Current Pharmaceutical Design, 2019, *25*, 2192-2198

RESEARCH ARTICLE

The Role of Autophagy and Death Pathways in Dose-dependent Isoproterenol-induced Cardiotoxicity

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ARTICLE HISTORY

Received: March 26, 2019
Accepted: June 4, 2019

DOI:
10.2174/1381612825666190619145025

Abstract: ***Background*:** Isoproterenol (ISO) is a non-selective β-adrenergic agonist. Our aims were to investigate the autophagy and cell death pathways including apoptosis and necrosis in ISO-induced cardiac injury in a dose-dependent manner.

***Methods*:** Male Sprague-Dawley rats were treated for 24 hours with I. vehicle (saline); II. 0.005 mg/kg ISO; III. 0.05 mg/kg ISO; IV. 0.5 mg/kg ISO; V. 5 mg/kg ISO; VI. 50 mg/kg ISO, respectively. Hearts were isolated and infarct size was measured. Serum levels of Troponin T (TrT), lactate dehydrogenase (LDH), creatine kinase isoenzyme MB (CK-MB) were measured. TUNEL assay was carried out to monitor apoptotic cell death and Western blot was performed to evaluate the level of autophagic and apoptotic markers.

***Results*:** Survival rate of animals was dose-dependently decreased by ISO. Serum markers and infarct size revealed the development of cardiac toxicity. Level of Caspase-3, and results of TUNEL assay, demonstrated that the level of apoptosis was dose-dependently increased. They reached the highest level in ISO 5 and it decreased slightly in ISO 50 group. Focusing on autophagic proteins, we found that level of Beclin-1 was increased in a dose-dependent manner, but significantly increased in ISO 50 treated group. Level of LC3B-II and p62 showed the same manner, but the elevated level of p62 indicated that autophagy was impaired in both ISO 5 and ISO 50 groups.

***Conclusion*:** Taken together these results suggest that at smaller dose of ISO autophagy may cope with the toxic effect of ISO; however, at higher dose apoptosis is initiated and at the highest dose substantial necrosis occurs.

Keywords: Isoproterenol, cardiotoxicity, necrosis, apoptosis, autophagy, β-adrenergic agonist.

1. INTRODUCTION

Isoproterenol [1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride, ISO] is a catecholamine and bearing a non-selective β-adrenergic agonist property. At low dose it can be used in heart block and cardiac arrest; however, at chronic or high doses, administration of ISO leads to the development of irreversible damage of the myocardium and ultimately causes infarct-like necrosis in heart muscles [1]. β-adrenergic overstimulation by ISO upset the balance between the oxygen demand and supply of the myocardium leading to pathological alterations [2]. The underlying mechanisms of ISO-induced cardiac injury are complex and multifactorial, but the major drawbacks of the ISO therapy are the generation of cytotoxic free radicals in myocytes followed by oxidative stress and lipid peroxidation, which leads to progressive mitochondrial damage, inflammatory cytokines production, ionic imbalance including intracellular Ca^{2+} overloading that results in cardiac injury [3]. Therefore, Ca^{2+} overload is in connection with the activation of the adenylate cyclase enzyme and the reduction of ATP levels [4]. In addition, further signaling pathways like NFκB, mitogen-activated protein kinases (MAPK) are also activated and promote cellular death. The aforementioned events of ISO-induced toxicity lead to cardiac fibrosis [3, 5, 6]. Previous studies on this matter showed that ISO-induced myocardial damage involves apoptosis; moreover, apoptosis seems to be an important complicating factor of myocardial injuries increasing the degree of myocyte cell death, which eventually leads to irreversible damages [7, 8].

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However, the role of autophagy and its connection with apoptosis and necrosis under this condition remains to be elucidated. Autophagy is an evolutionarily conserved pathway, which is critical in maintaining cell and tissue homeostasis, and involves the elimination of excess or damaged organelles under both physiological and pathological conditions [9], including impaired homeostasis, energy and oxygen starvation, and modification in metabolism [10]. It has been reported that cardiac hypertrophy is related to the accumulation of misfolded, exceed proteins and damaged organelles, including impaired mitochondria, but all these features can be cleared by autophagy, thus the appropriate elevation of autophagy antagonizes cardiac hypertrophy by increasing protein degradation [11]. This autophagic degradation process is essential for the cardiomyocytes and it may serve as a survival pathway during oxidative stress. In addition, it has been found that the impairment of autophagy promotes the accumulation of cellular aggregates and worsens cardiac function [12, 13]. However, regulatory mechanisms for autophagy, especially in the terms of cardiac hypertrophy, are still far from being completely understood [14]. Recent findings have revealed that autophagy and the two major types of death; apoptosis and necrosis have a well-balanced cross-talk, in a sequence in which autophagy precedes apoptosis [15]. Many stimuli that cause the induction of autophagy is followed by the induction of cell death pathways when the autophagy fails to adapt and cope with stress, or dysregulation occurs in the autophagic process [16]. Therefore, the disruption of the cross-talk between autophagy, apoptosis and necrosis leads to numerous important pathophysiological changes such as exceeded cardiomyocytes demise [16]. The fact that the physiological replacement of cardiomyocytes is an ineffective and slow process and cardiomyocytes are mostly nonrenewable further highlights the importance of the normal cross-talk of the above-mentioned pathways [17, 18].

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Thus, it has been an intense therapeutic interest to preserve the intact myocyte function and the prevention of cardiomyocytes loss under pathological conditions including cardiotoxicity. The aim of the present study was to investigate the autophagy and cell death signaling including apoptosis and necrosis in dose-dependent ISO-induced cardiac injury.

