Mitochondrial Molecular Basis of Sevoflurane and Propofol Cardioprotection in Patients Undergoing Aortic Valve Replacement with Cardiopulmonary Bypass

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Key Words

Cardioprotection • Sevoflurane • Propofol • Mitochondria • Ischemia/reperfusion

Abstract

Background/Aims: Study elucidates and compares the mitochondrial bioenergetic-related molecular basis of sevoflurane and propofol cardioprotection during aortic valve replacement surgery due to aortic valve stenosis. **Methods:** Twenty-two patients were prospectively randomized in two groups regarding the anesthetic regime: sevoflurane and propofol. Hemodynamic parameters, biomarkers of cardiac injury and brain natriuretic peptide (BNP) were measured preoperatively and postoperatively. In tissue samples, taken from the interventricular septum, key mitochondrial molecules were determined by Western blot, real time PCR, as well as confocal microscopy and immunohisto- and immunocyto-chemical analysis. **Results:** The protein

levels of cytochrome c oxidase and ATP synthase were higher in sevoflurane than in propofol group. Nevertheless, cytochrome c protein content was higher in propofol than sevoflurane receiving patients. Propofol group also showed higher protein level of connexin 43 (Cx43) than sevoflurane group. Besides, immunogold analysis showed its mitochondrial localization. The mRNA level of mtDNA and uncoupling protein (UCP2) were higher in propofol than sevoflurane patients, as well. On the other hand, there were no significant differences between groups in hemodynamic assessment, intensive care unit length of stay, troponin I and BNP level. **Conclusions:** Our data indicate that sevoflurane and propofol lead to cardiac protection via different mitochondrially related molecular mechanisms. It appears that sevoflurane acts regulating cytochrome c oxidase and ATP synthase, while the effects of propofol occur through regulation of cytochrome c, Cx43, mtDNA transcription and UCP2.

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Introduction

In patients with severe aortic valve stenosis increasing pressure loading of the left ventricle is subsequently followed by its hypertrophy. Increased left ventricle wall stress leads to relative coronary insufficiency associated with a transient depression of myocardial function postoperatively. Intraoperative aortic cross clamping might provoke ischemic/reperfusion injury and thus anesthetic preconditioning would be valuable in these clinical conditions. Anesthetic preconditioning is a phenomenon whereby transient heart exposure to the anesthetics leads to increased resistance on ischemia/reperfusion and it was confirmed repeatedly in last decade for volatile anesthetic, including sevoflurane. Transient exposure to volatile anesthetics, during coronary artery bypass surgery, prior to cardiopulmonary bypass, demonstrated reduction in the need for inotropic support. In a study of De Hert et al. [1] in patients underwent coronary artery bypass surgery, different anesthetic regimes were investigated and it was emphasized that duration of exposure to volatile anesthetics is of the most significance for anesthetic-mediated heart protection. They pointed out that the cardioprotective effects were more evident when anesthetics were administrated throughout whole procedure.

Besides volatile anesthetics, cardioprotective effects were clinically confirmed for intravenous anesthetics such as propofol [2, 3]. While some studies suggest that inhalation agents were superior to propofol [4-7], other have shown no difference between two anesthetic types [8] or even better cardioprotective capacities of propofol [9]. It seems likely that the efficacy of the two anesthetic modalities in cardioprotection and their relation are dependent on the severity and type of heart disease. The large clinical study, including different types of coronary patients, have reported that both sevoflurane and propofol posses some, although, different cardioprotective properties [9]. Most studies comparing two types of anesthetics were based on postoperative biochemical markers of myocardial injury [4-9]. However, very few studies compared the signaling cascade of volatile and intravenous anesthetics cardiac protection and molecular players involved [10].

Identifying underlying mechanisms of above mentioned protective capacity of anesthetics in the heart ischemia/reperfusion is the subject of intense research more than 20 years. It has been suggested that several endogenous signaling pathways, acting through protein kinase C and K_{ATP} channels [11, 12] and involving the

generation of reactive oxygen species (ROS) [13, 14], mediate the anesthetics antiischemic actions. Also, anesthetic preconditioning acts improving mitochondrial electron transport function [15, 16] and ATP level [17] after ischemia. So, it seems likely that mitochondria are key mediators of cardiac protective effects of anesthetics.

