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# Cardioprotective Effects Induced by Preconditioning with Halogenated Anesthetics

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**ABSTRACT**

**Background:** Numerous studies discuss the protective effects of halogenated anesthetics on myocyte injury induced by the ischemia-reperfusion syndrome of the heart. This mechanism is known as pharmacological preconditioning. **Aim of the study:** The objective of this study was to identify the effects of two volatile anesthetics frequently used in current clinical practice, Isoflurane and Sevoflurane, on the *in situ* heart. The study was performed on laboratory animals with induced brain death. **Material and methods:** The animals were divided into 3 groups, the control group (n = 8), IZO-PRE group (n = 8) and SEVO-PRE group (n = 8), on which the experimental protocol established for this study was applied. From a molecular point of view, the expressions of protein kinase C-epsilon (PKCε) and of glycogen synthase kinase – 3 beta (GSK-3β) were investigated. **Results:** Following the statistical analysis, we observed a significant reduction in the size of the infarcted area in the IZO-PRE group compared to the control group ($p < 0.0001$). Regarding the SEVO-PRE group, no reduction was observed ($p > 0.05$). The expression of GSK-3β is more pronounced in case of the SEVO-PRE group, at 5 minutes after reperfusion, and the effect disappears after 15 minutes. The expression of PKCε as a total form of the enzyme, occurs at 5 minutes after reperfusion in the SEVO-PRE group and late in the IZO-PRE group (after 15 minutes). **Conclusions:** Both anesthetics that were applied showed a cardioprotective effect. Isoflurane provided a better structural and morphological protection, but Sevoflurane resulted in a more effective protection in terms of functionality, significantly reducing the incidence of extremely severe life-threatening arrhythmias.

**Keywords:** sevoflurane, isoflurane, transplant

## BACKGROUND

In the organ transplantation field, a very intense and important topic is to find ways to increase the quality of organs that are taken from willing donors, in order to improve the recovery capacity of the transplant recipients.[1] From the moment of the organ removal until its actual transplantation, there is a variable period of ischemia, named cold-ischemia, responsible for the production

of cellular damage that involves a significant decrease in the functional performance of the organ.[2–10] The molecular disturbance produced in cold-ischemia is caused by the excessive production of pro-inflammatory molecules and free radicals. The injuries caused by ischemia are reflecting upon the mitochondrial activity, decreasing the energetic status for a prolonged time. Also, the microvascular system is affected both by the accumulation of cells responsible for inflammation, as well as by the accelerated biosynthesis of pro-inflammatory molecules.

Numerous studies highlight the protective effects of halogenated anesthetics on the cell injury induced by the ischemia-reperfusion syndrome on the heart.[11–14] This mechanism is known as pharmacological preconditioning.[15] Regarding the cardioprotection induced by volatile anesthetics, a number of studies report a series of features which include the ability of maintaining the reserves of ATP, decreasing the production of free radicals, reducing the $Ca^{2+}$ overload, opening the channels for $K^+$ and shortening the duration of the action potential. By corroborating these aspects, it can be concluded that the post-ischemic contractile dysfunctions are being reduced.[16–20] This refers to the fact that short periods of ischemia, with a duration small enough not to produce cardiomyocyte necrosis, alternated with reperfusion, offers myocardial protection against an infarction produced by an ulterior prolonged ischemia, called in the specialized literature ischemia "test" or ischemia "index".[2,11,21–25]

In this paper, we present a study regarding the effects of two volatile anesthetic agents, Isoflurane and Sevoflurane on the heart, *in situ*, by using laboratory animals in which brain death was induced. This paper also presents the molecular mechanisms involved in the phenomenon of cardioprotection induced by volatile anesthetics in the brain-dead organ donor.

## MATERIALS AND METHODS

### Animals

The experimental study was carried out with the approval of the Ethics Committee of the Emergency County Hospital "Pius Brinzeu" Timișoara, Romania and of the Animal Research Committee of the "Victor Babeș" University of Medicine and Pharmacy, Timișoara, Romania. We followed the national and international norms and principles of experimental research presented in The Guide for the Care and Use of Laboratory Animals published by the National Institute of Health – NIH Publication no. 85-23, revised in 1996.

