Cardiovascular Pathology 24 (2015) 94–101

Contents lists available at ScienceDirect

Cardiovascular Pathology

Original Article

Effects of isoflurane postconditioning on chronic phase of ischemia–reperfusion heart injury in rats

Ivan Agnić [a,*], Natalija Filipović [b], Katarina Vukojević [b], Mirna Saraga-Babić [b], Marija Vrdoljak [b], Ivica Grković [b]

[a] Department of Anesthesiology, University Hospital Split, Split, Croatia
[b] Department of Anatomy, Histology and Embryology, University of Split School of Medicine, Split, Croatia

ARTICLE INFO

Article history:
Received 20 February 2014
Received in revised form 9 September 2014
Accepted 9 September 2014

Keywords:
Myocardial infarction
Ischemia–reperfusion model
Chronic
VEGF
PECAM/CD31
Nestin

ABSTRACT

Introduction: The application of isoflurane in a postconditioning manner, during early reperfusion of ischemic myocardium, reduces the infarct size. Its favorable effect on highly vascularized granulation tissue formation is very important considering the fact that increased genesis of blood vessels in peri-infarct zone reduces the infarct size and improves cardiac function. Taking into consideration the influence of isoflurane on the subacute phase of infarct healing, by using different immunohistochemical markers, we wanted to explore whether isoflurane postconditioning influences the chronic phase of healing.
Methods: The size of infarcted region was measured, and comparisons between isoflurane-treated and control animals were made. Quality of infarcted area was assessed by detecting vascular endothelial growth factor (VEGF), platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31) as a marker of angiogenesis, and nestin as a marker of immature progenitor cells, and de novo formed blood vessels (vasculogenesis).
Results: There was no difference between the control and isoflurane-treated groups in VEGF and PECAM-1/CD31 expression. However, a large reduction in infarct size was found (68.1% of control). Also, a marked decrease of nestin expression in immature progenitor cells, along with a marked increase of the same marker in cardiomyocytes, (signs of myocardium regeneration), was found in experimental animals when compared to control animals that did not receive isoflurane treatment.
Conclusions: Based on our results, we can emphasize two morphologically detectable benefits of isoflurane postconditioning: a marked reduction in infarct size along with a more mature-looking infarct area in the chronic phase of infarct healing.

1. Introduction

Percutaneous coronary intervention and coronary artery bypass grafting (CABG) are therapeutic procedures that can be applied in order to reestablish blood flow in coronary arteries and reduce the size of cardiac infarction [1]. Despite its beneficial effects, reperfusion, paradoxically, increases the intensity of myocardial injury due to the effect on microvascular dysfunction, causing a stronger inflammatory response [2] and initiation of cellular death by apoptosis [3–5]. These effects inflict an additional damage to the cardiac muscle that is already affected by the ischemic injury. In order to reduce reperfusion injury of myocardium, various mechanical and pharmacological therapeutic procedures are available. They are usually applied immediately before, or at the very start, of the reperfusion and are referred to as postconditioning procedures [6].

In the last 15 years, postconditioning performed by the use of volatile anaesthetics came into use. With this approach, a level of infarct reduction similar to that achieved by ischemic postconditioning could be accomplished, with an added benefit of avoiding the risks of damage caused by balloon inflation in coronary vessels. The positive effect is a result of activation of enzyme paths explained in our last study [7].

It is known that postconditioning by isoflurane decreases the size of myocardial infarction, but most animal studies explored acute effects within a few hours from the beginning of reperfusion [8]. However, the process of myocardial infarction healing continues for several weeks and is divided into three overlapping phases: acute–inflammatory, subacute–proliferative, and chronic–maturation phase [9].

In our previous study, we established that postconditioning by isoflurane reduces the size of the myocardial infarct in the subacute phase of healing (day 4 after reperfusion), wherein a significantly higher number of blood vessels in granulation tissue of rats that were postconditioned by isoflurane were observed [7]. Taking into consideration that several studies indicated that the increased genesis of blood vessels in the peri-infarct zone reduces the infarct size and improves the cardiac function [10–12], by utilizing several immunohistochemical markers, we wanted to explore whether postconditioning with isoflurane influences the chronic phase of healing.

Funding: This study was funded by the Ministry of Science, Education, and Sports, Republic of Croatia (project nos. 216-2160528-0067 and 021-2160528-0507), whose support is gratefully acknowledged.

* Corresponding author at: Department of Anesthesiology, University Hospital Split, Spinčićeva 1, 21000 Split. Tel.: +385 91 2539497; fax: +385 21 557811.
E-mail address: agnic33@gmail.com (I. Agnić).

http://dx.doi.org/10.1016/j.carpath.2014.09.004
1054-8807/

Vascular endothelial growth factor (VEGF) strongly supports vasculogenesis ("de novo" formation of blood vessels from endothelial progenitors) and angiogenesis (i.e., sprouting and collateral growth of endothelial cells), microvascular permeability, and vasodilatation [13,14]. It is also important in the process of migration and proliferation of progenitor cells [15]. It has been demonstrated that myocardial reperfusion injury significantly increases the level of VEGF protein and its immune reactivity between days 3 and 7 after reperfusion, while the expression of VEGF significantly decreases on day 21 postinjury [16].

In our previous study, we demonstrated that postconditioning with isoflurane increases the expression of VEGF during the subacute phase (day 4 postinjury) of infarct healing in rats [17]. No data regarding the expression of VEGF and anesthetic postconditioning in the maturation phase of infarct healing can be found. Platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31) is a transmembrane glycoprotein that is expressed on the surface of endothelial cells [18]. It plays an essential role in angiogenesis [19], and its strong expression was found in emerging blood vessels during the healing of ischemic–reperfusion myocardial injury [20].

