloy and Yellon, 2007), and transcription factors, like hypoxia inducible factor (HIF), ultimately leading to several mitochondrion-protection/stabilization effects (Huffmyer and Raphael, 2009). The literature shows, beyond any doubt, that isoflurane post-conditioning results not only in significant reduction of myocardial infarction size, but also that both pre- and post-conditioning share some steps in signal transduction (Pagel, 2008; Frassdorf et al., 2009). However, the majority of studies, summarized in the above mentioned reviews, focus only on the acute effects of anesthetic post-conditioning, occurring within a few hours of reperfusion.

The process of healing of myocardial infarction can be divided into three overlapping phases with a distinct temporal dimension: the inflammatory phase, the proliferative phase and the maturation phase (Dobaczewski et al., 2010). The proliferative phase, occurring in rodents between the 2$^{nd}$ and 5$^{th}$ days post-infarct, is characterized by chemokine suppression, deposition of fibrous tissue and marked angiogenesis (Dobaczewski et al., 2010). Nahrendorf et al. found that there are two phases of monocyte/macrophage participation in MI healing with a so called "late" subset of these cells having not only attenuated inflammatory function but also a distinct role in stimulation of myofibroblast accumulation, deposition of collagen and angiogenesis, which cumulatively leads to the formation of highly-vascularized granulation tissue (Nahrendorf et al., 2007). In these circumstances new blood vessels can be formed in two different ways: by 'vasculogenesis', in which vessels occur from endothelial progenitors and 'angiogenesis', which includes sprouting and collateral growth of mural cells, forming so called 'collateral bridges' linking neighboring arterial networks (Chen et al., 2009). Angiogenesis also includes formation of pericyte-deprived "mother vessels" along with the growth of a rather rich network of capillaries (Nagy et al., 2003).

Recently, it was demonstrated that ischemia/reperfusion type injury causes a significant increase in the angiogenic factor, vascular endothelial growth factor (VEGF), with peak expression between the 3$^{rd}$ and 7$^{th}$ day post-reperfusion (Zhao et al., 2010). VEGF, known not only as a potent angiogenesis-triggering factor but also as an angiogenesis- and vasculogenesis-supporting factor, is activated by isoflurane preconditioning (Wang et al., 2006; Pagel, 2008), but no data regarding expression of VEGF and anesthetic postconditoning can be found.

Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) is a transmembrane glycoprotein expressed on the surface of endothelial cells, where its expression is largely concentrated at junctions between adjacent cells, on platelets and on most leukocyte subtypes (Woodfin et al., 2007). Recently, it was shown that PECAM-1/CD31 is an important regulator of proangiogenic properties of endothelial cells (Park et al., 2010), although its strong immunohistochemical expression in newly formed vessels, following ischemia-reperfusion injury, was described a decade ago (Ren et al., 2002). Interestingly, as in the case of VEGF, expression of PECAM-1/CD31 immunoreactivity in a volatile anesthetic post-conditioning model, has not yet been studied.

Nestin is a member of the class VI family of intermediate filament proteins representing a characteristic marker of multi-lineage progenitor cells indicating proliferating immature cells in tissue regeneration (Wiese et al., 2004). It has been shown that a subpopulation of Nestin positive cells contribute to myocardial regeneration by being able to differentiate not only to glial and neuronal cells, but also to vascular smooth muscle and even to cardiac myocites/myofibroblasts (Beguin et al., 2012; Calderone, 2012). Furthermore, Nestin immunoreactivity has also been detected in proliferating endothelial progenitor cells (EPCs) and in endothelial cells during active formation of new blood vessels in the ischemically damaged rodent and human heart, suggesting that Nestin expression can be utilized as an appropriate marker of "de novo" formed endothelial cells (El-Helou et al., 2008). Despite its prominent role in post-ischemic tissue regeneration, no data concerning the influence of anesthetic post-conditioning on Nestin immunoreactivity could be detected in the literature.

In view of the above, in particular the shortage of detailed histological evaluation of infarcted myocardium in the middle of the very active 'proliferative phase' of healing, our investigation is particularly focused on vasculogenesis and angiogenesis in granulation tissue four days after ischemia/reperfusion type injury.

## Materials and methods

### *Animal model*

All experimental protocols and procedures were approved by the Ethical Committee of the School of Medicine in Split and carried out according to laboratory animal guidelines (European Communities Council Directive of 24 November 1986). In this study we used Sprague-Dawley female rats (n=8) weighing 175-225 gr. Animals were obtained from the Facility for Experimental Animals, University of Split, Croatia. The rats were housed in individual plastic cages in a temperature-controlled environment, maintained on a 12:12 hours light-dark cycle, with rat chow and water available *ad libitum*.

Animals where anesthetized with a mixture of Ketaminol (Ketaminol 10, 80 mg/kg, Intervet International, Netherlands) and Xylazinum (Xylapan, 10 mg/kg, Vetoquintol, Switzerland), injected in the right