The population of this study consisted of 24 adult Sprague-Dawley rats, all male, weighing between 350–450 g, which were properly fed and hydrated until the day of the experiment. Solid food was suppressed 12 hours before the experiment, hydration being allowed ad libitum.

The animals were divided into 3 groups, the control group (n = 8), the IZO-PRE group (n = 8) and the SEVO-PRE group (n = 8) to whom the experimental protocol established for this study was applied.

### Experimental design

#### *Induction of anesthesia and monitoring of the vital functions*

For the induction of anesthesia the intraperitoneal route using a mixture of 80 mg/kg Ketamine (Vetased®) and 5 mg/kg Xylazine (Xylazine®) was used. In the first phase, the anesthetic mixture was administered intraperitoneal, avoiding the intravascular administration of anesthetic substances. The general anesthesia was maintained with Sevoflurane for the SEVO-PRE group and Isoflurane for the IZO-PRE group, by using the value of 1 MAC.

The physiological temperature was maintained by a heated thermostatic table. Two vascular surgical approaches were needed, one venous and one arterial. Continuous monitoring of the electrocardiogram was absolutely necessary and it was easy to achieve. The electrodes that registered the electrical signal, had the form of a needle, biopotential signals were collected from the chest, after the animal was anesthetized (Figure 1). The rat heart rate had a mean value of 300–350 beats/minute; therefore we selected the recording speed to be 50 mm/sec.[26]

The mean blood pressure was monitored invasively through the catheterization of the femoral artery with a 24 G catheter.

#### *Induction of brain death*

The rat preparation for inducing brain death began with the excision of a surface matching the parietal bones. After a meticulous hemostasis, the EEG electrodes were inserted. By using a scalpel, the periosteum was removed on a surface of 3–4 $mm^2$. The compression of the brain tissue was achieved through the insertion of a Swan-Ganz catheter of 7 F through the drill hole that was created. The catheter was introduced until the entire balloon was in the skull, then the balloon was swollen by about 0.5–1 mL of air, leading to damaging of the brain tissue through compression (Figure 1).

