Original Article

Effect of Simultaneous Use of Opium and Ischemic Preconditioning on Ischemia/Reperfusion Injury in the Rat Liver

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ABSTRACT

Background: Ischemia preconditioning (IPC) is known as a protective procedure against the injury induced by ischemia/reperfusion (IR) injury. There is also evidence that the administration of opioids may have the same effects on the injury.

Objectives: The aim of this study was to investigate the ameliorative effects of simultaneous use of opium and IPC on lobar IR injury in the rat liver.

Methods: Twenty-five adult male rats were randomly divided into five groups: 1) Sham-operated, 2) IR, 3) IR+IPC, 4) Opium+IPC+IR and 5) Naloxone+opium+IPC+IR. At the end of the reperfusion, blood and tissue samples were obtained to assay alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the blood, as well as to determine oxidative stress by measuring malondialdehyde (MDA) levels, total antioxidant capacity (TAC) values, and superoxide dismutase (SOD) and catalase (CAT) activities in the liver tissues.

Results: The levels of ALT, AST and MDA were significantly increased in the IR group compared to the sham-operated group ($P<0.05$). However, the application of IPC and IPC+opium significantly decreased the release of these enzymes, while the simultaneous application of opium and IPC had a stronger restorative effect on the IR injury ($P<0.05$). The recovery effects induced by opium+IPC in terms of TAC, SOD and CAT were also higher than that of the IPC alone. However, the use of naloxone significantly inhibited the protective effects induced by the opium.

Conclusion: The simultaneous use of opium and IPC is able to accelerate the protective effects of IPC on IR injury.

Keywords: Ischemia preconditioning (IPC), Ischemia/reperfusion (IR), Liver, Opium, Rat

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Introduction

Ischemia, the lack of blood flow to an organ, can cause malfunction of various organs in the body. Blood clots, vasoconstriction, embolism, tumors, congenital disorders, and surgery are among the factors that contribute to the blockage of vessels and the occurrence of ischemia. However, there is evidence that removing the blockage and restoring blood flow paradoxically causes more tissue damage known as reperfusion injury (Ye et al., 2020; Ghotbitabar et al., 2022; Nazari et al., 2024). Ischemia-reperfusion injury (IRI) in the liver is a common clinical phenomenon reported in many conditions, such as trauma and liver transplantation. After reperfusion, inflammatory cells, including neutrophils, appear through chemotaxis and release various pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and other local mediators (Wang et al., 2020). These cytokines induce the increased presence of other immune cells, such as CD4+ T lymphocytes. Neutrophils also stimulate the release of reactive oxygen species (ROS), which can disrupt the balance between the oxidant and antioxidant systems in the body (Wu et al., 2018). An increase in circulating ROS levels causes oxidative stress, which has been implicated in the induction of several types of tissue damage (Soares et al., 2019). Various procedures, including ischemia preconditioning (IPC), as a surgical technique, and the use of antioxidants, as a pharmaceutical tool, are used to reduce the IRI induced by oxidative stress (Lin et al., 2019). Ischemic conditioning may enhance antioxidant effects and reduce inflammation caused by IR in the liver. The protective effect of post-conditioning against IRI may be due to a reduction in inflammation and oxidative stress (Afshar et al., 2023).

IPC, as an important organ protection method, is applied by repeated short-term ischemia and reperfusion periods before long-term ischemia and reperfusion. The purpose of applying IPC is to provide organ tolerance to IR injury induced by oxidative stress (Zabala et al., 2019). There is evidence that the application of IPC can have a protective effect on various organs, such as the brain, heart, kidney, and liver (Annachhatre & Annachhatre, 2019). In a study conducted by Stokfisz et al, it was found that the application of IPC to the liver during transplantation significantly reduced IRI, as demonstrated by the reduction of liver enzymes, ROS, inflammation, and apoptosis. IPC is also able to inhibit inflammation by reducing various inflammatory cytokines, including TNF and interleukins, which leads to the prevention of oxidative stress by increasing the activity of antioxidants (Stokfisz et al., 2017).