To shed more light on the molecular mechanisms of volatile and intravenous (sevoflurane and propofol, respectively) anesthetics cardioprotection and the differences in their action, specifically on mitochondrial level, we investigated here anesthetic-specific expression profiles of mitochondrial bioenergetic-related molecules, including complex I, cytochrome *c*, complex IV, ATP synthase and uncoupling protein 2 (UCP2) as well as mtDNA transcription, in patient undergoing aortic valve replacement (AVR) due to aortic stenosis. Besides, the effects of sevoflurane and propofol on hemodynamics and biomarkers of cardiac injury (creatine kinase, creatine kinase MB and troponin I) in these patients were compared.

Materials and Methods

The study was approved by the Ethical Committee (Dedinje Cardiovascular Institute, Belgrade, Serbia), and written consent was obtained from all patients. Twenty two patients scheduled to undergo elective AVR due to severe aortic stenosis, aortic valve area (AVA) <1 cm², with cardiopulmonary bypass (CPB) were enrolled in this study. Preoperative exclusion criteria were: previous heart surgery (coronary, valvular or aortic reconstructive surgery), concomitant: coronary or valvular disease, aortic valve insufficiency, acute congestive heart failure, renal insufficiency (creatinine concentration >1.5 mg/dL), as well as presented carotid artery disease (stenosis >50%), severe hepatic disease (alanine or aspartate aminotransferase >150 U/L) and severe chronic obstructive pulmonary disease.

Study groups

Preoperatively, patients were randomly allocated to two different anesthetic regimens. In the first, sevoflurane group (11 patients), after anesthesia induction by intravenous drugs (midazolam-sufentanil), anesthesia was performed with sevoflurane and continuous infusion of sufentanil while in the second, propofol group (11 patients), for the induction and maintenance of anesthesia, intravenous drugs were used (continuous propofol-sufentanil infusion).

Anesthetic protocols

All cardiac preoperative medication, except for the angiotensin-converting enzyme inhibitors, was continued until the morning of surgery. Antiplatelet therapy with acetylsalicylic acid was stopped for one week. Premedication was the same in

all patients in both groups. Premedication of 5 mg midazolam, 6 mg morphine-sulphate and 0.5 mg atropine were given 20 min prior to surgery by intramuscular injection. Prior to induction of anesthesia routine monitoring has been established, including five-lead electrocardiography, capnography, pulse oxymetry and arterial (radial) pressure. After the induction of anesthesia, central venous and pulmonary artery catheters were placed and pressures were measured, while temperature was measured with naso-pharyngeal probe.

In propofol group, anesthesia was induced with 1-1.5 mg·kg⁻¹ of propofol, followed by 0.7-1 mcg·kg⁻¹ sufentanil, and muscle paralysis was obtained with 0.1 mg/kg pancuronium bromide. Anesthesia was maintained with propofol according to manual infusion regimen, 10 mg · kg⁻¹ · h⁻¹ for 10 min, 8 mg · kg⁻¹ · h⁻¹ for the next 10 min and 6 mg · kg⁻¹ · h⁻¹ and sufentanil continuous infusion 0.1-0.2 mcg · kg⁻¹ · h⁻¹ until the beginning of CPB.

In sevoflurane group, after the induction of anesthesia with midazolam 0.3 mg·kg⁻¹ and sufentanil 0.7-1 mcg·kg⁻¹, muscle relaxation with 0.1 mg/kg pancuronium bromide, anesthesia was maintained at 1-2 end-tidal minimal alveolar concentration (MAC) with sufentanil continuous infusion of 0.1-0.2 mcg · kg⁻¹ · h⁻¹ until the start of CPB. MAC is defined as the concentration of the volatile anesthetics in the alveoli that is needed to prevent movement (motor response) in 50% of subjects in response to surgical (pain) stimulus. Routine surgical technique and cardioprotective strategies were used in all patients of both groups. In addition, all patients received 500 mg methylprednisolone after the induction of anesthesia. All patients received 350 IU · kg⁻¹ heparin before the start of CPB and activated coagulation time was kept above 480 sec throughout the duration of CPB. After the aortic cross-clamping crystalloid cardioplegia was given. Also, glucose-insulin-potassium solution was used (30% glucose, insulin 2 IU · kg⁻¹ · L⁻¹ and KCl solution 80 mmol/L, infusion rate 1 mL · kg⁻¹ · h⁻¹). Blood salvage techniques included tranexamic acid and desmopressin administration, as well as intra-operative cell salvage. The perfusion rate was maintained between 2.0 and 2.4 L · m⁻² · min⁻¹ during moderate hypothermia (32 °C esophageal temperature), while hematocrit was maintained between 20 and 25%. The mean perfusion pressure was maintained at 50-70 mm Hg.