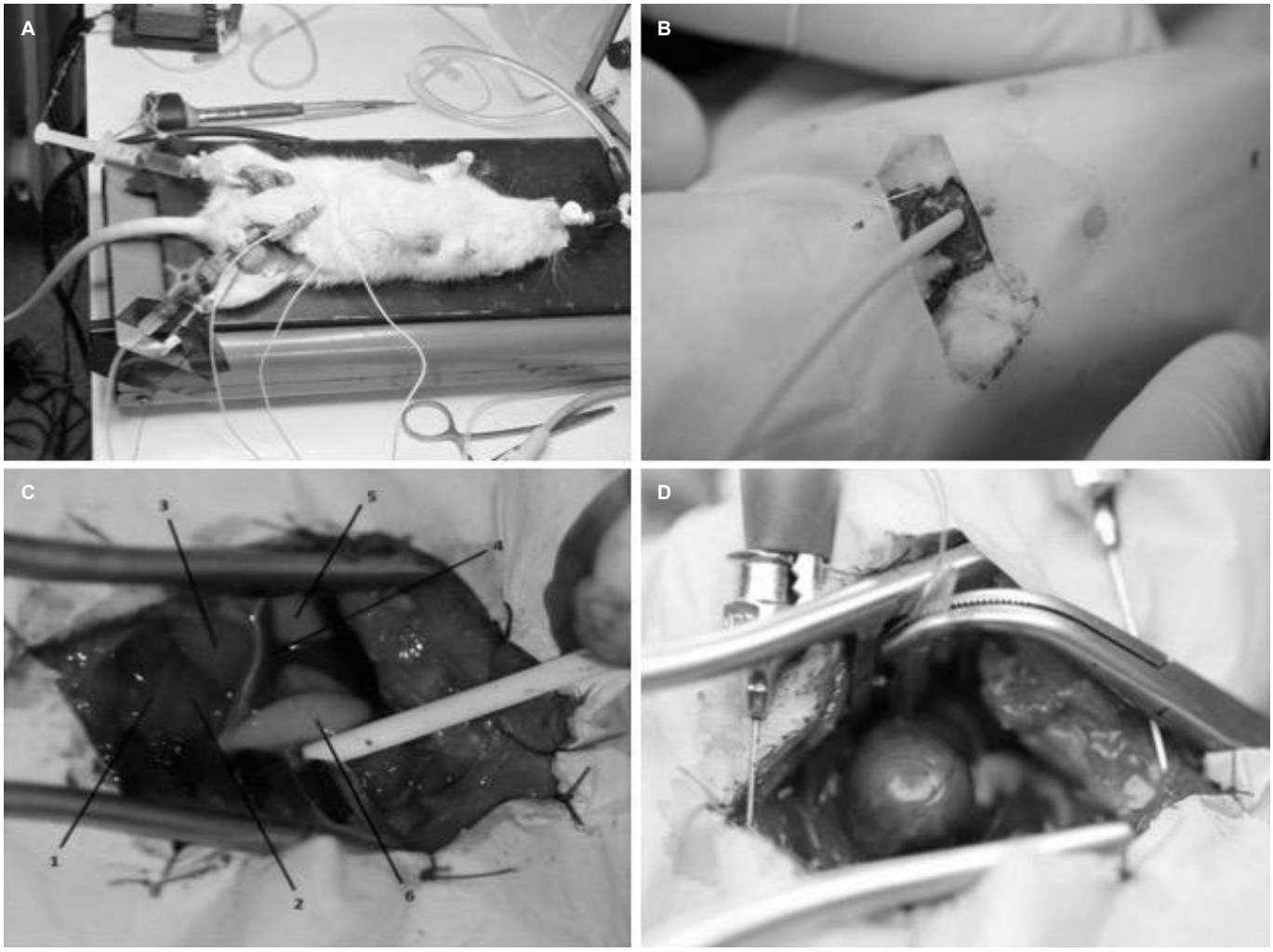


**FIGURE 1.** Surgical and experimental methods used in the study. **A** – The animal ready for experiment with vessels cannulated and connected to the monitoring systems. For the blood vessel cannulation, the skin is incised in the left inguinal region and the femoral vessels are highlighted; **B** – the compression of the brain tissue by inserting a catheter type Swan - Ganz 7F in the cranial cavity; **C** – the rat is placed in right lateral decubitus. The surgical region of the thoracic skin is prepared mechanically and antiseptically and isolated with an operating field. The skin incision was made along the 4th left intercostal space. Anatomical images of the whole operator field: 1 – left anterior descending coronary artery, 2 – left ventricular myocardium, 3 – left auricle, 4 – Azygos vein, 5 – thymus, 6 – the lung; **D** – pale myocardium, ischemia caused by coronary occlusion.

Brain death in the laboratory animal was confirmed by highlighting the EEG flat route in conjunction with clinical signs: the absence of cranial nerve reflexes, apnea test (brief disconnection of the ventilator, acid-base parameters), fixed mydriasis. The intracranial catheter was left there with the balloon inflated to assure hemostasis. After confirmation of brain death, we started the animal preparation for the *in vivo* study of the phenomenon of cardioprotection induced by volatile anesthetic agents.

### *Thoracotomy technique*

The skin incision was made along the 4$^{th}$ left intercostal space from the sternum to the posterior axillary line. The autostatic retractor was fitted with caution not to injure the lungs, thymus, heart and Azygos system, which are located in the posterior thoracic region. The pericardium was elevated with a fine anatomic forceps and sectioned with scissors, and then by dissection with a blunt scissor, it was excised completely. In the working field that was created, the following anatomical elements were identified: the left ventricle, left auricle and the pulmonary artery (Figure 1).

### *The heart preparation in order to achieve the experimental model*

Temporary occlusion of the left anterior descending coronary artery causes myocardial ischemia limited to the re-

