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Original article

DOI: 10.1515/rrlm-2016-0022

Exposure of Human Endothelial Progenitors to Sevoflurane Improves Their Survival Abilities

Efectele sevofluranului asupra proliferării și apoptozei progenitorilor endoteliali umani in vitro

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Abstract

Endothelial progenitor cells (EPCs) have prominent roles in vessel and tissue repair; however, their regenerative efficacy is diminished due to the poor survival in the hostile microenvironment of the injured organs. Recent data suggest a promising potential of volatile anesthetics for improving stem cell biology. Thus, we hypothesized that exposure to sevoflurane could stimulate growth and viability of cultured EPCs.

Total mononuclear cells were isolated from human umbilical cord blood by gradient centrifugation. After five days in culture, the cells were exposed for one or two hours to sevoflurane 2% or 4% in air/5% CO_2, or only to air/5% CO_2 (sham control) in a sealed modular chamber. 24 or 48 hours post-exposure, viability, proliferation and apoptosis were assessed using lactate dehydrogenase (LDH) leakage assay, a methyl tetrazolium salt (MTS) assay and FITC-annexin V/ propidium iodide (PI) staining, respectively.

LDH leakage was discretely lowered, whereas the levels of formazan were significantly increased ($p < 0.05$ for 1 h incubation with 4% sevoflurane at 24 hrs post-exposure, and with 2% sevoflurane at 48 h post-exposure) in the preconditioned cultures, proving no cytotoxic effects and increased proliferation in treated cells versus control samples. Early ($p < 0.05$) and late apoptosis ($p < 0.05$ only for 2% sevoflurane) were diminished following the procedure.

Thus, the commonly used sevoflurane anesthetic has protective effects on viability and proliferation of human early endothelial progenitor cells in vitro, suggesting a promising potential of anesthetic preconditioning for improving the regeneration of ischemic tissues.

Keywords: *priming, sevoflurane, progenitor cells, apoptosis, proliferation.*

Rezumat

Celulele progenitoare endoteliale (CPE) dețin roluri importante în regenerarea tisulară, însă potențialul terapeutic al acestora este diminuat de scurta supraviețuire în microclimatul indus de ischemie. Studii recente demonstrează proprietăți protectoare ale anestezicelor volatile asupra biologiei celulelor stem. În consecință, ne-am propus să

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investigăm efectele expunerii la sevofluran asupra viabilitații și proliferării CPE in vitro.
Culturi de celulele mononucleare izolate din sânge uman recoltat din cordonul ombilical au fost expuse în ziua a cincea la sevofluran 2% sau 4% în aer/ 5% CO_2, timp de una sau două ore, sau numai la aer/ 5% CO_2 (control) într-o incintă modulară închisă etanș. După 24 sau 48 de ore de la expunere au fost evaluate viabilitatea, proliferarea și apoptoza prin testul eliberării lactat dehidrogenazei (LDH), testul reducerii sărurilor de metil tetrazoliu (MTS) și respectiv testul dublei marcări cu anexina V-FITC/ iodura de propidiu.
Nivelurile LDH-ului eliberat în supernatant au fost discret scăzute, în vreme ce concentrația formazanului a crescut semnificativ în probele precondiționate, demonstrând absența citotoxiciații și stimularea proliferării dupa expunerea la anestezicul volatil. Atât apoptoza precoce cât și cea tardivă au fost diminuate în probele incubate cu sevofluran față de control.
În concluzie, sevofluranul, un anestezic volatil larg folosit în clinică, crește viabilitatea și proliferarea culturilor de celule angiogenice umane, sugerând un potențial promițător al precondiționării anestezice pentru regenerarea țesuturilor ischemice.