hamstring muscles. Endotracheal intubation was performed with a 17 gauge venous catheter. Rats were placed on a temperature-controlled heated table to maintain rectal temperature between 37 and 38°C. As described earlier in our publications, the trans-abdominal approach to the heart was utilized (Aljinovic et al., 2010). With the aid of a surgical microscope (Leica, M520 MC1, Switzerland), medial section of the diaphragm was performed through both tendinous and muscular parts, sparing the phrenic vessels. Simultaneously with the diaphragm incision, rats were connected to an animal ventilator (SAR 830, CWE Inc, USA). The lungs were ventilated with an air/oxygen mixture 60/40% on 56 breaths/min and inspiratory pressure between 10 and 12 cm/$H_2O$. After the pericardium was gently removed, the left anterior descending (LAD) coronary branch was identified by locating the corresponding vein on the heart surface between the left auricle and the pulmonary outflow tract. Once visually identified, an 8-0 nylon suture (Prolene, Ethicon, Norderstedt, Germany) was placed around the LAD. Both ends of the suture were threaded through a 5 cm long segment of polyethylene tubing (size PE-10, Instech Laboratories, PA, USA) to form a snare. Ischemia was induced by pulling the snare and clamping tube with a hemostat for 30 min. The isoflurane group (n=4) received 1.5% end-tidal concentration of isoflurane (1.0 minimum alveolar concentration [MAC]) for 15 minutes, starting from the last 5 minutes of ischemia to 10 minutes of reperfusion time. The gas concentrations were measured using a Dräger PM 8050 (Dräger, Lübeck, Germany) gas monitor. The control group received only air/oxygen mixture throughout the procedure. Myocardial ischemia was confirmed visually in situ by the local color change of the heart surface (pale appearance). ECG monitoring (Cardioline Delta 1, Cavareno, Italy) was used during the whole procedure and recorded after 10 and 30 minutes of ischemia, and in the same periods of reperfusion. Ischemia was confirmed with ST segment elevation and T wave inversion. After 30 minutes of ischemia, the snare was released, and the heart was allowed to reperfuse. Reperfusion was confirmed by the immediate hyperemia over the surface of the previously ischemic-cyanotic part of the heart and ECG normalization. Color change of the heart surface during ischemia and reperfusion was photo-documented using a Canon EOS 500D (Canon, Japan) digital camera, mounted on the surgical microscope. After completion of all surgical procedures, the diaphragm and the abdominal wall were closed in layers with fine sutures (Polyamide 6-0 and 7-0), as described previously.

Four animals died on the first postoperative day, probably due to massive myocardial infarction.

### *Tissue processing and histological staining*

Four days after the induction of ischemia-reperfusion injury, the animals were re-anaesthetized with the same combination of anaesthetics as for the operative procedure, the chest was opened with two parasternal cuts and hearts were extracted and cut into two parts dividing atria from the ventricles. Ventricles were fixed in 4% paraformaldehyde in phosphate buffer and 15% saturated picric acid and dehydrated in 100% ethanol. Measurements of infarct size were performed on two reference cross sections of every heart. The first cross section was made perpendicular to the axis of the heart exactly 2 millimetres below the inferior edge of the left auricule and the second section was made 2 millimetres lower (towards the apex of the heart) than the first section. Histological sections (4-6 $\mu$m thick) were made from the ventricular tissue embedded in paraffin wax so that planes of section were represented on a series of histological sections on slides. After deparafinisation, sections were rehydrated in ethanol and water.

For Mallory trichrome staining, sections were incubated with hematoxylin for 5 minutes, differentiated with tap water and afterwards treated with acid fuchsin for 1 minute. Following a few rinses with distilled water, sections were differentiated with 1% phosphomolybdic acid for 1 minute. After a brief rinse with distilled water, sections were stained with anilin blue for 15 minutes, again briefly rinsed with distilled water, differentiated with 1% acetic acid for 1-5 minutes and dehydrated in ethanol and xylol.

To detect infarcted areas and anatomical relationships between different parts of the rat hearts, haematoxylin and eosin staining and Mallory trichrome staining were performed. Slides at two different planes of section were photographed with a Canon PowerShot A480 (Canon, Japan) photographic camera using Super macro settings. Digital images were then transferred into Adobe Photoshop CS3 software (Adobe, USA) and an area of infarct was selected using Magic Wand Tool. This tool allows a precise selection of irregularly shaped objects on a digital image based on differences in tone and color. Once demarcated from the healthy myocardium, the infarcted area was 'shifted' away from the image and its total pixel content was expressed as the surface area (in $mm^2$) calculated with the aid of a standard sized grid that was photographed together with the section of the ventricles. Once the infarcted area was 'removed' from the image, the surface area of the remaining part of the left ventricular wall and the septum was calculated by converting pixels into $mm^2$. Hence, there were two sets of data at two cross-section levels (that were averaged) for every infarct, the absolute value of its surface area and the proportion of the left ventricular wall that was infarcted in each particular specimen.

Sections for immunoflourescence staining were cooked in sodium citrate buffer (pH 6.0) for 17 min at 95°C in a microwave oven. After being cooled to room temperature, sections were washed in PBS and incubated with goat serum (Normal Goat Serum, X0907 DAKO, Glostrup, Denmark) for 1 hour. After multiple washes in PBS, sections were separately incubated with polyclonal

rabbit anti-VEGF primary antibody (dilution 1:200; ab46154, Abcam, Cambridge, UK), polyclonal rabbit anti-Nestin antibody (1:200, ab93157, Abcam, Cambridge, UK) and purified mouse anti-rat CD31 antibody (1:100, BD Pharmingen, CA, USA) for 1 hour in a humidified chamber. Primary antibodies were diluted in Dako REAL antibody diluent (Dako Denmark A\S, Denmark). For double immunofluorescent antibody staining, a combination of Nestin-CD31- primary antibody co-localization was used. After multiple washes in PBS, sections were incubated for 1 hour with secondary antibodies. The secondary antibodies used were: Streptavidin Alexa Fluor 488 conjugate, 508205, Invitrogen, Oregon, USA at 1:500 dilution; and biotinilated goat anti-rabbit IgG, ab64256, Abcam, Cambridge, UK; biotinilated goat anti-mouse IgG-B, sc-2039, Santa Cruz Biotechnology Inc., SantaCruz, CA, USA; Rhodamine, AP124R, Jackson Immuno Research Lab., PA, USA; Rhodamine, sc-2095, Santa Cruz Biotechnology Inc., SantaCruz, CA, USA; all at 1:200 dilution. Following secondary antibody incubation, the sections were washed in PBS and counterstained with DAPI to stain nuclei.