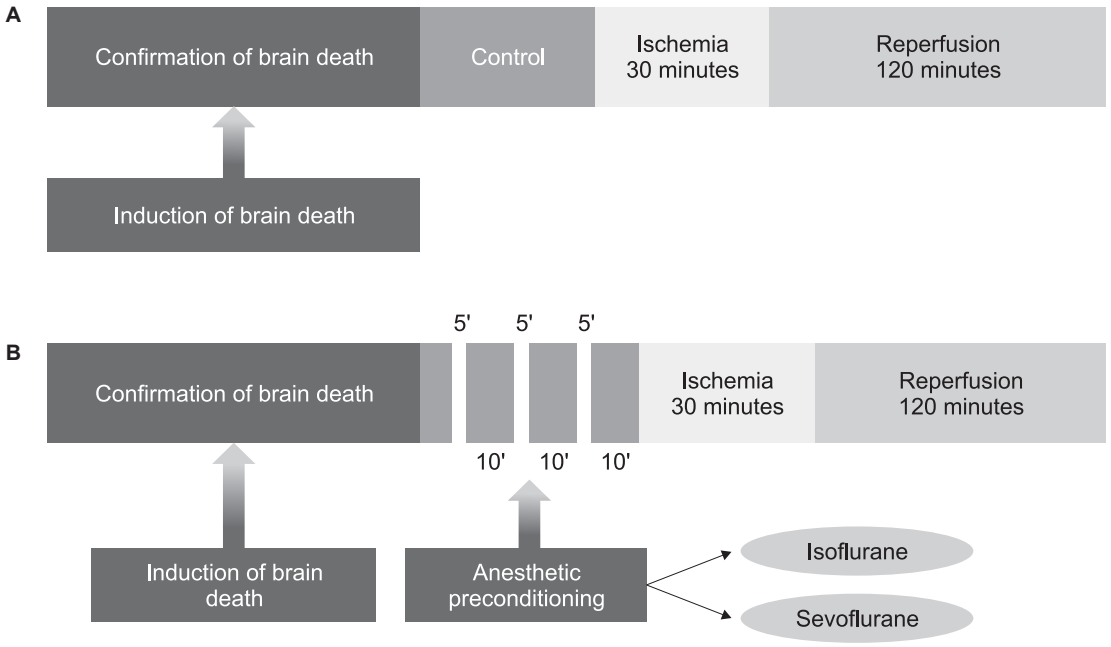


**FIGURE 2.** Experimental protocol. **A** – protocol applied to the control group; **B** – protocol applied to the Isoflurane group (IZO-PRE) and to the Sevoflurane group (SEVO-PRE)

gion tributary to it. The appearance of a whitish color on the tissue from the myocardial territory tributary to the anterior descending coronary artery confirmed a complete occlusion (Figure 1).

After induction and confirmation of brain death, three episodes of 5 minutes each of exposure to Isoflurane (conc. 1.5%) or Sevoflurane (conc. 3.5%) were obtained, depending on the group. The anesthetic administration was alternated with 10 minutes washing, as a protocol of anesthetic preconditioning. According to the literature, this protocol was associated with maximal cardioprotection in rats. All batches have been exposed to an ischemic period over 30 minutes and then 2 hours of reperfusion.[10,23,27] In Figure 2, the experimental protocol is schematically presented.

## Quantification of the experimentally achieved infarction

The most used method for quantifying the infarction in experimental cardiology, reproduced *in vivo*, is the method of double coloring with Evans blue and 2,3,5-triphenyltetrazolium chloride.[28,29] At the end of the two hours of reperfusion, a colloidal pigment — Blue Evans (Evans blue, Sigma E2129) was administered in bolus through the femoral vein, in the amount of 4–5 ml for the delimitation of the infarct risk area. The heart is quickly dissected, the right ventricle is removed and the mentioned areas are excised. The obtained heart samples are washed with normal saline to remove the excess dye, then are weighed, packed in transparent foil, labeled and stored in the freezer for later evaluation. In order to determine the area of necrosis by using 2,3,5-triphenyltetrazolium chloride, the frozen pieces were sliced to a thickness of 2 mm, 5–7 slices were required.[30] The process was followed by the incubation of the slices for 25 minutes at 37 ° C in 2,3,5-triphenyltetrazolium chloride (Sigma T8877, 2% w/v), dissolved in a phosphate buffer and adjusted to a pH = 7.42, in order to reflect the necrotic area. The arrhythmogenic score in myocardial ischemia was also analyzed.[31]

## Western-blot technique for determining the kinases of the cardioprotective sequence

In this paper, we monitored the expression of two proteins: protein kinase C, epsilon isoforms (PKCε) and glycogen synthase kinase – 3 beta (GSK-3β), both as total forms and as the ratio between the phosphorylated form/total form. Each of these enzymes were isolated from the rat heart during the experiments proposed by Western Blot technique.[32–35] Regarding the Western Blot analysis, the myocardial tissue must be taken and frozen quickly enough to avoid kinase degradation under the action of the existing proteases from the tissue. To achieve this goal, the heart was excised just before stopping in asystole, rinsed quickly with cold saline solution at 4°C, then suddenly introduced in liquid nitrogen (-196°C). The samples once frozen, were kept in the freezer at -70°C.