Cuvinte cheie: *precondiționare, sevofluran, celule progenitoare, apoptoza, proliferare.*

Received: 23th December 2015; Accepted: 3th May 2016; Published: 27th May 2016

Introduction

Endothelial progenitor cells play important roles in regenerating ischemic tissues, either by being mobilized from the bone marrow and recruited in the injured area (1)(5), or following their exogenous administration as autologous or allogeneic cell transplants (2)(3)(4). Injected EPCs can differentiate into mature endothelial cells and stimulate angiogenesis, vasculogenesis and cardiac function by inserting in the injured vessels or through paracrine secretion of angiogenic factors (5)(6). However, stem cell engraftment for the treatment of tissue ischemia has a limited benefit due to impaired survival and function of donor cells in the damaged area (7), observation that led to the development of several methods for improving their post-transplantation behavior (8).

An increasing body of experimental data demonstrates cardioprotective and proangiogenic effects of preconditioning with volatile anesthetics, observed at organ and cellular levels (9)(10)(11). Recently, it has been suggested that the protective outcomes of anesthetic preconditioning may involve the modulation of stem cell dynamics and function. Enhanced mobilization of bone marrow endothelial progenitors following exposure to sevoflurane was reported by our group in rodents (12), and cultured EPCs isolated from healthy human volunteers preconditioned with sevoflurane exhibited a better colony-forming capacity (13). Moreover, in vitro priming with the same volatile anesthetic increased the number of colony-forming units and the expression of mRNA vascular endothelial growth factor (VEGF) in cultures of EPCs isolated from human umbilical vein blood (13) and improved the viability and function of human mesenchymal stem cells (MSCs) (14).

On these grounds, and considering the importance of EPCs survival abilities for the healing of ischemic tissues, we investigated the effects of in vitro exposure to the broadly used anesthetic sevoflurane volatile anesthetic on the viability and proliferation of endothelial progenitors isolated from human umbilical cord blood.

Methods

EPCs isolation

Umbilical cord blood samples were collected from placentas of healthy women aged 22 – 38 years with no signs of infection and gestational ages between 38/0 and 40/2 weeks, immediately after elective cesarean delivery. The study protocol was

approved by the ethics committee of Filantropia Hospital Bucharest, Romania and all subjects gave written informed consent. EPCs were cultured as previously described (15)(16). Total mononuclear cells were isolated by Biocoll (Biochrom, Germany) density gravity centrifugation. After three washing steps, cells were resuspended in Endothelial Growth Medium 2 Bullet Kit (EGM2, Lonza, Belgium) supplemented with 20% fetal bovine serum (FBS), and plated on fibronectin coated culture dishes (96-, 24-well plates or 8-well glass slides) at a density of 1 x 10^6 cells/ cm^2. Nonadherent cells were removed after 4 days, and the media were then changed every other day. Cultures obtained by this method contain a heterogenic population of cells, termed early endothelial progenitors or cultured angiogenic cells, recognized to promote angiogenesis rather by paracrine influence than through endothelial commitment (17)(18). Cells were preconditioned in day 6, and assays were performed 24 or 48 hours afterwards.

EPCs characterization

Cultured EPCs were characterized by immunofluorescence as adherent cells double positive for aceltylated low density lipoproteins (AcLDL) uptake and Ulex Europaeus Agglutinin I (UEAI) binding, using previously described techniques (16)(19). The capacity of these cells to express endothelial markers such as KDR (kinase insert domain receptor), vWf (von Willebrand factor), CD31 and eNOS (endothelial nitric oxide synthase) and to facilitate vascular repair in vivo was previously demonstrated (20)(21). On day 7, adherent cells cultured on 8-well chamber slides (Thermo Scientific Nunc, USA) were incubated firstly with DiI-AcLDL (2.5 mg/mL, Life Technologies, USA) for 2 hours in a cell culture incubator, washed, fixed with CellFIX (BD Biosciences, USA), and then stained with FITC-UEAI (10 mg/ mL, Sigma, USA) for 1 hour. After washing, the nuclei were marked with DAPI (4',6-diamidino-2-phenylindole) and 6 high power fields (x 200 magnification) were analyzed using an inverted fluorescent microscope (Nikon TE300), equipped with a Nikon DS-Qi1 camera and NIS Elements software (Nikon Instruments, USA). More than 95% of the adherent cells stained positive for both FITC-UEAI and DiI-AcLDL (**Figure 1**).