The use of appropriate exogenous antioxidants is another method of reducing IRI by preventing the production of ROS that occurs with the onset of reperfusion. The administration of bucillamine in the early stages of reperfusion can significantly inhibit the severity of IR injury in the transplanted liver (Amersi et al., 2002). Gross et al. observed that opioids may be useful as antioxidants and may also play a role in the protective mechanism of IPC (Gross et al., 2009). The role of the delta opioid receptor (δ-OR) in the protective effects of IPC has been reported (Dragasis et al., 2013). It has been shown that the use of nitinol as an opioid antagonist for delta receptors, inhibited the effect of methadone and morphine to reduce the size of myocardial infarction induced by reperfusion (Gross et al., 2009). It has also been shown that agonist stimulation of delta receptors may be associated with antioxidant effects (Yarahmadzehi et al., 2020). Furthermore, Jian et al. found that different opioid compounds, such as berberine, papaverine, morphine, and protopine have antioxidant and anti-inflammatory effects (Jian et al., 2019). Therefore, we hypothesized that the simultaneous use of opium and IPC can accelerate the protective effects against IR-induced injury in the rat liver.

Materials and Methods

Animals and experimental groups

Twenty-five adult male Wistar rats weighing 200-250 g were obtained from the institutional animal facility. Animals were maintained under a 12-hour dark-light cycle in a temperature-controlled environment (24±2 ˚C) with standard chow and water provided ad libitum. All experiments were performed according to the standard procedures outlined in institutional guidelines. The rats were randomly divided into five experimental groups as follows:

Control (sham-operated) group: After anesthesia, laparotomy was performed and the liver was exposed, no ischemia was established and after the abdomen was closed, the animal was left in normal condition for 180 minutes.

IR group: In this group, 60 minutes of ischemia and then 120 minutes of reperfusion were performed in the anesthetized rat. IPC+IR group: IPC was applied using a cycle of 10 minutes of ischemia followed by 10 minutes of reperfusion, and then the liver was exposed to 60 minutes of ischemia plus 120 minutes of reperfusion.

Opium+IPC+IR group: Fifteen min before IPC induction, 30 mg/kg of opium was injected intraperitoneally (IP) and then, the procedure was performed as in group 3.

Naloxone+opium+IPC+IR group: The procedure performed on the rat in this group was the same as in group 4, except that 15 minutes before opium injection, 3 mg/kg of naloxone (Toliddarou Co., Iran) was injected intravenously (IV) into the rats.

Opium was obtained from the Police Anti-Drug Bureau and analyzed for purity and constituents using the gas chromatography-mass spectrometry (GC-MS) method. The obtained opium contained approximately 30% alkaloids (morphine 15%, thebaine 4.4%, codeine 5.2% and papaverine 3.7%), while the remainder consisted of non-alkaloid organic and inorganic substances, with 15.5% water (moisture).

Surgical procedure for induction of IR and application of IPC

Before each experiment, the rats were fasted for approximately 15 h and then anesthetized with 80 mg/kg ketamine (Alfasan, Netherlands) plus 5 mg/kg xylazine (Alfasan, Netherlands) prepared as a cocktail and injected IP. After administering 300 units of heparin to each rat through the femoral vein, a laparotomy was performed. The method used to induce lobar IR was previously described by Arab et al. (2009). Briefly, the liver was exposed by cutting the ligaments attached to the ventricular wall. To induce warm lobar ischemia, the portal vein, hepatic artery, and bile duct were clamped with a microvascular clamp for 60 minutes. After the removal of the clamp, reperfusion was started and continued for 120 minutes. IPC was applied using a cycle of 10 minutes of ischemia followed by 10 minutes of reperfusion before performing prolonged (60 minutes) ischemia.

Sampling

At the end of the reperfusion period, blood was collected from the rats' hearts using a 5 mL disposable syringe. The blood samples were centrifuged at 2500 rpm for 10 minutes, and the serum was separated and then frozen at -20 ˚C until analyzed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST). After blood sampling, the rats were euthanized by the injection of a large dose of thiopental, and tissue samples were immediately collected from the left and middle lobes of the liver. These samples were stored in a freezer at -70 ˚C and were used to measure the levels of malondialdehyde (MDA), total antioxidant capacity (TAC), superoxide dismutase (SOD) and catalase (CAT) activities.