Staining controls included omission of primary antibodies from the staining procedure, which resulted in no staining of tissue. After final rinsing in PBS, all sections were mounted (Immuno-Mount, Shandon, Pittsburgh, PA, USA), air-dried and coverslipped. The tissues were viewed and photographed using Olympus BX51 (Tokyo, Japan) microscope equipped with Olympus DP71 camera and processed with Cell A Imaging Software for Life Sciences Microscopy (Olympus, Japan).

### *Quantification of VEGF, Nestin and CD31 positivity*

The number of VEGF and Nestin/CD31 positive cells was evaluated quantitatively by two independent investigators and classified as negative (no stained cells) and positive (stained cells). Counts were made throughout the granulation tissue in the infarction wound in the control and isoflurane group. DP-SOFT version 3.1 software was used to divide each chosen section of granulation tissue into squares of 50x50 $\mu$m at x40 magnification. Positive cell profiles were counted in squares that were completely covered with cells. The cells located on the left and upper border of squares were not taken into account, only those on the right and lower border as we have described previously (Aljinovic et al., 2010). To avoid counting the same cell twice every third section was counted from proximal, distal and middle part of infarcted heart wall for each heart. In each area of the granulation tissue (50x50 $\mu$m), the percentage of VEGF positive and number of Nestin/CD31 double positive cells in control and isoflurane group was calculated. The percentage of VEGF positive cells was expressed as mean ± SD, while the number of Nestin/CD31 positive cells was expressed per mm$^2$. The counts of Nestin and CD31 positive vessels were made throughout the whole infarction tissue in the series of images at x40 magnification by two independent investigators and classified as negative (no stained vessels) and positive (stained) vessels. The number of positive vessels in infarction was expressed per mm$^2$. For statistical comparison between groups we used t-test (GraphPad Software, La Jolla, CA, USA) after we confirmed normal distribution of the data. Significance was accepted at $P<0.05$. The impact of post-conditioning with isoflurane on the infarct size and expression of immunoreactivity for the above markers was also expressed as a percentage difference relative to the controls (Skyschally et al., 2009).

## Results

### *Intraoperative observations*

In all cases, the ligation of LAD at the level of the inferior border of the left auricle caused immediate pallor of the surface of the heart, affecting the anterior wall of the left ventricle (Fig. 1A), with no noticeable color changes on the surface of the right ventricle. Within 10 minutes of ligation, a characteristic and distinct elevation of the S-T segment was noticed on ECG recording (Fig. 1B). There was no color change of the ischemic area during the entire period of ligation, but following the release of the suture, the area immediately turned red, indicating (Fig. 1C) successful reperfusion. Within 10 minutes of reperfusion, signs of ischemia gradually diminished on the ECG in both groups of animals (Fig. 1D). There was no difference in the rate of postoperative recovery and no behavioral differences could be noticed between the two groups of animals in the immediate postoperative period.

### *Qualitative differences in ischemic areas*

Cross sections of whole rat hearts revealed both the position and size of infarcted myocardial segments. In both the control and isoflurane-treated animals, infarcted areas were clearly visible at small magnification following staining with Hematoxylin and eosin (HE) as well as with Trichrome Mallory stain. Histological changes were noticeable as distinct areas characterized by the loss of cardiomyocytes in combination with infiltration of inflammatory cells and granulation tissue (Fig. 1E,F,H,I). Interestingly, there was no reduction of thickness of the myocardial wall in segments affected by ischemic changes. Central parts of infarcted areas in control animals showed characteristic amorphous necrotic detritus and signs of hemorrhage (Fig. 1K). However, sections from isoflurane-treated animals showed a different appearance of the central infarction zone; areas of necrotic detritus and hemorrhage were smaller (Fig. 1L) or totally absent (Fig. 1M). Trichrome Mallory staining was performed to detect eventual fibrous organization of the scar area, signs of which could be detected in neither control nor isoflurane-