Protocols for in vitro preconditioning with sevoflurane

In day 6, culture dishes were placed in an airtight modular incubator chamber (Billups-Rothenberg, USA), connected through a flow-meter (Billups-Rothenberg, USA) to a sevoflurane vaporizer (Penlon, UK) and to a source of air and 5% CO_2 mixture. The chamber was purged with sevoflurane at a concentration of 2 vol % or 4 vol % in air/ 5% CO_2 for 10 min at a flow of 10 L/ min, according to manufacturer's recommendations, sealed and placed thereafter in a cell culture incubator for one or two hours. Sevoflurane concentration was verified with a Riken FI-21 system (Riken Keiki, Japan). Sham-control samples were exposed to air/ 5% CO_2 only. The effects of sevoflurane were assessed 24 and 48 hours afterwards.

Lactate dehydrogenase release assay

Release of lactate dehydrogenase (LDH), an indicator of membrane integrity, was determined with a Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific, USA), following the manufacturer's protocol. In short, 1 x 10^6 cells/ cm^2 cultured in fibronectin coated 96-well plates were primed in day 6, and 24 or 48 hours later the assay was performed. The culture media was changed before the preconditioning procedure. Lysis Buffer as positive control was used to completely lyse the cells and release the maximum LDH.

To determine the LDH leakage of treated cells, the supernatant was transferred in 96-well plate, and 50 µl of LDH reaction solution was added to the cells for 30 min at room temperature and in the dark. The absorbance was measured at 492 nm using a Microplate Reader V 1.0 Asys Hitech Expert 96 (Biochrom, UK) and data were analyzed with the software provided (Kim Version 5.45.0.1; Daniel Kittrich). Sample absorbance was expressed relative to maximum LDH release values. LDH leakage in preconditioned EPCs cultures was expressed as percentage of sham control samples.

Cell proliferation by MTS Assay

Cell growth and viability were determined by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-suofophenyl)-2H-tetrazolium, inner salt] assay using the CellTiter 96H AQueous One Solution Cell Proliferation Assay kit (Promega, USA) and following manufacturer's instructions. 1 x 10^6 cells/ cm^2 were seeded in fibronectin 96-well plates and after 24 or 48 hours following the preconditioning protocol, 20 µl of MTS solution was added to each well. Plates were incubated for an additional 4 hours at 37 ºC,