Assay of enzyme release

An Alan Eppendorf (Germany) autoanalyzer system was used to measure the release of ALT and AST from hepatocytes. Serum samples were thawed and an autoanalyzer was used to measure ALT and AST levels using the Trucal-u calibrator. Once all the necessary solutions for measuring the enzymes were prepared, the device was set to perform the analysis via the computer, and the serum levels of these enzymes were measured using commercial kits from Byrex (Fars Co., Iran). Oxidant/antioxidant assessment

The liver samples taken from the both left and median hepatic lobes were used to determine TAC, MDA and the activity of the SOD and CAT in the liver. The level of MDA was used to estimate the amount of lipid peroxidation in the liver exposed to different treatments. The MDA level was measured according to the method developed by Placer et al. (1966). The working reagent solution was 20% trichloroacetic acid (TCA) (Merck, Germany) and 0.6% thiobarbituric acid (TBA) (Merck, Germany) in 0.25 N hydrochloric acid (HCL) (Merck, Germany). The homogenized tissue sample was mixed with a working solution and placed in a boiling water bath for 30 minutes.

After cooling, it was centrifuged at 5000 rpm for 5 minutes and then the absorbance of the supernatant was measured at 535 nm using a spectrophotometer (Biotech, USA). The activity of SOD was estimated by the method developed by Kono (1978), which is based on a color reaction and the ability of SOD to inhibit the formation of superoxide radicals. The TAC of tissue samples was measured according to the method developed by Benzie and Strain (1999). This method is based on the reduction of ferric TPTZ (2, 4, 6-tri(2-pyridyl)-1, 3, 5-triazine) complex to ferro TPTZ under acidic conditions.

The absorbance of the blue-colored solution obtained from the reactions of the latter complex was measured spectrophotometrically (Biotech, USA) at a wavelength of 593 nm. The activity of CAT in the samples was measured by the method of Koroliuk et al. (1988). Tris-HCl buffer (0.05 mmol/L, pH 7.8, Merck, Germany) containing 10 mmol/L hydrogen peroxide was mixed with homogenized tissue samples. After 10 minutes, 4% ammonium molybdate was added to the solution to produce the color reaction. The optical absorbance of the colored mixture was measured at a wavelength of 410

Table 1. The levels of AST and ALT (IU/L) in different experimental groups

Groups	Mean±SD	
	AST (IU/L)	ALT (IU/L)
Control	29.13±8.19[d]	27.27±8.52[d]
IR	108.13±12.33[a]	118.67±17.68[a]
IR+IPC	71.3±9.19[b]	78.9±15.19[b]
Opium+IPC+IR	49.67±12.32[c]	49.04±13.21[c]
Naloxone+Opium+IPC+IR	68.95±6.92[b]	76.11±13.84[b]

Abbreviations: AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; IR: Ischemia-reperfusion; IPC: Ischemia preconditioning.

Note: Different superscripts in each column indicate significant differences (P≤0.05) between the various groups.

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The homogenized tissue sample was mixed with the working solution and placed in a boiling water bath for 30 minutes. After cooling, it was centrifuged at 5000 rpm for 5 minutes, and the absorbance of the supernatant was measured at 535 nm using a spectrophotometer (Biotech, USA). The activity of SOD was estimated using the method developed by Kono (1978), which is based on a color reaction and the ability of SOD to inhibit the formation of superoxide radicals. The TAC of tissue samples was measured according to the method developed by Benzie and Strain (1999). This method is based on the reduction of ferric TPTZ (2, 4, 6-tri(2-pyridyl)-1, 3, 5-triazine) complex to ferrous TPTZ under acidic conditions. The absorbance of the blue-colored solution obtained from the reactions of the latter complex was measured spectrophotometrically (Biotech, USA) at a wavelength of 593 nm. The activity of CAT in the samples was measured using the method of Koroliuk et al. (1988). Tris-HCl buffer (0.05 mmol/L, pH 7.8, Merck, Germany) containing 10 mmol/L hydrogen peroxide was mixed with homogenized tissue samples. After 10 minutes, 4% ammonium molybdate was added to the solution to produce the color reaction. The optical absorbance of the colored mixture was measured at a wavelength of 410 nm using a spectrophotometer.