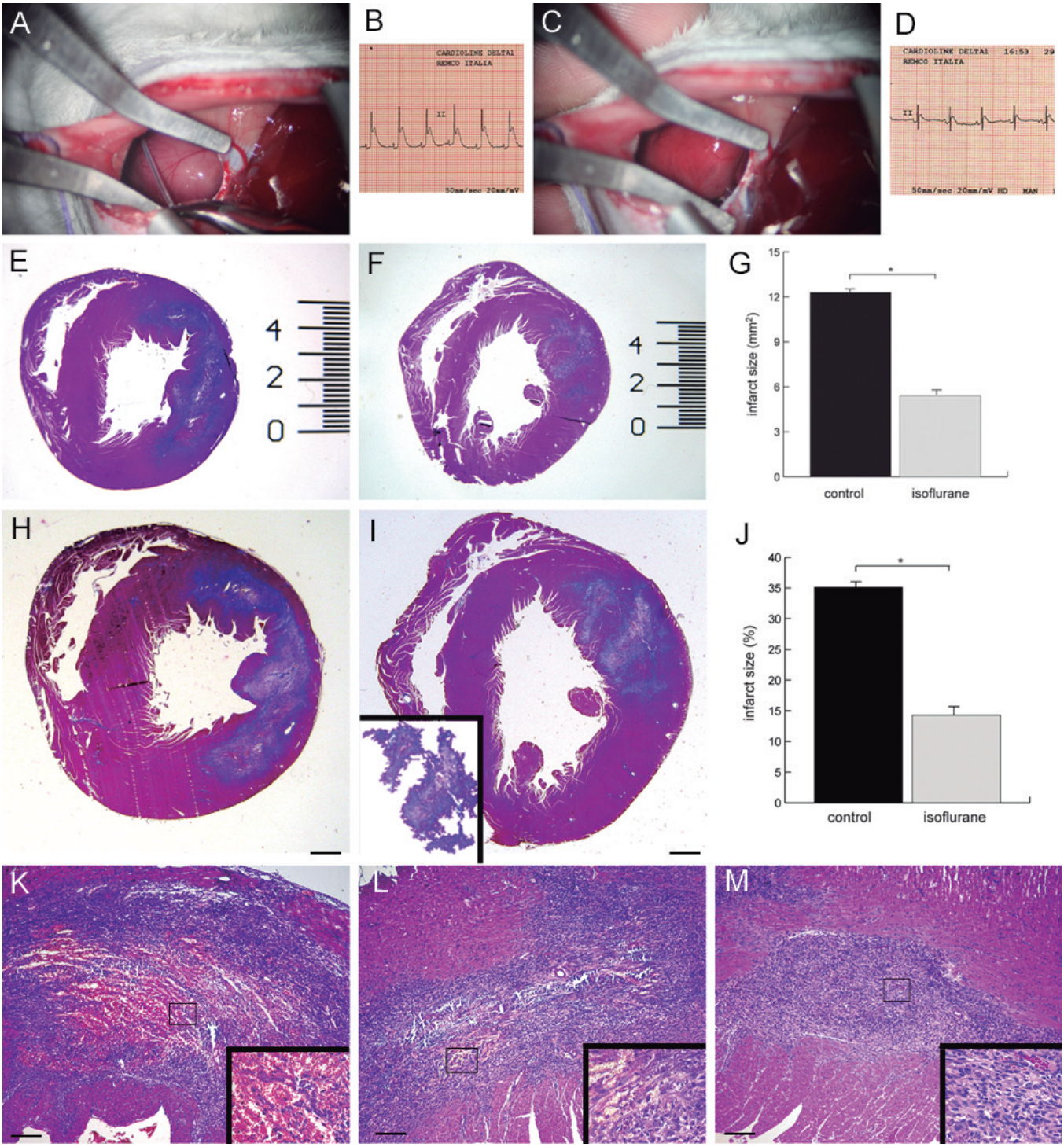


**Fig 1.** Pale appearance of the anterior wall of the left ventricle following ligation of the left anterior descending (LAD) artery **(A)** and an immediate elevation of the S-T segment on the ECG recording **(B)**. Characteristic 'rush' of blood in the pale area indicating successful reperfusion **(C)** and following normalization of the ECG recording **(D)**. Trichrome Mallory staining of cross sections of control (**E and H**) and isoflurane treated (**F and I)** animals and comparison of infarct sizes expressed as absolute values in $mm^2$ (**G**) and as percentages of the left ventricular wall **(J)**. Insert on I shows the infarct area when digitally 'removed' from the ventricular wall. Histological section through the infarcted heart wall in the control group **(K)**: two thirds of the wall thickness is occupied by granulation tissue containing large central area with necrotic detritus and hemorrhages (insert). Histological section through the heart walls of the isoflurane treated animals (L and M): one third **(L)** or less **(M)** of the wall thickness is filled with granulation tissue, containing only very small areas of necrotic detritus and hemorrhages (inserts). Scale bars: H, I, 1 mm; K, L, M, 25 $\mu$m.

treated animals at this early stage of healing. In addition, it was also noticed that, regardless of its size, there was usually a layer of normal-looking myocardium separating the infarcted area from the ventricular cavity and epicardium. However, occasionally, small parts of infarcted areas interrupted a layer of variable thickness and reached the epicardium or endocardium, which was more prominent in the control group.

*Size of infarcted areas*

A very precise calculation of infarcted surface areas was obtained using our quantification method. The size of infarcted surface areas was expressed as both the absolute size in millimeters square and as a proportion of infarcted segments of the left ventricular surface, with the interventricular septum included (expressed as mean