Standard median sternotomy and pericardiectomy were performed. After the aortic cross-clamping and aortotomy, stenotic aortic valve has been excised. Tissue sample of myocardium has been taken from the interventricular septum and it was immediately frozen in liquid nitrogen and stored at -70°C.

After the valve replacement, reperfusion time on CPB was set at 50% of the aortic cross clamp time, patients were rewarmed to a temperature of 36.5°C, the heart was paced in atrioventricular sequential mode at a rate of 90 beats/min, and the patients were weaned from CPB. If it was needed, inotropic or vasopressor therapy was started. Heparin activity was neutralized with protamine at a ratio of 1 mg protamine for 100 IU of heparin. After the surgical procedure anesthesia was maintained with sufentanil (0.1-0.2 mcg · kg⁻¹ · h⁻¹) and propofol (4-6 mg · kg⁻¹ · h⁻¹) in propofol group and sevoflurane (0.5-1 end-tidal

MAC) in sevoflurane group. After the surgery, patients were transferred to the intensive care unit (ICU). Postoperatively they were further sedated until sufficiently rewarmed and hemodynamically stable and then weaned from the ventilator and extubated, when following parameters were achieved: no significant dysrhythmias, hemodynamic stability, without major bleeding, with temperature >36.5°C, with adequate level of consciousness and satisfied pain control as well as without significant inotropic support.

Hemodynamic data analysis

Global hemodynamic data, mean arterial pressure (MAP), pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), cardiac output and cardiac index (CO, CI), and systemic vascular resistance (SVR), pulmonary vascular resistance (PVR), left ventricular stroke work index (LVSWI) and right ventricular stroke work index (RVSWI) were measured and recorded just before the start of surgery (T0), at the end of procedure (T1), 6 h (T6) and 24 h (T24) after the arrival in the ICU.

Biochemical analysis

In all patients blood was sampled for determination of creatine kinase (CK), creatine kinase MB (CKMB) and troponin I (TI) concentration 16 h and 24 h after the operation. For troponin I, sensitivity of the assay was 0.04 ng/mL. Values of brain natriuretic peptide (BNP) were also determined preoperatively and 7 days after the procedure.

Data about aortic cross clamp time, total reperfusion time, CPB time, duration of mechanical ventilation and time spent in ICU were also recorded and processed.

RNA extraction and real time PCR

Total RNA was prepared from 100 mg of isolated interventricular septum using TRIzol method (Invitrogen, Life Technologies, CA, USA) as previously described [18].

Real time PCR was performed using SYBR Green technology on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, California).

Primer used were: forward 5'-GAC CTA TGA CCT CAT CAA GG-3' and reverse 5'-ATA GGT GAC GAA CAT CAC CAC G-3' for UCP2 and forward 5'-CAC CCA AGA ACA GGG TTT GT-3' and reverse 5'-TGG CCA TGG GTA TGT TGT TAA-3' for mtDNA. As an internal standard for amplification, the expression of RPLP (forward 5'-CAG ATT GGC TAC CCA ACT GTT-3' and reverse 5'-GGC CAG GAC TCG TTT GTA CC-3') was quantified in parallel. The primers were purchased from Metabion International AG, Martinsried, Germany.

Prior optimization was conducted for each set of primers, which consisted of determination of optimal primer and MgCl₂ concentration, the template concentration and verification of the efficiency of the amplification and non-specific amplification. PCR amplification was performed in duplicate in a total reaction volume of 20 µL. The reaction mixture consisted of 3 µL diluted template, 10 µL iQTM SYBR Green Supermix 2x, 200 nM forward and reverse primers and 0.4 µL ROX. Real time PCR protocol include an initial step of 95°C (3 min), followed by denaturation at 95°C for 10 s, annealing

at 59°C for 15 s (UCP2) or 58°C for 30 s (mtDNA) and extension at 72°C for 30 s (UCP2) or 15 s (mtDNA). Results were normalized to RPLP transcription to compensate for variation in input RNA amount and efficiency of reverse transcription.