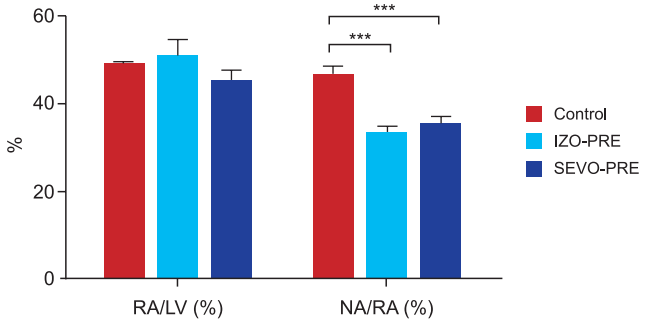


**FIGURE 3.** RA/LV — risk area at the groups preconditioned with Isoflurane and Sevoflurane; NA/RA — infarct area at the groups preconditioned with Isoflurane and Sevoflurane. RA/LV — represents the risk area (RA) as percentage from the left ventricle (LV), NA/RA — represents the reduction of the infarct size, expressed as the ratio between the necrosis area (NA) and the risk area (RA).

### Statistical analysis

The experimental data is presented as mean ± standard deviation (SD). Statistical analysis was performed using the statistical software Prism 6 for Mac OS X v.6.0. (GraphPad Software, Inc., San Diego, CA) and Microsoft Office Excel for Mac 2011 v.14.4.8. (Microsoft Corporation, Bucharest, Romania). In order to quantify the experimental infarct size, to assess the differences of the infarct size and of the risk area, an analysis of covariance (ANOVA) was performed by using the infarct size as a dependent variable and the risk area as a covariate, followed by post-hoc Tuckey test. A p value >0.05 was considered statistically insignificant, p <0.05 statistically significant (*), p <0.01 highly significant (**) and p <0.001 extremely significant (***).

## RESULTS

The effects of Isoflurane and Sevoflurane were studied on the three groups of brain-dead rats as follows: the control group (n = 8) in which no anesthetic was applied in the preconditioning protocol, the IZO-PRE group (n = 8) which had received Isoflurane (1.5% Isoflurane preconditioning in oxygen), and the SEVO-PRE group (n = 8) which had received Sevoflurane (3.5% sevoflurane preconditioning in oxygen). The risk areas were studied and the infarcted areas were expressed as a percentage from the risk areas on the lots in which the preconditioning protocol was applied, and compared with the control group submitted only to the ischemia/reperfusion protocol in the absence of any protective interventions. Expressing the risk area as a percentage of the left ventricle does not show differences between the 3 groups.

Following the statistical analysis, we observed a significant reduction in the size of the infarcted area, in both preconditioning groups compared to the control group (p <0.0001) (Figure 3).

Quantification of the necrosis areas shows a significant reduction in the mean percent values.

Regarding the development of the mean arterial pressure and heart rate during the experiment, we observed