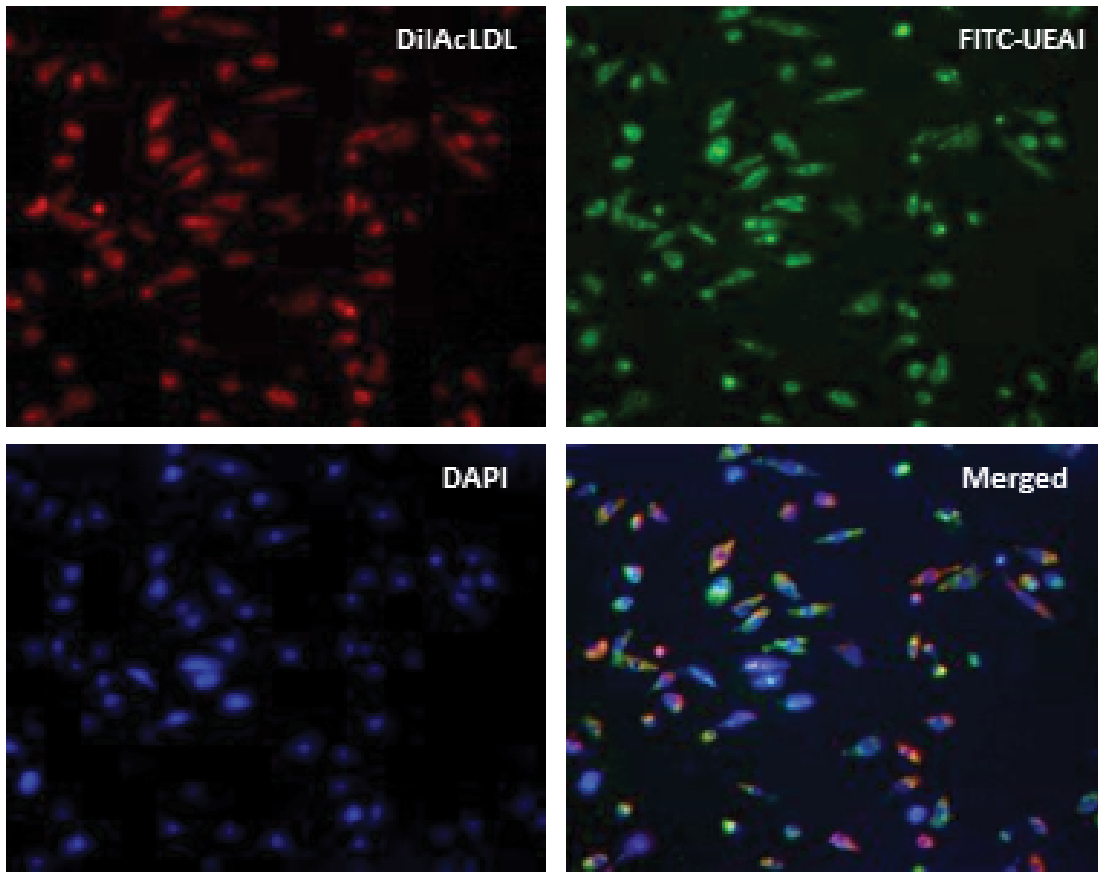

Figure 1. Endothelial progenitor cell characterization. Adherent cells are positive for the uptake of DilAcLDL and the binding of FITC-UEAI. DAPI staining shows that nuclei are present in all cells DilAcLDL+/FITC-UEAI+. Magnification, x200.

and the absorbance at 492 nm was recorded thereafter using a Microplate Reader V 1.0 Asys Hitech Expert 96 (Biochrom, UK) with the provided software (Kim Version 5.45.0.1; Daniel Kittrich). The absorbance of control cells was considered 100% viability and the values of treated cells were calculated as percentage of control.

Detection of apoptosis

Cell apoptosis was assessed via an FITC Annexin V Apoptosis Detection Kit I (BD Pharmigen, USA). In short, 24 hrs after the priming procedure the cells were gently detached with 0.5 mM EDTA from fibronectin 24-well plates, collected and prepared for flow cytometry according to the manufacturer's instructions. After incubation in the dark at room temperature for 15 minutes, data were acquired with a BD FACSCanto II flow cytometer (Becton Dickinson, USA) and the analysis was performed using a BD FACSDiva 6.1. software. Cells stained for annexin V-FITC only were considered positive for early apoptosis, whereas those stained for both annexin V-FITC and propidium iodide (PI) were identified as late apoptotic or necrotic. Only the 2 hours exposure protocol was tested.

Statistical analysis

Data analysis was performed using a GraphPad InStat software package (GraphPad Software Inc., USA). Results are expressed as mean ± SEM. Differences between treatments were computed using ANOVA parametric tests (Tukey-Kramer multiple comparisons test). A $p < 0.05$ was considered statistically significant.

Results

Comparative analysis of LDH leakage showed similar levels in cultures treated with sevoflurane and control samples (95.99% ± 1.60 and 94.42% ± 1.30 vs. 100% sham control for 1h exposure to 2% sevoflurane, at 24 hrs and 48 hrs respectively; 96.25% ± 1.30 and 95.17% ± 0.03 vs. 100% sham control for 2 hrs exposure to 4% sevoflurane, at 24 h and 48 h respectively; **Figure 2**), demonstrating that none of the used protocols is cytotoxic.