SDS-PAGE and Western blotting

Immunoblot analyses were performed on isolated interventricular septum as described previously [18]. Primary antibodies against: subunit 9 of NADH-ubiquinone oxidoreductase (NDUFA9) for complex I (2.5 µg/mL), cytochrome *c* (1.5 µg/mL), subunit IV of cytochrome *c* oxidase (COXIV) for complex IV (0.1 µg/mL), ATP synthase subunit 9 for complex V (0.8 µg/mL) and connexin 43 (Cx43) (1:8000) were purchased from Abcam, Cambridge, UK. Quantitative analyses of immunoreactive bands were done by ImageQuant software. Volume represents the sum of all pixel intensities within a band; 1 pixel = 0.007744 mm². The density of the each protein signal was normalized to that of the internal control, β -actin (Abcam, Cambridge, UK). The mean values from the sevoflurane group were taken as 100% and that from propofol were expressed as percentages with respect to sevoflurane.

Confocal microscopy, immunohisto- and immunocytochemistry

After aortic valve excision tissue samples, taken from the interventricular septum, were cut into small pieces, fixed in 2.5% glutaraldehyde in a 0.1M phosphate buffer (pH 7.2), and postfixed in 2% osmium tetroxide in the same buffer. The specimens were dehydrated through a series of alcohol solutions of increasing concentration and embedded in Araldite. The blocks were trimmed and cut with diamond knife (Diatome, Switzerland) on an U6 ultramicrotome (Leica, Austria). Semi-fine sections were used for immunohisto- and thin sections mounted on nickel grids for immunocytochemistry. For cytochrome *c* staining, semi-fine sections were immunostained according to the avidin-biotin-peroxidase method [19], using primary antibody against cytochrome *c* in concentration of 10 µg/mL (Abcam, Cambridge, UK) and examined with a light microscope (Leica, Austria).

Connexin 43 was detected with anti-Cx43 antibody (1:1000; Abcam, Cambridge, UK) in combination with rhodamine-conjugated secondary antibody (1:200; Abcam, Cambridge, UK) by confocal microscopy. Confocal images were acquired with a confocal laser scanning microscope LSM510 (Carl Zeiss Microimaging GmbH, Jena, Germany) and a Windows NT operating system. The examination of fluorescent immunolabeled samples was performed under Ar laser lamp.

Grids were washed two times with TBS (200 mM Tris, 1.5 M NaCl, pH 7.4), blocked with 5% BSA in TBS, for 1 h at ambient temperature, and then incubated with primary antibodies against cytochrome *c* and Cx43. Sections were washed ten times in TBS and incubated with 10 nm gold-conjugated secondary antibody (Abcam, Cambridge, UK). After washing with TBS and water the sections were dried and examined using Philips CM12 transmission electron microscope (FEI, The Netherlands/USA).

The specificity of the immune reactions was tested by replacing the primary antibody with a nonimmune rabbit serum

or by incubating the sections with the secondary antibody alone.

Other assays and statistics

All clinical data are expressed as mean $\pm$ standard deviation. T-test was used for comparisons between the subgroups for continuous variables, whereas categorical variables and continuous variables with non-Gaussian distribution were compared using Mann-Whitney U test (a probability value of $P < 0.05$ was considered significant). Statistical analysis was done using SPSS 17.0 statistical package.

Protein content was estimated by the method of Lowry et al. [20] using bovine serum albumin as a reference. Analysis of variance (ANOVA) was applied for within-group comparison of the data from molecular analysis. If the F test showed an overall difference, Tukey's *t*-test was used to evaluate significance of the differences. Statistical significance was accepted at $P < 0.05$.

Results

Patients characteristics and intraoperative data

Baseline demographic and echocardiographic characteristics of enrolled patients are shown in Table 1. Total of 22 consecutive patients were enrolled in the study. There were 11 patients in sevoflurane and 11 patients in propofol group. There were no differences between the groups with respect to baseline characteristics. Briefly, patients were predominantly males in their early sixties with moderate reduction in effort tolerance (NYHA II class). They had low predicted surgical mortality, as indicated by logistic Euroscore. Patients in both groups had preserved left ventricular ejection fraction as indicated by echocardiography, with pure severe aortic stenosis measured by aortic valve area and mean systolic gradient over aortic valve.

All patients received St. Jude mechanical aortic valve. Cardiopulmonary bypass and aortic cross-clamp time was similar between sevoflurane and propofol groups.