Statistical analysis

Biochemical assay data are expressed as the Mean±SD of at least five experiments in each group. The data were analyzed by ANOVA using SPSS software, version 27, and the significance of differences between experimental groups was tested using Tukey's post hoc test. A P<0.05 was considered statistically significant.

Results

The effects of IPC+opium on enzyme release

Significant differences were observed between the different experimental groups in terms of enzyme release (ALT and AST). As shown in Table 1, a significant increase in ALT and AST levels was observed in the IR group (118.67±17.68 and 108.13±12.33, respectively) compared to the control group (27.27±8.52 and 29.13±8.19, respectively) (P<0.005). However, the use of IPC was associated with a significant reduction (P<0.0001) in ALT and AST levels in the group receiving the treatment compared to the IR group (78.9±15.19 vs 118.67±17.68 and 71.3±9.19 vs 108.13±12.33, respectively).

The simultaneous application of opium and IPC enhanced the protective effects of IPC, as shown by various assessment parameters, including changes in biochemical values. The levels of ALT and AST in the opium+IPC+IR group decreased significantly (P<0.01-0.001) from 78.9±15.19 to 49.04±13.21 and 71.3±9.19 to 49.67±12.32, respectively. No significant

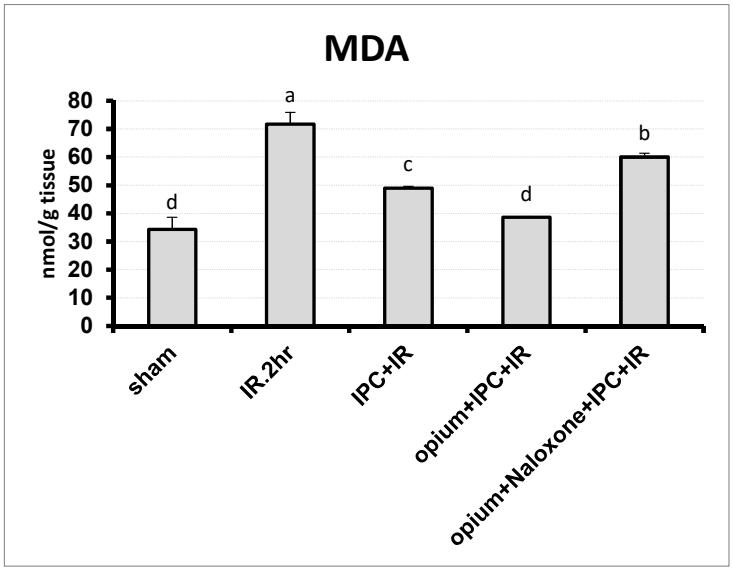

Figure 1. The levels of MDA; nmol/g in different experimental groups

Note: The data are expressed as Mean±SD obtained from at least 5 experiments in each group. Different superscripts in each column indicate significant differences (P≤0.05) between the various groups.

increases in the levels of ALT and AST were observed in the naloxone+opium+IPC+IR group compared to the opium+IPC+IR group.

The effects of IPC+opium on oxidative changes induced by IR

There was a significant difference in oxidative stress indices (MDA, SOD, CAT, TAC) among the different experimental groups. As Figure 1 shows, MDA levels significantly increased in the IR group compared to the sham-operated (control) group (71.63±4.31 vs 34.33±4.25, P<0.0001). However, there was a significant decrease