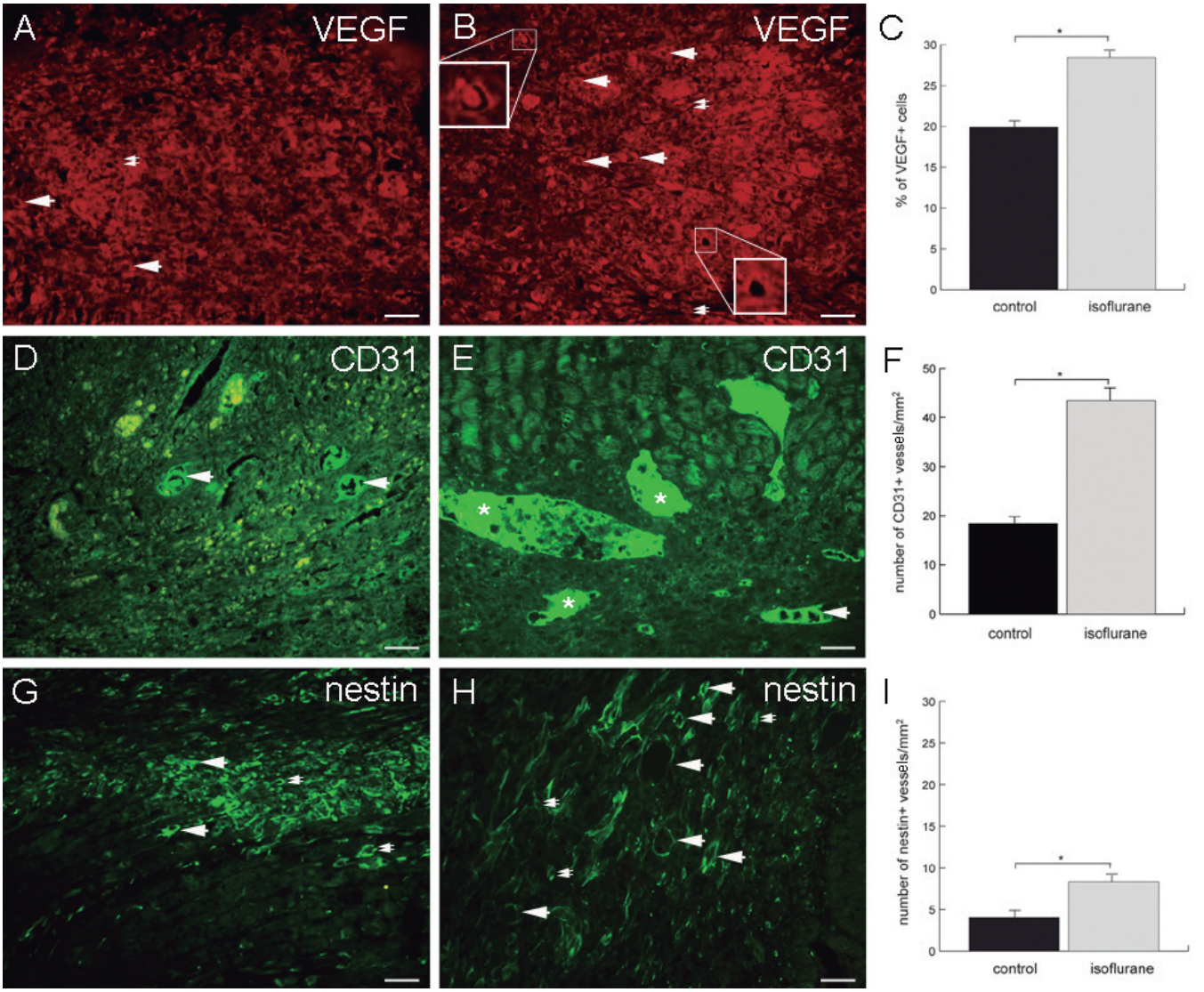


**Fig. 2.** Histological section through infarcted areas of the heart in the control **(A, D, G)** and isoflurane treated **(B, E, H)** animals. VEGF immunorecativity (red stain) is observed in ovoid cells (single arrows) and blood vessels (double arrows) in granulation tissues of control group **(A)** and in ovoid cells (arrows), blood vessels (enlarged areas) and spindle shaped cells (double arrows) in the granulation tissues of isoflurane treated animals **(B)**. CD31 immunoreactivity (green stain) is seen in the small blood vessels and so-called "glomerular bodies" (arrows) in the granulation tissue of control group **(C)** and in the numerous blood vessels, including so-called "mother vessels" (asterisk) in the granulation tissue of isoflurane treated animals **(E)**. Nestin immunoreactivity characterized single cells (double arrows) and walls of blood vessels (arrows) in the heart granulation tissue of control animals **(G)** and isoflurane treated animals **(H)**. Comparison of expression of VEGF **(C)**, CD31 **(F)** and Nestin **(I)** between control and isoflurane treated animals. Scale bar: 25 $\mu$m.

± SD). Isoflurane-treated animals had an average infarct size of 5.4 $mm^2$±2.0 (N=4) and it represented 14.3±2.0% of the total left ventricular surface and septum. In contrast, non-treated (control) animals developed infarcts of average size 12.3 $mm^2$±1.7 (N=4), representing 35.1±9.9%. When these values were compared between the two groups of animals, it appeared that isoflurane treated animals had significantly smaller infarcts than control animals regardless of which values were taken into consideration (Fig. 1G,J); the P value for comparison of absolute surface areas and proportions of affected ventricular surface was P<0.0001 and P=0.0018, respectively. When the impact of isoflurane on infarct size is expressed as a percent difference in relation to controls (like in (Skyschally et al., 2009)), it appears that there is a 59.3 % reduction when proportions of infarcted segments are taken into account and 56.1% reduction when absolute size in $mm^2$ are compared.

*Immunohistochemical findings*

VEGF-expression

VEGF-immunoreactivity was detected in normal, non-affected animals and in ischemic myocardium of both groups of animals. VEGF-immunoreactivity was also detected in the cytoplasm of cells inside heart granulation tissue containing blood vessels of both the control (Fig. 2A) and isoflurane treated animals (Fig. 2B). The highest level of VEGF expression was detected in big ovoid cells, probably corresponding to macrophages and progenitor cells (Fig. 2A,B). In addition, VEGF-immunoreactivity was also found in endothelium of blood vessels (Fig. 2A,B) and in spindle shaped cellular profiles likely to represent the myofibroblasts (Fig. 2B). The proportion of VEGF positive cells in granulation tissue of ischemic areas in isoflurane group was 28.4±4.8, which was statistically