Hemodynamic data and biomarkers

Hemodynamic data in all patients were obtained using standard right heart catheterization with Swan-Ganz catheter. As shown in Table 2, values for SVRI, CI, LVSWI and PCWP were similar between the groups preoperatively, at the end of operation, and after 6 and 24 hours after the end of operation. Conversely, patients in sevoflurane group had higher values of CK 24 hours after surgery. Troponin I was similar in all patients 16 and 24 hours following surgery (Table 3).

	Sevoflurane (n = 11)	Propofol (n = 11)	P value
Preoperative data			
Sex (male)	5/11 (45%)	8/11 (73%)	0.67
Age (years)	63 ± 13	63 ± 9	0.98
NYHA class	2 ± 0	1.9 ± 0.3	0.33
Euroscore (logistic)	5.21 ± 2.24	5.18 ± 1.88	0.91
Body mass index	26.9 ± 6.1	28.9 ± 2.25	0.32
Hypertension	8/11 (73%)	7/11 (64%)	0.84
Diabetes	1/11 (9%)	2/11 (18%)	0.81
Ejection fraction (%)	58 ± 4	57 ± 12	0.72
Aortic valve area (cm ²)	0.65 ± 0.14	0.68 ± 0.13	0.63
Peak gradient (mm Hg)	111 ± 28	111 ± 22	0.78
Mean gradient (mm Hg)	67 ± 20	67 ± 15	0.94
Medications			
Sufentanil	11/11 (100%)	11/11 (100%)	1.00
Dose (mcg/kg)	26.98 ± 17.30	21.70 ± 15.18	0.45
Intraoperative data			
CPB time (min)	90.09 ± 25.48	91.82 ± 21.31	0.86
Aortic cross-clamp (min)	70.00 ± 21.38	65.73 ± 10.52	0.55

Table 1. Patients characteristics. Data are means ± SD, median (range), or number of patients (%). Abbreviations: NYHA, New York Heart Association class; CPB, cardiopulmonary bypass.

	Sevoflurane (n = 11)	Propofol (n = 11)	P value
SVRI (dynes · s · cm ⁻⁵ · m ⁻²)			
Preoperative	1529 ± 408	1576 ± 320	0.76
End of operation	1340 ± 344	1441 ± 604	0.81
6h post operation	1044 ± 318	1072 ± 343	0.81
24h post operation	848 ± 177	645 ± 358	0.57
CI (L · min · m ⁻²)			
Preoperative	1.99 ± 0.44	2.36 ± 0.69	0.15
End of operation	2.35 ± 0.71	2.44 ± 0.55	0.45
6h post operation	2.90 ± 0.95	2.85 ± 0.63	0.85
24h post operation	3.29 ± 0.28	3.14 ± 0.70	0.51
LVSWI (g · m · m ⁻²)			
Preoperative	31.36 ± 11.03	37.36 ± 20.98	0.44
End of operation	26.26 ± 11.62	22.02 ± 6.36	0.30
6h post operation	29.81 ± 10.32	27.07 ± 10.26	0.53
24h post operation	31.08 ± 14.02	24.45 ± 7.26	0.20
PCWP (mm Hg)			
Preoperative	11.55 ± 3.91	10.91 ± 2.55	0.65
End of operation	11.45 ± 3.42	10.27 ± 2.32	0.35
6h post operation	11.91 ± 2.77	11.82 ± 3.28	0.94
24h post operation	11.09 ± 2.58	11.45 ± 4.63	0.14

Table 2. Hemodynamic data. Data are given as means ± SD. Abbreviations: SVRI, systemic vascular resistance index; CI, cardiac index; LVSWI, left ventricular stroke work index; PCWP, pulmonary capillary wedge pressure.

Postoperative course and complications

All patients were followed until hospital discharge. There were no deaths in both groups in this period.

Similarly, there was no difference in the use of inotropes, duration of mechanical ventilation, stay in ICU and duration of hospitalization between sevoflurane and

	Sevoflurane (n = 11)	Propofol (n = 11)	P value
CK (U/L)			
16h post operation	945 ± 925	343 ± 213	0.14
24h post operation	2560 ± 3805	618 ± 682	0.045
CK MB (U/L)			
16h post operation	82 ± 72	56 ± 51	0.70
24h post operation	111 ± 129	31 ± 45	0.25
Troponin I (ng/mL)			
16h post operation	5.24 ± 6.31	3.75 ± 4.01	0.80
24h post operation	3.56 ± 5.60	1.79 ± 1.97	0.84
BNP (pg/mL)			
preoperative	244 ± 342	248 ± 193	0.69
7 days post operation	583 ± 628	372 ± 226	0.62

Table 3. Biomarkers of cardiac injury and brain natriuretic peptide. Data are given as means ± SD. Abbreviations: CK, creatine kinase; BNP, brain natriuretic peptide.