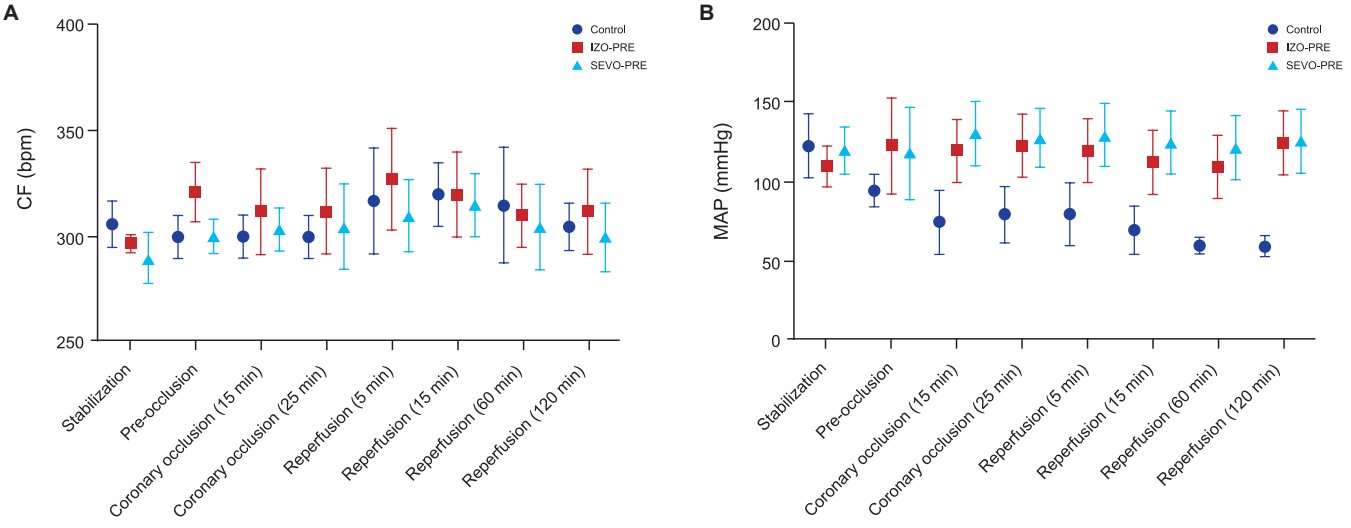


**FIGURE 4.** Evolution of the cardiac frequency (CF) and the mean arterial pressure (MAP) at different times of the experiment. **A** – FC evolution; **B** – MAP evolution. For explications see the text.

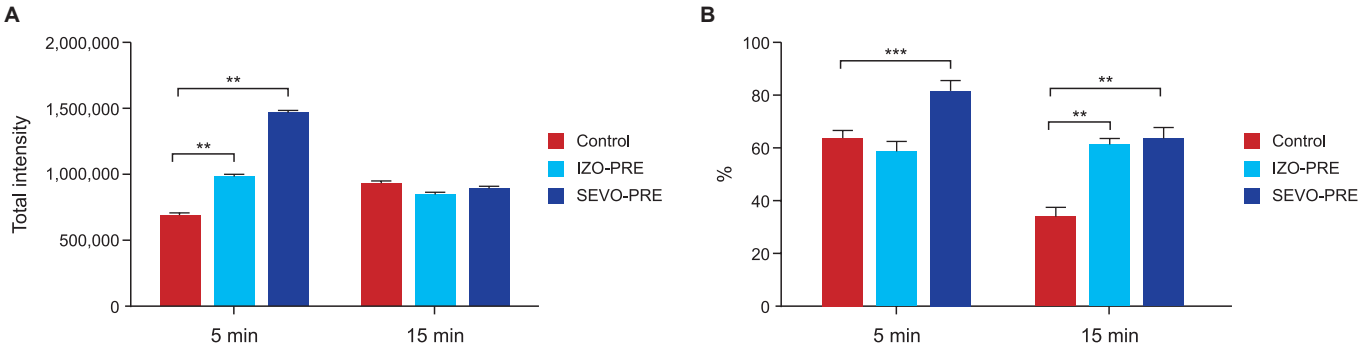


**FIGURE 5.** Graphical representation of total GSK-3β expression **(A)** and procentual representation of phosphorylated/total GSK-3β at 5 and 15 minutes after reperfusion **(B)**

a significant reduction of the absolute values during the experiment in the control group. Also, it can be seen that during reperfusion, this parameter had a quasi-constant evolution for both groups, without significant differences compared to the value registered at the end of ischemia.

Following the statistical analysis, no significant differences were shown regarding the recovery of the functional parameters at reperfusion between the groups treated with Isoflurane or Sevoflurane at a certain time of reperfusion, therefore both anesthetic agents are equally effective in cardioprotection (Figure 4).

In both conditioning experimental protocols, at 5 minutes and at 15 minutes we observed a significant increase in total GSK-3β compared with the control group ($p < 0.01$). The ratio between the total form and the phosphorylated form is significantly increased at 5 minutes after reperfusion in the SEVO-PRE group and at 15 minutes after reperfusion in the IZO-PRE group (Figure 5).