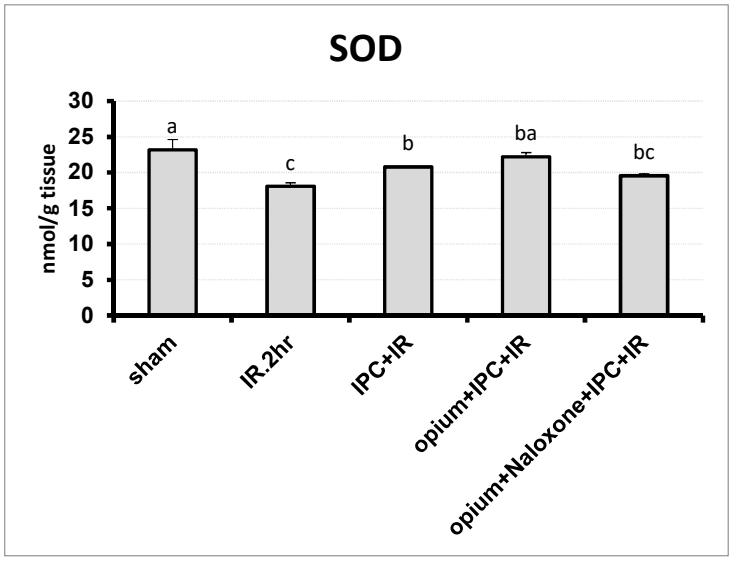

Figure 2. The activity levels of SOD; nmol/g in different experimental groups

Note: The values are expressed as Mean±SD obtained from at least 5 experiments in each group. Different superscripts in each column indicate significant differences (P≤0.05) between the various groups.

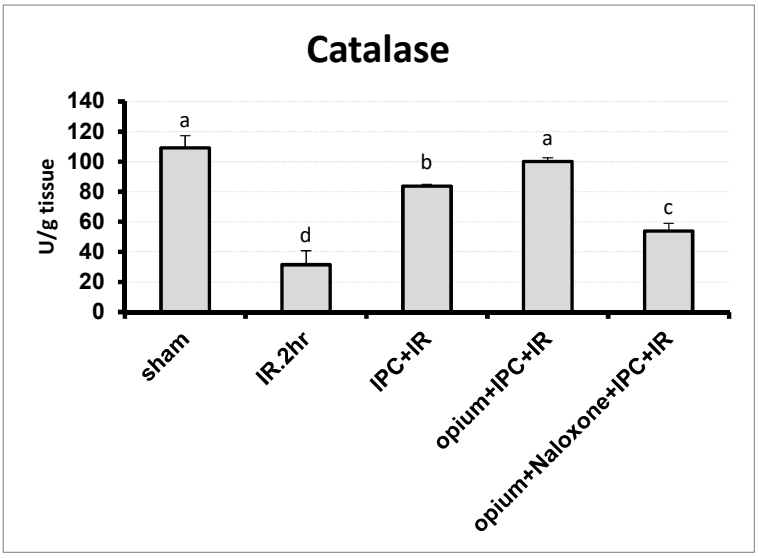

Figure 3. The activity levels of CAT (U/g) in different experimental groups

Note: The data are expressed as Mean±SD obtained from at least 5 experiments in each group. Different superscripts in each column indicate significant differences (P≤0.05) between the various groups.

(P<0.0001) in SOD (18.08±0.49 vs 23.16±1.45) (Figure 2), CAT activities (31.51±9.24 vs 109.1±8.08) (Figure 3) and TAC values (46.49±1.77 vs 88.49±5.04) (Figure 4) in the IR-treated group compared to the control group. MDA levels in the IPC and IR groups were 48.98±0.61 and 71.63±4.3, respectively, showing a significant reduction (P<0.0001) in the opium+IPC+IR group.

The results showed that SOD and CAT activity and TAC values significantly (P<0.05-0.001) increased in the IPC group (20.77±0.11 vs 18.08±0.49, 83.66±1.31 vs 31.51±9.24 and 68.38±1.07 vs 46.49±1.77, respectively). MDA levels decreased significantly in the opium+IPC+IR group (P<0.01-0.001) from 48.98±0.61 to 38.6±0.28. Also, CAT activity and TAC values significantly increased in the opium+IPC+IR group compared