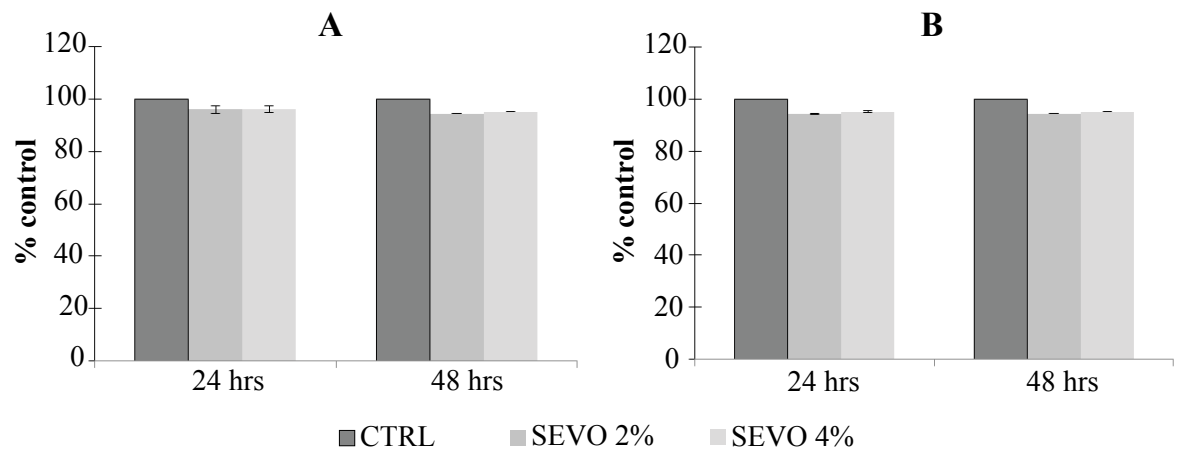

Figure 2. LDH leakage of EPCs exposed to sevoflurane at 24 hours and 48 hours follow-up. A) One hour in vitro exposure. B) Two hours in vitro exposure. Data are presented as percentage of control ± SEM of four independent experiments; p > 0.05 versus control; CTRL – control, SEVO – sevoflurane.

The overall viability of the culture assessed with the MTS method was increased by sevoflurane (**Figure 3**), showing a statistically significant proliferative effect for the 1 h exposure protocol. 2% sevoflurane induced a better proliferative effect at 48 hrs post-exposure vs sham control (104.13% ± 4.36 at 24 hrs post-exposure, ns., and 124.13% ± 11.46 at 48 h, $p < 0.05$), whereas the effect of incubation with a double concentration reached a plateau at 24 h follow-up (117.69% ± 4.42 at 24 hrs, $p < 0.05$, and 117.04% ± 7.23 at 48 hrs, vs. 100% control). Proliferation of cultures preconditioned for 2 hrs had an ascending profile over time, with a more prominent increase at 48 hrs follow-up in comparison to the 1h exposure, without reaching statistical significance, though (113.55% ± 7.81 and 132.36% ± 15.34 vs. 100% sham control for priming with 2% sevoflurane, at 24 hrs and 48 hrs respectively; 105.30% ± 5.12 and 130.31% ± 5.79 vs. 100% control after exposure to 4% sevoflurane, at 24 hrs and 48 hrs, respectively). Many studies reported antiapoptotic effects of sevoflurane on neuronal cells (22)(23), cardiomyocytes (24)(25) and recently on mesenchymal stem cells (14). Thus, we tested whether anesthetic preconditioning could reduce programmed death of cultured early EPCs using annexin V and PI staining. Indeed, EPCs cultures exposed for 2 hrs to sevoflurane had a significantly lower percentage of cells in early and late apoptosis versus sham samples, at 24 hrs follow-up (**Figure 4**).