In another group of animals, we harvested the hearts immediately at the end of the preconditioning protocol, in the absence of prolonged ischemia and final reperfusion, in order to investigate whether the preconditioning protocol per se is responsible for the phosphorylation of the enzyme. Comparing the results with a sham group (subjected only to thoracotomy and to the wire placement under the anterior descending coronary artery, for a time period equivalent to the length of 3 episodes of preconditioning after the brain death induction) we observed that the anesthetic preconditioning, alone, significantly increased the ratio between the phosphorylated and the total form. Similar determinations were performed on PKCε expression, the results are presented in Figure 6.

## DISCUSSIONS

In 1988, Warltier *et al.* have demonstrated that treatment with Isoflurane had improved the systolic function of the left ventricle after 15 minutes of acute occlusion of the left anterior descending coronary artery. The contractile function of the myocardial territory afferent to the occluded artery in dogs that had been pretreated with anesthetic, had fully recovered at 5 hours after reperfusion, whereas in the untreated group the achieved recovery was only 50%.[15,21,22,36] Thus, Kersten *et al.* have administered Isoflurane to dogs for an hour before a regional ischemia had

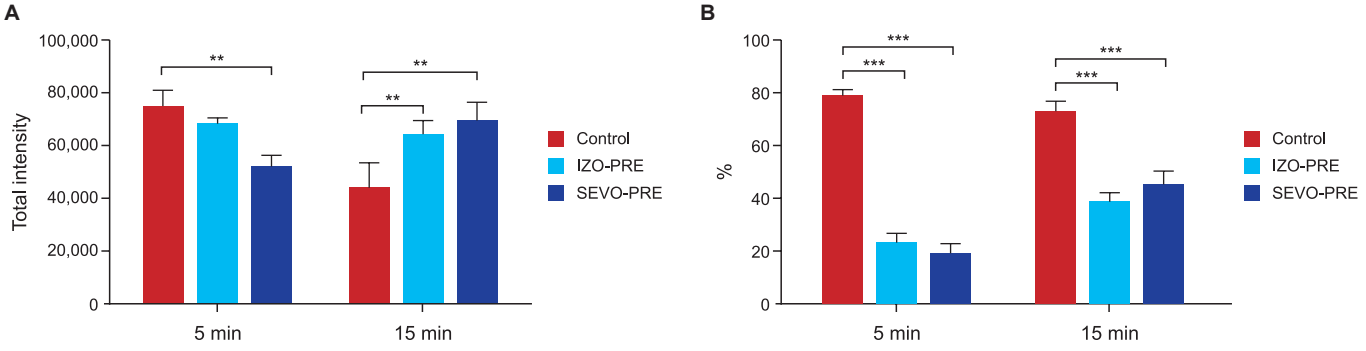


**FIGURE 6.** Graphical representation of total PKCε expression **(A)** and procentual representation of phosphorylated/total PKCε at 5 and 15 minutes after reperfusion **(B)**

been induced for 60 minutes, consequently obtaining a reduction of the infarct size from 25% to 12%.[37,38]

Following these experiments, a significant reduction of the infarcted size area was achieved in the preconditioned lots compared to the control ones. The role of intracellular signaling mediated by kinase was extensively studied in the cases of protection offered by both pre- and post-conditioning with volatile anesthetics. The purpose was to check the involvement of two kinases belonging to the RISK pathway, GSK-3β and PKCε in the cardioprotection offered by preconditioning anesthesia with the two volatile anesthetics, on the model of regional ischemia/reperfusion *in vivo* in the brain-dead animal.

One of the most investigated kinases was the protein kinase C (PKC) with its many isoforms. The team led by Schlack has demonstrated that low doses of Isoflurane stimulates the activation of PKCε and leads to a reduction of the infarcted size area, which is superior to the protection offered by high doses on both models of ischemia and reperfusion, *in vitro* and *in vivo*.[39] These authors have demonstrated, on a rat heart *in vivo*, that both the administration of Isoflurane 0.4 MAC and xenon in three episodes of 5 minutes each before the ischemia-reperfusion protocol, had resulted in a significant reduction of the infarct area. They considered the cardioprotection as being mediated by an increased phosphorylation and a translocation of PKCε. Furthermore they have identified the P38 MAP kinase as the action target of PKCε in the case of isoflurane and xenon preconditioning.[39] The most important fact is that different concentrations of the administered anesthetic may have different effects on the proteins involved in signaling. Thus, for Isoflurane, only the low doses protect the heart through the phenomenon of preconditioning, and this effect is mediated by increased phosphorylation and translocation of PKCε.