	Sevoflurane (n = 11)	Propofol (n = 11)	P value
Inotropic support	2/11 (18%)	3/11 (27%)	0.64
Mechanical ventilation (hours)	15.00 ± 2.97	13.36 ± 3.85	0.28
Acute renal failure	2/11 (18%)	0/11 (0%)	0.14
Respiratory failure	1/11 (9%)	1/11 (9%)	1.00
Bleeding	4/11 (36%)	1/11 (9%)	0.12
ICU stay (hours)	21.40 ± 21.71	15.73 ± 20.79	0.86
Hospitalization (days)	13.55 ± 8.00	11.27 ± 6.75	0.48
In hospital death	0/11 (0%)	0/11 (0%)	1.00

Table 4. Postoperative data. Data are means ± SD, median (range), or number of patients (%). Abbreviations: ICU, intensive care unit.

Fig. 1. Protein content of complex I, complex IV and ATP synthase in cardiomyocytes of patients receiving sevoflurane and propofol. The signals from representative Western blot are shown (A). Data obtained after quantification of specific bands, and expressed as % of sevoflurane group taken as 100%, represent the mean ± SD (B). *comparison between sevoflurane- and propofol-receiving patients, ***, $P < 0.001$; **, $P < 0.01$.

propofol group. Incidence of respiratory and renal failure, as well as incidence of bleeding, was similar between the groups (Table 4).

Protein levels of mitochondrial respiratory complexes

As evident from Fig. 1A, sevoflurane and propofol patients showed no differences in the cardiac protein level

Fig. 2. Cytochrome *c* protein level and its immunolocalization in cardiomyocytes of patients receiving sevoflurane and propofol. (A) Representative immunoblot of cytochrome *c* (left); Corresponding densitometric readings presented as a percentage of sevoflurane group taken as 100% (right). The quantification results represent the mean $\pm$ SD. *comparison between sevoflurane- and propofol-receiving patients, *, $P < 0.05$. (B) Immunolocalization of cytochrome *c* in cardiomyocytes (representative sections of the two groups). There was higher immunopositivity of cytochrome *c* in cardiomyocytes of propofol (P) than sevoflurane (S) receiving patients (inset, immunogold 20-nm particles). DAB-immunohistochemistry, mag. x100, orig. Inset: immunogold, mag. x31000. mt-mitochondria.

Fig. 3. The protein level and localization of connexin 43 (Cx43) in cardiomyocytes of patients receiving sevoflurane and propofol. (A) The signals from representative Western blot are shown (left). Corresponding data obtained after quantification of Cx43 bands are represented the mean $\pm$ SD and expressed as % of sevoflurane group taken as 100% (right). *comparison between sevoflurane- and propofol-receiving patients, ***, $P < 0.001$. (B) Confocal laser scan micrographs of the immunofluorescence of Cx43 localization (representative sections of the two groups). High expression of Cx43 and their relocalization into mitochondria (inset B-up, immunogold 20-nm particles) besides its gap junction presence (inset B-down, inset A) was observed in cardiomyocyte of propofol (P) patients. Immunofluorescence, mag. x100, orig. Inset: immunogold, mag. x31000. mt-mitochondria.

of complex I, but significant differences in complex IV and ATP synthase protein levels. Quantification of proteins bands (Fig. 1B) showed higher protein level of complex IV and ATP synthase in sevoflurane than in propofol patients.

*Protein content of cytochrome *c**

Immunoblot analysis revealed higher protein level of cytochrome *c* in propofol than sevoflurane receiving patients (Fig. 2A). The superior effect of propofol on cytochrome *c* expression over sevoflurane was confirmed

Fig. 4. mtDNA transcript content in cardiomyocytes of patients receiving sevoflurane and propofol. Results are given as mean $\pm$ SD for mtDNA normalized to RPLPO transcription. *comparison between sevoflurane- and propofol-receiving patients, ***, $P < 0.001$.