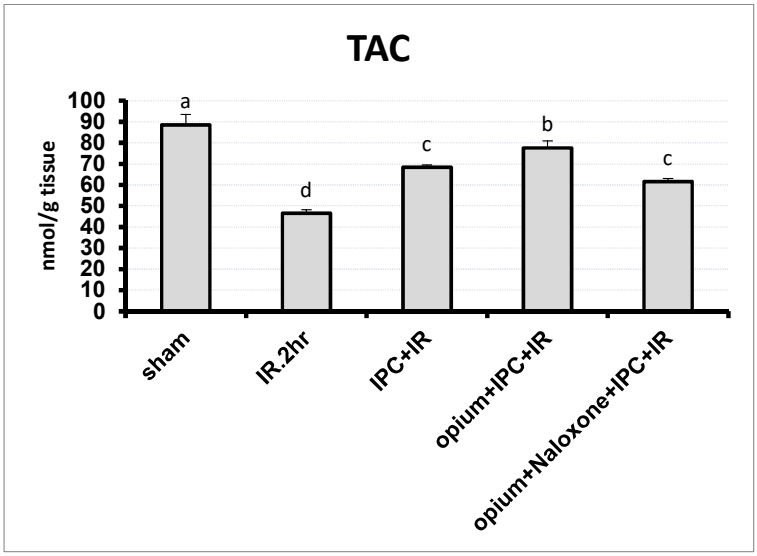

Figure 4. The values of TAC; nmol/g in different experimental groups

Note: The values are expressed as Mean±SD obtained from at least 5 experiments in each group. Different superscripts in each column indicate significant differences (P≤0.05) between the various groups.

to the IPC+IR group (100.09±2.38 and 77.58±3.31, respectively; P=0.01). However, the application of naloxone, as an opioid antagonist, inhabited the changes induced in the values of some parameters.

A significant increase in MDA levels was observed in the naloxone+opium+IPC+IR group compared to the opium+IPC+IR group (60.04±1.33 vs 38.6±0.28, respectively; P<0.001). A significant reduction (P=0.001) was also observed in SOD and CAT activities and TAC values in the naloxone-treated animals compared to the opium+IPC+IR group (19.54±0.3 vs 22.22±0.57, 53.81±5.17 vs 100.09±2.38 and 61.61±1.42 vs. 77.58±3.31, respectively). There was a significant increase in the MDA level, which serves as a marker of lipid peroxidation, in the rats exposed to 1 hour of ischemia followed by 2 hours of reperfusion compared to the group receiving opium and IPC. Additionally, there was a significant decrease (P<0.0001) in SOD and CAT activities and TAC values in the IR-treated group compared to the opium+IPC group.

Discussion

The present study was designed to investigate the protective effects of the simultaneous use of opium and IPC on IR-induced injury. It was hypothesized that while the application of IPC is able to reduce IRI, the administration of opium before the application of 10-minute ischemia and 10-minute reperfusion cycle may significantly accelerate the protective effects of IPC. This suggests that opioids can be involved in the protective mechanism of the IPC method.

IRI in the liver is a pathophysiological phenomenon associated with surgery, liver transplantation, hemorrhage, hypovolemic shock and partial hepatectomy. Injury is the major cause of death in surgery and liver transplantation due to the production of various proinflammatory agents, including cytokines, circulating chemokines, and ROS, leading to damage in distant organs (Shen et al., 2020). Shen et al. (2020) showed that liver exposure to IR is associated with increased liver enzymes and organ failure leading to liver dysfunction. They showed that the livers exposed to 1 hour of ischemia followed by 2 hours of reperfusion showed a significant increase in serum levels of ALT and AST, as well as tissue changes due to oxidative stress. The results of the present study are consistent with the results of many other studies.

However, it was found that the simultaneous application of IPC and opium before ischemia was able to inhibit the increased levels of AST and ALT induced by IR in the liver. Kim et al. (2020) found that the application of IPC, consisting of 10 minutes of hepatic ischemia followed by 15 minutes of reperfusion before 45 minutes of ischemia, was able to inhibit the elevated levels of AST and ALT. Lin et al. also reported that the application of IPC before hepatic ischemia can improve the function of this organ by increasing the antioxidant activities (Lin et al., 2019).