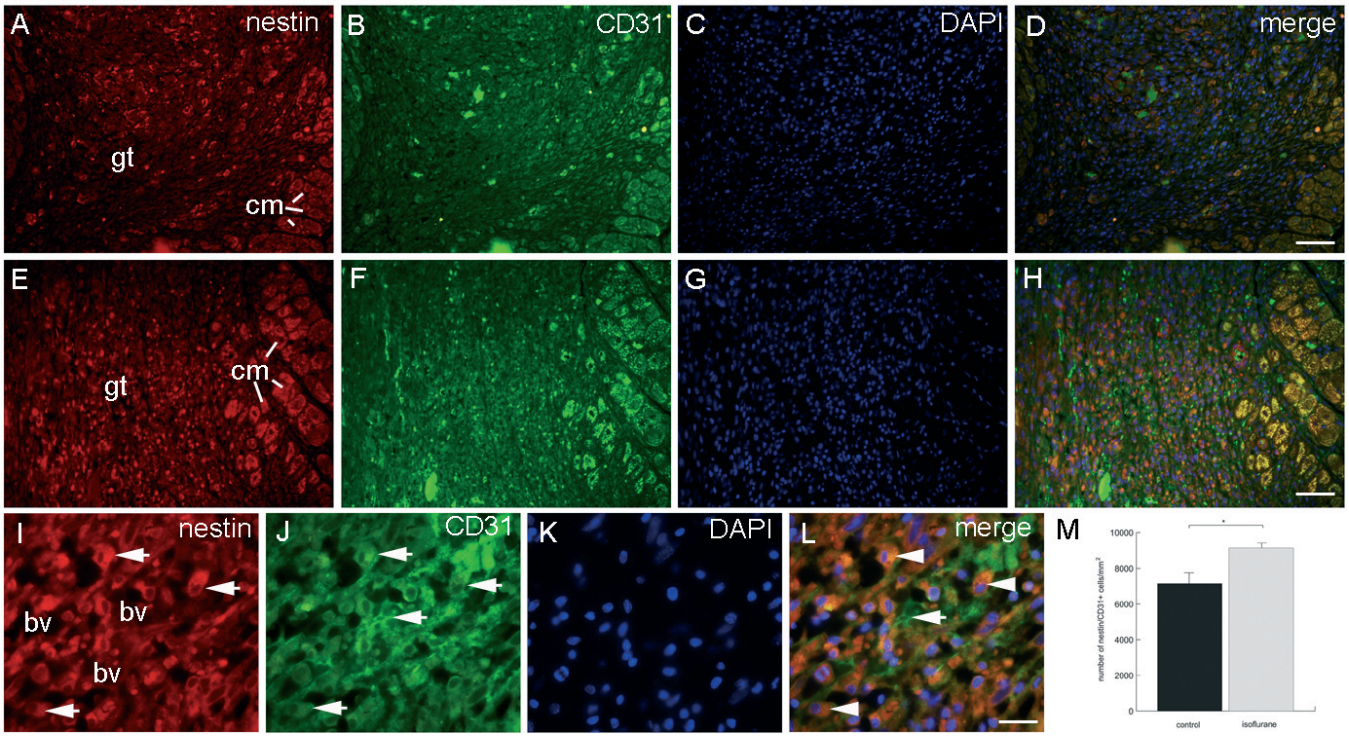


**Fig. 3.** Co-localization of CD31 and Nestin immunoreactivity in the infarct granulation tissue of the control animals **(A-D)** and isoflurane treated animals **(E-H, I-L).** Nestin **(A)** and CD31 immunoreactivity **(B)** was detected in the granulation tissue (gt) and lining cardiomyocites (cm) of the infarcted area of the control group. After merging with nuclear DAPI stain (**C**), co-expression of nestin /CD31 was detected in cells within granulation tissue and walls of small blood vessels (**D**). More intense immunoreactivity to Nestin (**E**) and CD31 **(F)** was observed in granulation tissue (gt) of infarcted areas of isoflurane treated heart tissues. After merging with nuclear DAPI stain (**G**), more intense co-expression of Nestin/CD31 was detected in numerous cells and small blood vessels within the granulation zone (**H**). Higher magnification of isoflurane treated infarct granulation zone showing immunoreactivity for Nestin in large ovoid cells (arrows) situated between blood vessels (bv) **(I)**. CD31 immunolocalization characterizes both large cell and blood vessels (arrows) in the same zone **(J)**. After merging with DAPI nuclear stain **(K)**, large ovoid cells show co-localization of Nestin/CD31 (arrowheads), while CD31 reactivity characterizes walls of blood capillaries (arrow) **(L)**. Comparison of Nestin/CD31 co-expression **(M)** between control and isoflurane treated animals. Scale bars: A-H, 25 $\mu$m; I-L, 10 $\mu$m.

higher (P<0.0001) than the proportion in ischemic areas of non-treated control animals 19.8±6.3 (Fig. 2C).

When the impact of isoflurane on the proportion of VEGF immunoreactivity is expressed as percent difference in relation to controls it appears that there is a 43.3 % increase.

CD31-expression

Non-ischemic heart tissue of both groups of animals rarely showed signs of CD-31 expression in the endothelium of blood vessels. Ischemic areas of heart in both the control (Fig. 2D) and isoflurane (Fig. 2E) groups of animals, displayed enhanced CD31 expression in blood vessels of granulation tissue, particularly in the endothelium of small capillaries. CD31-immunoreactivity was prominent in the larger vessels of the isoflurane group (called mother vessels) (Fig. 2E, asterisk), with characteristic bridging (Fig. 2E), and in blood vessels of the control group, forming so-called 'glomeruloid bodies' (Fig. 2D). The mean number of CD 31-immunoreactive vascular profiles of granulation tissue in isoflurane treated animals was 43.4±7.7 per $mm^2$ and it was statistically higher (P<0.0001) than the mean number of capillaries in control animals 18.4±2.9 per $mm^2$ (Fig. 2F). When the impact of isoflurane on the increase of CD31 immunoreactivity is expressed as percent difference in relation to the control group, it appears that the increase is more than double the value of the control group, that is, there is a 136% increase.