Nestin is an intermediary filament protein that has been used as a marker of immature progenitor cells capable of developing into different subpopulations of cells important to the process of infarct healing [21]. It is also a marker of proliferating endothelial progenitor cells in human and rat heart damaged by ischemia [22,23], as well as for "de novo" formed endothelial cells and blood vessels (vasculogenesis). It has been demonstrated that postconditioning by isoflurane increases the number of endothelial progenitor cells and blood vessels (formed by both processes: vasculogenesis and angiogenesis) during the subacute phase of healing [7]. To the best of our knowledge, the influence of postconditioning, using isoflurane, on the chronic phase of myocardial infarct healing has not been investigated until now. Hence, we have used the abovementioned markers to explore the influence of isoflurane postconditioning on the appearance and properties of myocardial infarct 14 days after ischemia–reperfusion injury.

2. Materials and methods

2.1. 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). Sprague–Dawley female rats ($n=8$) weighing 175–225 g were used. They 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, on a 12-h:12-h light–dark cycle. The standard rat chow (4RF21 GLP; Mucedola srl, Settimo Milanese, Italy) and water were available ad libitum.

The operation procedure was performed as we described in a previous study [7]. Briefly, rats were anesthetized with a combination of Ketaminol (Ketaminol 10, 1.2 ml/kg, Intervet International, Netherlands) and Xylazinum (Xylapan, 0.4 ml/kg, Vetoquintol, Switzerland). A mixture of anesthetics was injected into the right hamstring muscles. Ischemia was induced for 30 min. Starting from the last 5 min of ischemia up until 10 min into reperfusion time (15 min in total), to the isoflurane group ($n=4$) we gave 1.5% end-tidal concentration of isoflurane (1.0 minimum alveolar concentration), measured by using a Dräger PM 8050 (Dräger, Lübeck, Germany) gas monitor. The control group ($n=4$) received only an air/oxygen mixture. Cardiac ischemia was confirmed visually by the pale appearance of the heart surface and also by ST-segment elevation and T-wave inversion on electrocardiogram (ECG) (Cardioline Delta 1, Cavareno, Italy). After 30 min of ischemia, reperfusion was allowed and confirmed by immediate hyperemia over the surface of the heart, as well as by normalization of the ECG trace. The procedure was photographed using a Canon EOS 500D (Canon, Japan) digital camera mounted on a surgical microscope (Leica, M520 MC1, Switzerland).

2.1.1. Tissue processing and histological staining

Complete ischemia of the left ventricle, confirmed intraoperatively, was the main criterion for inclusion of the animal into the next phase of the study. The animals were left to survive until day 14 after reperfusion, when they were sacrificed and their hearts harvested. After fixation in Zamboni's fixative [4% paraformaldehyde and 0.20% picric acid in 0.1 M phosphate-buffered saline (PBS) at pH 7.4], the ventricles were rinsed, dehydrated, and embedded in paraffin wax. Tissues were serially sectioned (4–6-μm-thick sections) and mounted on glass slides. After deparafinization, sections were rehydrated in ethanol and water. Mallory trichrome staining was used as described previously [7].

To detect infarcted areas and to identify histological differences of infarcted areas between groups, slides 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 as we described previously [7]. In brief, the infarcted area was demarcated and "shifted" away from the image, and its total pixel size and the pixel size of the surface area of the left ventricular wall and the septum were expressed as the surface area (in mm^2; calibrated by standard size grid that was photographed with the section). The size of the infarct was expressed as the absolute value of its surface area and the percentage of the left ventricular wall and septum.

Following deparafinization, antigen retrieval for immunoflourescence staining was done by heating in sodium citrate buffer (pH 6.0) for 17 min at 95 °C in a microwave oven. The following primary antibodies were used: 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) overnight in a humidified chamber. After multiple washes in PBS, sections were incubated for 1 h with secondary antibodies. The secondary antibodies used were as follows: Streptavidin Alexa Fluor 488 conjugate (508205; Invitrogen, OR, USA, at 1:500 dilution); biotynilated goat anti-rabbit IgG (ab64256, Abcam, Cambridge, UK); and goat anti-mouse IgG antibody rhodamine, AP124R (Merck KGaA, Darmstadt, Germany), both diluted at 1:200. The sections were then washed in PBS and counterstained with 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei. Alternatively, after incubation with primary nestin, CD31, and VEGF antibodies and washing, sections were incubated with a biotynilated goat anti-rabbit IgG secondary antibody (ab64256, Abcam, Cambridge, UK) or goat anti-mouse IgG-B (sc-2039, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), both diluted at 1:100, for 20 min at room temperature. Detection was performed by using streptavidin peroxidase system (K0690, Dakocytomation, Carpinteria, CA, USA) and diaminobenzidine (DAB) reaction. Nuclei were counterstained with hematoxylin. Staining controls in both cases, immunohistochemistry and immunofluorescence, included omission of primary antibodies from the staining procedure, which resulted in the absence of staining in tissue. After final rinsing in PBS, all sections were air-dried, mounted (Immuno-Mount, Shandon, Pittsburgh, PA, USA), and coverslipped. DAB-stained sections were dehydrated and coverslipped. The sections were viewed and photographed using Olympus BX51 (Tokyo, Japan) microscope equipped with Olympus DP71 camera. Photographs were processed with Cell A Imaging Software for Life Sciences Microscopy (Olympus, Japan).

2.1.2. Quantification of VEGF, nestin, and nestin/CD31 immunoreactivity

Photographs were analyzed by two independent investigators. VEGF-, nestin-, and nestin/CD31-positive cells were classified as negative (no stained cells) and positive (stained cells) cells and quantified throughout the infarction area.