Fig. 5. Real time PCR analysis of mRNA expression of uncoupling protein (UCP2). Results are given as mean $\pm$ SD for UCP2 normalized to RPLPO transcription. *comparison between sevoflurane- and propofol-receiving patients, *, $P < 0.05$.

by immunohistochemistry showing strong immunoreactivity in cardiomyocytes of propofol receiving patients (Fig. 2B).

The content and localization of Cx43 protein

Anesthetics effects on Cx43 protein expression in the heart had not been previously explored. We have found that propofol receiving patients showed higher Cx43 protein content than sevoflurane patients (Fig. 3A). In both cases, sevoflurane and propofol, fluorescence revealed the presence of membrane immunostaining for Cx43 in cardiomyocytes (Fig. 3B). However, in propofol administered group, in addition to membrane staining for Cx43, we observed higher intracellular accumulation of cytoplasmic and mitochondrial Cx43 expression than in sevoflurane receiving patients.

The mRNA levels of mtDNA and UCP2

Real-time PCR analysis showed the higher transcripts content of mtDNA (Fig. 4) and UCP2 (Fig. 5) in propofol than in sevoflurane patients group.

Discussion

Many clinical studies have been demonstrated cardioprotective role of anesthetic in patients with coronary disease who undergo coronary artery bypass surgery, with significant advantage for volatile anesthetics (sevoflurane) opposite to intravenous

anesthetic (propofol). In the present study, patients undergoing aortic valve replacement due to significant aortic valve stenosis, in anesthesia maintained in two different anesthetic regimes, did not show significant difference in hemodynamic data during and after the surgery as well as during postoperative period. However, the results confirmed the role of both volatile and intravenous (sevoflurane and propofol, respectively) anesthetics in anesthetic preconditioning and postconditioning in these patients. Moreover, it seems likely that sevoflurane and propofol lead to cardiac protection via different energy-related mechanisms at mitochondrial level. The molecular targets of sevoflurane are cytochrome *c* oxidase (complex IV) and ATP synthase, while the action of propofol seems to occur through regulation of cytochrome *c*, Cx43, mtDNA transcription and UCP2.

Our results have shown that there are no differences in the cardiac protein level of complex I between two patients groups but the protein levels of complex IV and ATP synthase were higher in sevoflurane than in propofol patients. We hypothesize that observed high protein levels of these two components of the respiratory chain could be related to the protective effects of sevoflurane in ischemic conditions that have been repeatedly shown previously. Mechanisms of preconditioning-like effects of sevoflurane were mainly explained as the protection from the consequences of electron transport chain inhibition during ischemia, i.e. ROS generation, Ca^{2+} overload as well as ATP depletion [17, 21, 22]. The present study suggests the

Fig. 6. Simplified scheme of the potential mitochondrial sides of action of sevoflurane and propofol. Sevoflurane directly affects ATP synthesis through regulation of cytochrome *c* oxidase (complex IV) and ATP synthase. Propofol controls mitochondrial membrane integrity and ROS production, through regulation of content and localization of cytochrome *c* and connexin 43 (Cx43) and uncoupling (UCP2 protein), as well as mtDNA transcription.

molecular basis of such energy-preserving effects of sevoflurane in ischemic heart. Also, it seems that this cardioprotective-related molecular adaptation is more characteristic for sevoflurane than for propofol action.

On the other hand, the cardioprotective effects of propofol have a long time been mainly related to its antioxidative properties and its benefit in protecting the myocardium from reperfusion injury [23, 24]. Several possible mechanisms of propofol action, including free radical scavenging and activation of anti-apoptotic pathway, have been proposed [25]. However, in recent years other targets of propofol cardioprotection such as protein kinase C [26], glycogen synthase kinase 3 β [27] and nitric oxide synthase [28] have been suggested. Our results suggest that mitochondria are targets for propofol-induced cardioprotection. In contrast to complex IV and ATP synthase protein level, higher in sevoflurane than in propofol group, cytochrome *c* protein level was higher in patients receiving propofol. It has been reported that cytochrome *c* is detached from the

inner mitochondrial membrane during cardiac ischemia/reperfusion resulting in the loss of respiratory activity [29]. In the present study propofol receiving patients showed strong mitochondrial localization of cytochrome *c*, in addition to the higher protein level comparing to sevoflurane group. These results speak in favor propofol role in maintaining mitochondrial membrane potential in terms of cardioprotection from ischemia-reperfusion injury.