In our study we used 1 MAC of Isoflurane and Sevoflurane for the induction of anesthesia conditioning, which corresponds to an intermediary concentration between those used by Schlack and his team.[39]

Using immunohistochemical techniques, Uecker *et al.* have confirmed that the protection induced by isoflurane is achieved through the activation of PKC.[40] They have observed that the preconditioning induced by volatile anesthetics lead to PKCδ and PKCε translocation into the nucleus. On the mitochondrial level, it acts upon PKCδ, and in the sarcolemma and the intercalated discs on PKCε. After administration of Isoflurane only the phosphorylation of PKCδ occurred, and not of PKCε. The administration of PKC blockers (Chelerythrine and Rottlerin) blocked the activation of PKC and consequently, the cardioprotection.[40] In accordance with this study and compared with the classical studies regarding preconditioning, the cardioprotective effect was measured more as an improvement in the post-ischemic functional recovery than as a reduction of the infarct area. Furthermore, only one preconditioning protocol was used (Isoflurane administration for 15 minutes in a concentration of 1.5 MAC) and the myocardial tissue samples were taken at a single time (after the administration of the preconditioned stimulus). The mitochondria represent a major target of the apoptotic proteins that can cause either direct destruction of the mitochondrial membranes or indirectly lead to the opening of the mitochondrial permeability transition pore. PI3K phosphorylation leads to bad results in its sequestration at the mitochondrial level and to the inhibition of apoptosis. Similarly, the activation of PI3K-Act prevents the apoptosis produced by Bax, by inhibiting the conformational changes required for the translocation. It was also shown that the activation of PI3K phosphorylates inactivates pro-caspase-9, thus blocking the formation of caspase-9, one of the many enzymes responsible for implementing the apoptotic signal. PI3K and PDK1 are powerful activators of other protein kinases including the PKC isoforms and tyrosine kinase (PTK), which are heavily involved in the cardioprotection obtained during the anesthetic preconditioning. In our case, we have quantified the expression of PKC isoform epsilon at two moments after reperfusion, 5 and 15 minutes, achieving the following results: a large increase in the ratio of phosphorylated/total PKCε at 5 minutes after reperfusion and a decrease at 15 minutes. Recent studies have shown that the inactivation of the synthase kinase of glycogen-3β has a privileged position in the intracellular transmission sequence of cardioprotection, immediately proximal to the opening of the mitochondrial permeability transition pore. Multiple intracellular signaling pathways that induce cardioprotection converge towards GSK-3β and inactivate this kinase by phosphorylation, a phenomenon associated with a reduced probability of opening the mitochondrial permeability transition pore, and with a reduced necrotic and apoptotic cell death. From this perspective, GSK-3β is considered a new therapeutic target in cardioprotection at reperfusion.

In conclusion, the phosphorylation of GSK-3β plays a critical role in the intracellular signaling mechanisms that protect the cardiomyocytes against the death induced by post-ischemic reperfusion. This is associated with the inactivation of the enzyme and a reduced cardiomyocyte necrosis. Currently the pore structure remains to be elucidated. We know that it remains closed under "de-energizing" conditions that occur during ischemia, but it opens at reperfu-

sion due to calcium overload and excessive formation of free radicals. The opening of the pore results in the collapse of the mitochondrial membrane potential ΔΨm, decoupling of the respiratory chain and cytosol release of the factors responsible for cell death, such as cytochrome c, apoptosis inducing factor AIF, Smac/DIABLO and endonuclease G.[38–40]

## CONCLUSIONS

Isoflurane provides better protection structurally and morphologically, in this case, the area of necrosis was lower compared to the Sevoflurane group. On the other hand, Sevoflurane resulted in a more effective protection regarding the functionality, significantly reducing the incidence of extremely severe life-threatening arrhythmias. Regarding the secondary indicators of cardioprotection, we observed that the heart rate was changed discretely with a weak statistical significance. The expression of GSK-3β was more pronounced in the case of SEVO-PRE and the expression of PKCε as total form of the enzyme appeared at 5 minutes after reperfusion in the SEVO-PRE group.

## CONFLICT OF INTEREST

None declared.

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