Discussions

The necessity of improving survival of endogenous or injected stem cells in ischemic tissues was emphasized by numerous animal and clinical studies. Our goal was to optimize a method that allows studying in vitro the effects of volatile anesthetics on EPCs viability and function, and that might as well have a good potential for helping transplanted cells to better cope with the rough ischemic microenvironment of the host tissue.

None of the tested preconditioning protocols was cytotoxic, as demonstrated by the LDH

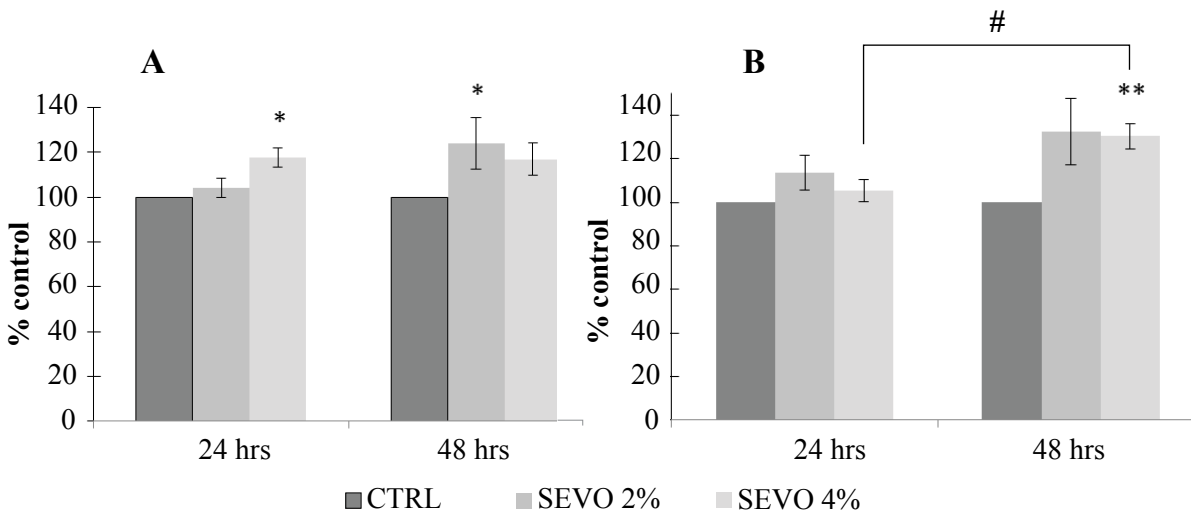

Figure 3. Proliferation of EPCs incubated with sevoflurane at 24 hours and 48 hours follow-up. A) One hour in vitro exposure. B) Two hours in vitro exposure. Data are presented as percentage of control ± SEM of four independent experiments. *P < 0.05 versus control, **p = 0.068 versus control, #p < 0.05 48 hrs versus 24 hrs follow-up; CTRL – control, SEVO – sevoflurane.

leakage and MTS assays. Furthermore, the levels of metabolic activity in the primed cultures were substantially increased, proving an expansion of the cellular population induced by sevoflurane. Our data are in line with the findings of Luchinetti et al, who reported that discontinuous exposure to 2% sevoflurane increases the colony forming units in EPCs cultures (13). However, decreased proliferation in MSCs exposed to serum deprivation and hypoxia could not be significantly improved by in vitro preconditioning with 2% sevoflurane for 2 hrs (14). It is important to note that in our setting the effect of sevoflurane on EPCs proliferation maintained and even increased over time (significantly higher values at 48 versus 24 hrs following the procedure, **Figure 3**), suggesting long lasting outcomes provided by the anesthetic preconditioning.