Nestin-expression

Non-ischemic myocardium of both animal groups showed no signs of Nestin expression, although some blood vessels in normal myocardium showed Nestin expression. Nestin positive cellular profiles were present in ischemic heart areas of control (Fig. 2G) and isoflurane treated animals (Fig. 2H), probably corresponding to progenitor cells and immature cells of various lineages within the granulation tissue. In control animals (Fig. 2G), we found smaller numbers of Nestin positive vessels compared to the isoflurane group, where Nestin immunoreactivity in capillaries and larger vessels was significantly higher (Fig 2H). The mean number of Nestin-immunoreactive vascular profiles in granulation tissue of isoflurane treated animals was 7.7±1.7 per $mm^2$ and it was statistically higher (P<0.0001) than the average number in control animals (4.4±1.0 per $mm^2$).

When the impact of isoflurane on the increase of Nestin immunoreactivity is expressed as percent difference in relation to control animals, it appears that there is a 77.9% increase.

*Co-localisation of CD31 and Nestin*

We found no co-localisation of Nestin and CD31 immunoreactivity in non-ischemic heart tissue. In ischemic heart tissue of both groups of animals, co-expression of CD31 and Nestin immunoreactivity was found in cells within granulation tissue, as well as in the endothelium of blood vessels. CD31/Nestin positive cells, characterized with uniform green and red fluorescing cytoplasmatic staining (appearing orange when images were merged) was found to be more prominent in the isoflurane group (Fig. 3 E-H,I-L) than in the control group (Fig. 3A-D). The number of immunoreactive cells co-expressing CD31 and Nestin (not counting CD31/Nestin positivity in already distinguished blood vessels) in granulation tissue of isoflurane treated animals was 9136±145 per $mm^2$ and it was statistically higher (P<0.0001) than the number of co-expressing cells (7155±412 per $mm^2$) in control animals. (Fig. 3M).

## Discussion

We demonstrated that a significant, positive effect of isoflurane, applied in a 'post-conditioning' manner, can be found in our sub-acute ischemic injury model. Furthermore, we believe that the results of our experimental approach can be compared with the proven benefits of anesthetic pre-conditioning, as well as ischemic pre-/post-conditioning, and that we can offer some explanations for the histological differences found in the background of infarcts occurring in isoflurane treated animals compared to non-treated animals.

Our original diaphragmectomy-based approach resulted in excellent visibility of the anterior (sternal) surface allowing not only visualization but also evaluation of both the size of infarct and the intensity of reperfusion that could be precisely recorded and measured using a digital camera mounted on the microscope. We believe that our modification (Aljinovic et al., 2010) of the trans-diaphragmatic approach to the heart described by Huikeshoven et al. (2000) is a tangible approach for these, rather demanding, experiments.

There is a large body of evidence showing that volatile anesthetics have strong protective effects on both reversible and irreversible ischemic myocardial injury (Tanaka et al., 2004). Volatile anesthetics can be applied in two different ways; just like intermittent ischemic episodes of so-called ischemic pre-/post-conditioning, either before the ischemic episode or after it, during reperfusion. Although it was assumed that anesthetic and ischemic pre-conditioning share the same transduction pathway, it has been proven that anesthetic-induced preconditioning has more efficient and predictable cardioprotective properties (Sergeev et al., 2004).

We wondered if that is the case for anesthetic vs. ischemic post-conditioning too. In our study we found that the infarct size, four days after ischemia-reperfusion, was 56% smaller in isoflurane treated animals than in the control group when values of surface area counts were compared. Ischemic post-conditioning in most cases also leads to a significant reduction of

infarction. A comprehensive literature review, taking into consideration 40 publications on the subject of ischemic post-conditioning in rats (in vivo), showed that, in cases where duration of ischemia was between 30 and 45 minutes (in our case it was 30 minutes), significant infarct size reduction- between 23 and 60%- occurred (Skyschally et al., 2009). In most cases, the duration of reperfusion was only 2 hours and ischemic damage was presented and evaluated as an ischemic 'area at risk', detected by the absence of perfusion-induced labeling of affected myocardium. Interestingly, in nine of the above mentioned studies, the duration of reperfusion was 24 hours, and in five of those the reduction of area of risk was minimal and non-significant (Skyschally et al., 2009). Not a single, ischemic, post-conditioning study with reperfusion time longer than 24 hours was reported in the above review. Since the 'area at risk' changes very little, or does not change at all in relation to the left ventricular surface areas between control and post-conditioning animals (Skyschally et al., 2009) one can expect that the effect of post-conditioning (expressed as % of control) on the size of infarct would be similar if not the same value regardless of whether it is related to the area of risk or to the entire left ventricular area, which makes our results comparable with results from the literature. So far, investigations using isoflurane in a post-conditioning manner showed a positive effect regarding infarct size (Pagel, 2008), but focus mainly on a period of only a few hours after reperfusion. We demonstrated that the effect of isoflurane post-conditioning (markedly decreased infarct size), can clearly be detected four days after reperfusion. This effect of isoflurane post-conditioning is an important prognostic result in light of the fact that, in clinical medicine, a small infarct size (not surprisingly) appears to be a major determinant of favorable left ventricular remodeling (Masci et al., 2011). It seems that both timing of post-conditioning and concentration of anesthetic are crucial for the experimental outcome (Kin et al., 2004; Frassdorf et al., 2009). We chose a particular post-conditioning time (last 5 minutes of ischemia time and the first 10 minutes of reperfusion time) and isoflurane concentration (1.0 MAC), since it was proven that these concentration settings achieve the best myocardial sparing in short term experiments (Obal et al., 2001; Chiari et al., 2005; Frassdorf et al., 2009). In rabbits, the exposure to 1.0 MAC isoflurane during early reperfusion (3 minutes before and 2 minutes after beginning of reperfusion) achieved significant reduction of infarct/area of risk size of 43% (Chiari et al., 2005), which is still less than in our experiments. Hence, keeping in mind that there is probably a noticeable species difference, we can postulate that, extending isoflurane post-condition from 5 to 15 minutes in the above experiment would result in larger myocardial sparing.