Besides cytochrome *c*, propofol receiving patients also showed higher protein level as well as mitochondrial localization of Cx43, molecule not classically declared as mitochondrial, than sevoflurane patients. Cx43 is a major cardiac gap-junction channel protein that has been implicated in the electric coupling of cardiac muscle cells [30]. However, it has been recently shown that Cx43 can be localized in the mitochondrial membrane of cardiomyocytes when its cardioprotective properties were observed. Boengler et al. [31] have found that ischemic preconditioning induced increase of Cx43 localization in mitochondria. The underlying mechanism

of protective role of mitochondrial Cx43 in the heart is not fully elucidated, but several potential mechanisms, including keeping the mitochondrial permeability transition pore in closed state,³¹ regulation of ROS production [32] and maintaining of mitochondrial membrane integrity and cytochrome *c* localization [33], have been suggested. Besides, the observed antiarrhythmic effects of anesthesia, in term of cardioprotection, were recently connected to their effect on Cx43 localized at cytoplasmic membrane. Hirata et al. [10] have found that propofol, but not sevoflurane, preserves phosphorylated-Cx43 protein (active form) during myocardial ischemia in rats and suggest this as one of the mechanisms of propofol antiarrhythmic effect. Our results extended these data on the anesthetics effects on protein expression of Cx43 suggesting superior effect of propofol over sevoflurane.

We have also observed the markedly higher mitochondrial DNA-encoded mRNA, i.e. mitochondrial gene transcription in patients who received propofol than in those receiving sevoflurane. Heart failure is frequently associated with qualitative and quantitative defects in mtDNA. The failing hearts and ischemic heart disease are characterized by decreased mtDNA copy number and mtRNA transcript as well as reduced oxidative capacity due to low respiratory complexes enzyme activities [34, 35]. On the other hand, mtDNA replication and transcription can change in order to meet different energy demand, as well as under different physiological and environmental conditions [36]. For example, the increase in mtDNA content was observed in aging when oxidative mtDNA damage occurs [37]. This increase was thought to compensate for respiratory function decline since mtDNA encoding 13 subunits of the oxidative phosphorylation complexes [38, 39]. For our knowledge there is no data, till now, on the anesthetics effect over mtDNA transcription and/or copy number. Our results suggest mtDNA as a new mitochondria-related molecular target of propofol that could contribute cardiac protective effect based on restoration of disturbed oxidative metabolism during ischemia-reperfusion.

Besides the differences in the expression profile of the respiratory chain complexes between patients receiving two types of anesthetics, one more mitochondrial energy-related protein showed differences between groups. There was the higher level of UCP2 transcript

in propofol than in sevoflurane group. It is now well established that uncoupling proteins have a significant physiological role in the regulation of mitochondrial proton gradient and consequently ROS production [40]. In rat cardiomyocytes, UCP2 overexpression confers tolerance to oxidative stress via diminishing mitochondrial Ca^{2+} overload and reducing ROS generation [41]. In that context, it is not surprising that this molecule was suggested to be the part of mitochondrial molecular adaptation during heart preconditioning [42]. McLeod et al. [42] reported that ischemic preconditioning phenotype, i.e. tolerance to anoxia-reoxygenation injury and increased ROS production, is abolished following UCP2 depletion. Mitochondrial uncoupling has been also shown to mediate anesthetic-induced heart preconditioning [43]. Our results suggest that such mitochondria-related mechanism of cardioprotection is more prominent in propofol than sevoflurane action.

Keeping in mind all limitations of human studies, regarding the absence of controls because of ethical considerations, some important facts derive from this clinical study. Namely, there are no significant differences between the effects of sevoflurane and propofol on the hemodynamic and biochemical markers of myocardial damage, as well as postoperative outcome in the patients undergoing AVR due to aortic stenosis. However, there are anesthetic-specific expressional pattern of key mitochondrial bioenergetic-related molecules suggesting different possible mechanisms for sevoflurane- and propofol-mediated cardioprotection (Fig. 6). Further study aimed to determine the physiological relevance of these findings are in progress, but it appears that sevoflurane directly affects ATP synthesis (through regulation of cytochrome *c* oxidase and ATP synthase), while the effects of propofol occur through regulation of mitochondrial membrane integrity and ROS production (cytochrome *c*, UCP2 and Cx43), as well as mtDNA transcription.

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