Developing a strategy that mitigates apoptotic cell death of angiogenic cells is of central importance for the infracted heart treatment. Halogenated anesthetics were proven to exert pro- or antiapoptotic effects on various cell types and in diverse experimental settings. Sevoflurane post-conditioning decreases apoptosis acting on the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/ mTOR pathway in ischemia/ reperfusion (I/R) rat hearts (25). Combination

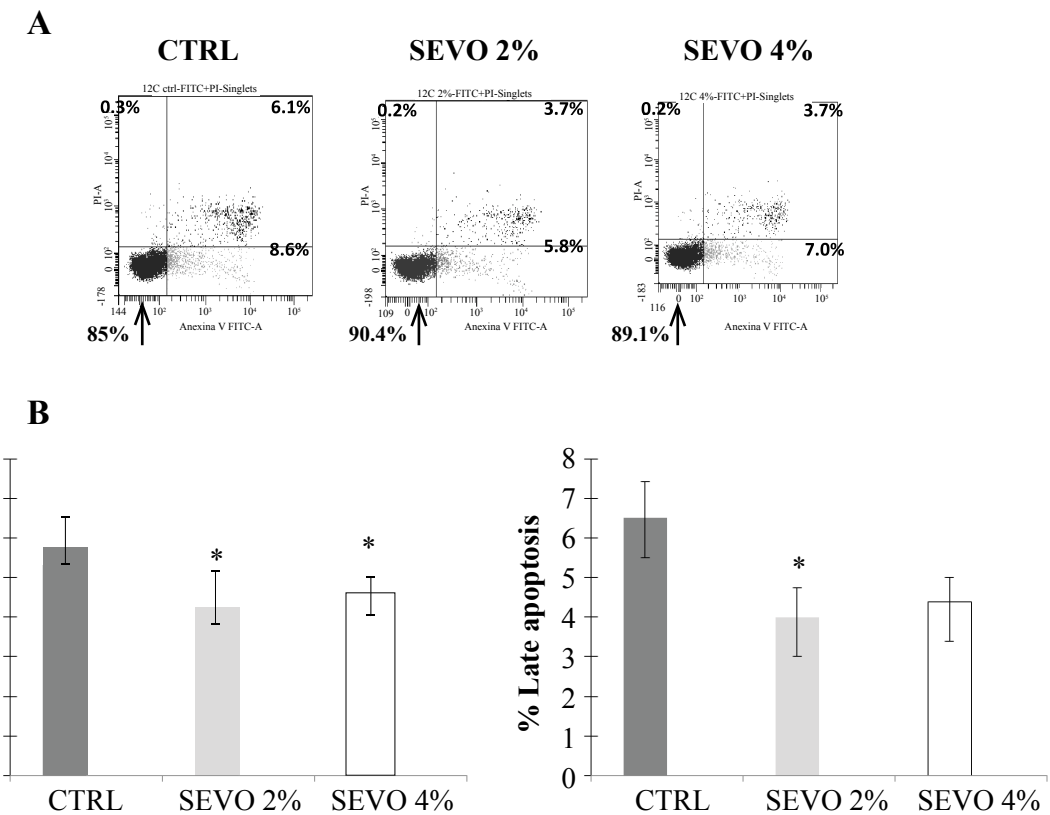

Figure 4. Early and late apoptosis of EPCs is reduced by in vitro exposure to sevoflurane. A) Flow cytometry of a representative experiment for assessing EPCs early (annexin V+/ PI-) and late (annexin V+/ PI+) apoptosis. B) Quantification of EPCS apoptosis. Data are expressed as mean ± SEM of five independent experiments. *P < 0.05 versus control; CTRL – control, SEVO – sevoflurane.

therapy with sevoflurane and propofol exerts anti-apoptotic effects via Bcl-2/Bax pathway in rat myocardium exposed to I/R (24), and apoptosis and cellular autophagy is reduced, whereas lysosomal function is improved in rat myocytes following sevoflurane postconditionig (26). Also, apoptosis induced in hypoxic and serum deprived MSCs can be alleviated following in vitro preconditioning with 2% sevoflurane for 2 hrs. In line with these observations, we demonstrate here for the first time a consistent antiapoptotic effect of sevoflurane on EPCs cultures. Interestingly, exposure to 2% sevoflurane was more efficient in lowering apoptosis and improving proliferation than to the 4% concentration.