Since it is well recognized that effective infarct healing depends on the creation of microvasculature networks providing regenerating tissue with nutrients and oxygen (Ren et al., 2002), we focused on morphological characteristics of microvasculature in the infarct areas and also on detection/quantification of several well established angiogenic factors.

The cardio protective effect of isoflurane appears to be at least partially mediated through extracellular signal-regulated kinase upregulation (Feng et al., 2006; Krolikowski et al., 2006) which, via induction of hypoxia-inducable factor 1-α (HIF 1-α), upregulates VEGF. We detected an overall 43.4% increase in expression of VEGF immunoreactivity in infarcted areas of isoflurane post-conditioned animals that was distributed in a variety of granulation tissue cells, from big ovoid and spindle shaped cells to endothelial cells in small blood vessels likely representing macrophages, myofibroblasts and endothelial progenitor cells that are reported to be expressing VEGF in hypoxic conditions (Chintalgattu et al., 2003; Urbich et al., 2005; van Amerongen et al., 2007). Our findings are in complete agreement with immunoblotting experiments in which isoflurane, applied in the pre-conditioning manner, produces large, time-dependent increases in VEGF expression, varying from 36% to 63% in acute reperfusion periods (Wang et al., 2006). Hence, we can postulate that in our experiment, isoflurane-induced increases in VEGF, like in pre-conditioning scenarios, may be combined with the production of reactive oxygen species (Tanaka et al., 2004) leading to 'positive feedback' stimulation of pro-survival antiapoptotic kinases.

Since one of the main characteristics of the proliferative phase of infarct healing is neo-angiogenesis /vasculogensis (Dobaczewski et al., 2010), in order to quantify specific angiogenic responses, we used PECAM-1/CD31 and Nestin immunoreactivity, which were proven to be useful early markers for this aspect of infarct healing (Ren et al., 2002; Ismail et al., 2003; Calderone, 2012). Having a distinct role in angiogensis as an essential endothelial adhesion molecule, PECAM-1/CD31 is particularly useful during inflammatory and early proliferative phases of healing (Park et al., 2010). In our experiments, both PECAM-1/CD31 and Nestin showed marked increases in their expression in post-conditioned animals, with a 136% and 77.9% increase when compared to controls, respectively. This pattern of expression of PECAM-1/CD31 appears to be typical for the early stage of infarct healing throughout the first week of reperfusion (Ren et al., 2002), even without any sort of 'conditioning', strengthening its validity as a very selective marker for neoangiogenesis in post-ischemic regeneration. The fact that the density of PECAM-1/CD31 positive vascular profiles in treated animals was more than double the density of control animals, and that it was found in larger/more mature vessels, suggests a potential dual effect of isoflurane post-conditioning: increased angiogenesis by sprouting with collateral growth of mural cells, and quicker maturation of newly

formed blood vessels in the granulation tissue. It appears that at this subacute/proliferating stage of infarct healing the main mechanism for re-establishment of blood supply is angiogenesis, and it seems that isoflurane post-conditioning speeds up that process.

Being aware that Nestin is a marker of more versatile progenitor cells, some of which are capable of differentiating to vascular cells in the early phase of scar formation and healing (Calderone, 2012), we focused only on capillaries and small blood vessels in both treated animals and controls. The density of Nestin vascular profiles was only one fifth of the density of PECAM-1/CD31 positive vascular profiles in both control and isoflurane treated animals. Although there was a marked increase of Nestin in treated animals, it was only about half of that observed for PECAM-1/CD31. Nestin was found in small, 'younger-looking' vessels linking their origin with endothelial progenitors, which is what we know as vasculogensis (de-novo formation) rather than angiogensis. It appears that both of these processes occur in the proliferative phase of infarct healing, that there is a slight time delay between them (vasculogenesis occurs later) and that isoflurane post-conditioning has a positive effect on both of them.

Finally, we wanted to see if isoflurane post-conditioning is a powerful enough stimulus to 'force' resident populations of cardiac Nestin positive cells (not already differentiated capillaries and blood vessels), possessing an intrinsic ability to differentiate to vascular, neuronal or glial cells in infarcted heart (Calderone, 2012), towards a vascular path. Hence, we performed double immunolabelling for Nestin and PECAM-1/CD31 and found a large degree of co-localization in infarcted granular tissue areas with an increase of 27.7% in isoflurane post-conditioned animals. Very high co-localization of PECAM-1/CD31 and Nestin was recently found in a study by Suzuki et.al., who interpreted this finding as de-novo formation of immature endothelial cells derived from endothelial progenitor cells (Suzuki et al., 2010). We would like to suggest that undifferentiated, rather large cells in the infarct healing areas, which co-express PECAM-1/CD31 and Nestin, are potential 'building materials' for vasculogenesis, which, at this stage of healing, have not reached their full potential.

Collectively, our data demonstrates that isoflurane post-conditioning, like preconditioning, enhances VEGF expression, probably by sharing the same mechanism, since it has a significant positive effect on neovascularisation and a comparable effect (with ischemic post-conditioning) on reduction of infarct size even four days after reperfusion. In other words, not only is the infarct much smaller, but it is also much more organized/vascularised. This multi-faceted positive effect of isoflurane post-conditioning should be explored further in light of its possible role in clinical practice, especially in light of commonly performed percutaneous coronary interventions (PCI) with stenting.

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