The efficacy of opium and IPC in controlling oxidative stress has been investigated in various experimental models. Shen et al. (2020) showed that when the liver is exposed to IR, various indicators of oxidative stress, including MDA, are increased and the level of SOD and CAT activities are decreased, as observed in the present study (Shen et al. (2020). However, SOD and CAT activities and TAC values significantly increased in the opium+IPC group compared to the IR group. It can be concluded that the changes in oxidative/antioxidant indices in the groups exposed to 1 hour of ischemia followed by 2 hours of reperfusion indicate the occurrence of oxidative stress in the acute phase of the hepatic warm IR model. These results are consistent with the findings of other studies, including those by Yarahmadzehi et al. (2020) and Li et al. (2014).

The protective effects of opium may be due to the prevention of oxidative stress by increasing antioxidant activities. There is evidence that various opioid compounds, including papaverine, morphine and protopine are able to exert antioxidant and anti-inflammatory effects (Jian et al., 2019). Studies show that opioids can play an important role in reducing IR-induced injury in various organs, including the liver (Wang et al., 2012). Morphine has demonstrated promising effects in patients with myocardial infarction by exerting an anti-inflammatory effect (Dorsch et al., 2016). Fentanyl was also able to reduce infarct size and increase cardiac contractile function. In addition, remifentanil, an ultrashort-acting opioid, has shown similar cardioprotective effects to fentanyl (Annachhatre & Annachhatre, 2019). Yarahmadzehi et al. reported that opium was effective in reducing ischemia-induced brain injury by increasing antioxidant and anti-inflammatory activities, ultimately leading to a neuroprotective effect (Yarahmadzehi et al., 2020).

Recent studies suggest that endogenous opioids are involved in the protective effects of IPC in the treated organs. Husain et al. (2009) reported that the activation of opioid receptors is required to induce the protective effects of IPC in the retina and showed that the use of a broad-spectrum opioid agonist, such as morphine, can reduce retinal ischemic injury (Husain et al., 2009). In the present study, the administration of opium before the application

of IPC increased the levels of antioxidant activities in the liver tissue exposed to IRI. Yarahmadzehi et al. reported that the protective effects of various opium alkaloids (morphine, papaverine, protopine, and berberine) against postischemic brain cell death may be exerted through the anti-inflammatory mechanism (Yarahmadzehi et al., 2020). It has been shown that the therapeutic effects of berberine, as a natural compound against diabetes mellitus and insulin resistance, may be partially attributed to its antioxidant and anti-inflammatory activities (Li et al., 2014). It has also been shown that the neuroprotective effects of papaverine are exerted through anti-inflammatory and antioxidant properties (Solmaz et al., 2022). Finally, there is evidence that morphine induces both oxidant and antioxidant activities by producing ROS and increasing SOD and glutathione reductase activities in peripheral blood cells (Zhang et al., 2004).

Conclusion

The simultaneous application of opium and IPC can accelerate the reduction of hepatic enzymes and oxidative stress induced by IR in rat liver, as well as improve antioxidant activities. These accelerating effects of opium are probably due to the antioxidant role of morphine and other alkaloids present in opium. Thus, the simultaneous use of opium and IPC can be more effective in reducing IRI. It can be concluded that the simultaneous use of opium and IPC may provide new insights into the mechanisms of action of IPC and could be a promising method for protecting the liver against IR injury in the future.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the University of Tehran, Tehran, Iran (Code: IR.UT.VETMED.REC.1402.062).

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The paper was extracted from the PhD dissertation of Maryam Karami, approved by the Department of Comparative Biosciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

Authors' contributions

Conceptualization and supervision: Hossein Ali Arab; Methodology: Hossein Ali Arab, Maryam Karami and Javad Jamshidian; Data collection: Maryam Karami; Data analysis: Hossein Ali Arab, Javad Jamshidian and Asma Kheirollahi; Funding acquisition and resources: Hossein Ali Arab and Asma Kheirollahi; Investigation, and writing: All authors.

Conflict of interest

The authors declared no conflict of interest.

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