Stem cells exposed in vitro to anoxic or hypoxic environment are more resistant when transplanted to ischemic organs, exhibit decreased apoptosis and produce increased angiogenesis and functional benefits in the host organ (27)(28)(29). Adding VEGF to cultured EPCs increased their survival, and VEGF mRNA levels were higher in hypoxic MSCs, as well as in EPCs primed with sevoflurane (28)(29)(13). Our results show enhanced growth and viability in cultured angiogenic cells exposed to sevoflurane. Hence, anesthetic preconditioning can be a good alternative to hypoxic priming and a feasible strategy for improving donor cell resistance in the ischemic tissues.

Compared to other volatiles, sevoflurane is largely used in cardiac and non-cardiac surgical interventions due to its faster and smoother induction and recovery. As it was previously proven, in vivo preconditioning with this anesthetic is able to augment the number of circulating bone marrow derived progenitor cells (12), and possibly, in light of our present findings, could also improve the survival of recruited or resident endogenous EPCs in ischemic or injured tissues.

A larger sample size would have led undoubtedly to more consistent results; however, we could establish a preconditioning protocol that produces a clear protective response, which can be used in future studies for investigating the effects of sevoflurane on EPCs functional properties. Another limitation is that proliferation was determined in the global cell population of a heterogenic culture, whereas more specific results could have been provided by counting the cells double positive for DilAcLDL/FITC-UEAI (30), or the colonies derived from progenitors using colony forming unit (CFU)-assays (13)(19). We preferred, nonetheless, this approach reasoning that an MTS assay, paralleled by the measurement of LDH leakage, is more objective than cell or colony count.

Conclusions

In vitro exposure to sevoflurane stimulates proliferation and reduces apoptosis of cultured angiogenic cells isolated from human umbilical cord blood. No cytotoxic effects were observed following exposure to 2% or 4% sevoflurane, for one or two hours. Preconditioning with 2% sevoflurane for two hours seems to have the best impact on cell growth and viability.

Acknowledgement

The authors thank Dr. Anca Panaitescu and Dr. Ruxandra Cigaran from Filantropia Hospital, "Carol Davila" University of Medicine and Pharmacy Bucharest, Romania for the blood sample collection, and express their special gratitude to all patients for accepting to be enrolled in this study. No competing interests exist with respect to authorship, research and/or publication of the present work. The financial support was provided by the Romanian Ministry of National Education, PN-II-RU-TE-2012-3-0463 project, grant number TE-RU-41/ 29.04.2013.

Abbreviations

AKT - protein kinase B
Bax - Bcl-2 - associated X protein

Bcl-2	- B-cell lymphoma 2
CFU	- colony forming unit
DAPI	- 4',6-diamidino-2-phenylindole
Dil-AcLDL	- aceltylated low density lipoproteins labeled with 1,1\'-dioctadecyl – 3,3,3\',3\'-tetramethyl-indocarbocyanine perchlorate
EGM	- endothelial growth medium
eNOS	- endothelial nitric oxide synthase
EPC	- endothelial progenitor cells
FBS	- fetal bovine serum
FITC	- fluorescein isothiocyanate
KDR	- kinase insert domain receptor
LDH	- lactat dehydrogenase
MSCs	- mesenchymal stem cells
mTOR	- mammalian target of rapamycin
MTS	- 3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl) -2-(4-suofophenyl)-2H- tetrazolium, inner salt
PI3K	- phosphatidylinositol 3-kinase
PI	- propidium iodide
SEM	- standard error of the mean
UEAI	- Ulex Europaeus Agglutinin I
VEGF	- vascular endothelial growth factor
vWf	- von Willebrand factor

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