2. MATERIALS AND METHODS

2.1. Animals and Ethics Statement

The experiments were accomplished using adult male rats (Charles River Laboratories International, Inc. Sulzfeld, Germany). All animals were housed and treated according to the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication no. 86-23, revised in 1996). Maintenance and treatment of animals taken part in this study were additionally approved by the Institutional Animal Care and Use Committee of the University of Debrecen, Debrecen, Hungary. The animals were housed in wire-bottomed cages maintained on 12:12-h light-dark cycle throughout the study and provided with laboratory rodent chew pellets and water ad libitum. Approval number: DE MAB/64-1/2015.

2.2. Chemicals

(−)-Isoproterenol hydrochloride and 2,3,5-Triphenyltetrazolium chloride were purchased from Sigma (St. Louis, MO, USA). Stain-Free gels were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Antibodies against Beclin-1, LC3B, p62 and Caspase-3 were obtained from Cell Signaling Technology (Boston, MA, USA). TUNEL assay (*In Situ* Cell Death Detection Kit, TMR red) was bought from Roche (Mannheim, Germany).

2.3. Study Design and Treatment Protocol

Male Sprague-Dawley rats with a body weight range of 300-350 g were randomly divided into six groups as follows I. vehicle (n=24), II. 0.005 mg/kg ISO (n=21), III. 0.05 mg/kg ISO (n=25), IV. 0.5 mg/kg ISO (n=31), V. 5 mg/kg ISO (n=34), and VI. 50 mg/kg ISO (n=41). ISO was freshly prepared in saline, and rats were injected with a single intraperitoneal ISO injection (i.p.) in each group. The different doses of ISO used in the present study were chosen based on our previous *ex vivo* experiments [19]. Animals were sacrificed 24 h after the ISO injection.

2.4. Isolated Heart Preparation and Infarct Size Measurement

After 24-hours of the treatment with vehicle or ISO, rats were anesthetised with i.p. injection of ketamine (100 mg/kg)/xylazin (10 mg/kg), and blood samples were collected from the left jugular vein. After administration of heparin (1000 IU/kg) as an anticoagulant, thoracotomy was performed and hearts were excised and placed on ice-cold oxygenated Krebs–Henseleit bicarbonate buffer (KHB buffer) (118.5 NaCl, 4.7 KCl, 2.5 CaCl2 x $2H_2O$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 10.0 glucose (in mM). The aorta was cannulated and perfused in Langendorff "non-working" mode with KHB buffer. After 10 min of aerobic perfusion triphenyl tetrazolium chloride (TTC) staining was carried out to access the infarct size. Briefly, following 10 min of aerobic perfusion the hearts were perfused with a 50 mL 1% (*w*/*v*) solution of TTC in a phosphate buffer (pH 7.4) and the samples were stored at −20 °C for subsequent analysis. The frozen sections were cut, subsequently scanned on an Epson J232D flat-bed scanner (Seiko Epson Corporation, Nagano, Japan), blotted dry and weighted. The infarcted area (white coloration) and the risk area (entire scanned section) were analyzed using planimetry software (Image J, National Institute of Health, Bethesda, MD, USA). Estimates of the infarcted zone magnitude were subsequently obtained by multiplying the infarcted areas by the weight of each slice. The resulting numbers represent the weight of the risk zone and the infarcted zone. Outcomes were expressed as a percentage of the weight of the infarcted tissue and the weight of the risk zone.

2.5. Assessment of Serum Enzymes

After 24 h of the ISO injection blood samples were collected from the left jugular vein, and the serum was separated for measurement of Troponin-T (TrT), lactate dehydrogenase (LDH), creatine kinase MB isoenzyme (CKMBLC). For the *in vitro* quantitative evaluation of the serum enzymes electrochemiluminescence immunoassay "ECLIA" was used obtained from Roche Diagnostics GmbH (Mannheim, Germany) and measured by "Cobas 8000" modular analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

2.6. TUNEL Assay

To detect apoptosis, we used the terminal deoxynucleotidyl transferase (TdT) nick end labelling test by the *In Situ* Cell Death Detection Kit, TMR (fluorescein-labeled cell markers) red (Roche, Mannheim, Germany). DNA fragmentation, the sign of the early stage of apoptosis, can be detected by labeling the free 3'-OH termini with modified nucleotides in an enzymatic reaction. The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes the template-independent polymerization of deoxyribonucleotides to the 3'-end of single- and double-stranded DNA. Harvested heart tissues were fixed in 4% formalin for 24 hours at 4 °C, embedded in paraffin, and cut into 4.5 micron thick sections. All tissue sections were placed on Superfrost Plus glass slides (Thermo Scientific, Rockford, IL), then sections were deparaffined in xylene and acetone afterward rehydrated in 70% ethanol and water. The sections were boiled in citrate buffer pH 6.0 for 6 min, then cooled at room temperature for 10 min, thereafter washed two times for 5 min in phosphate-buffer saline (PBS pH 7.4). Finally, sections were incubated with TdT (terminal deoxynucleotidyl transferase) in a humidified box, at 37 °C for 1 hour. After washing, to identify nuclei, we used DAPI (4′,6-diamidino-2-phenylindole), which emits blue fluorescence upon binding to AT regions of DNA (Thermo Fisher Scientific, Waltham, MA, USA). The slides were washed with PBS, air-dried, and subsequently covered with mounting medium and glass slide covers. The moviol solution was used as a mounting medium. Fluorescence microscopic images were obtained by a Zeiss Axioscope A1 microscope with HBO100 illuminator (Zeiss, Jena, Germany). After merging the blue and red channels, purple spots were associated with the apoptotic nucleus, while blue spots were identified as the non-apoptotic nucleus (ZEN 2012 software, Zeiss, Jena, Germany). Apoptosis was quantified by the ratio of TdT-positive nuclei / total nuclei in each section.

2.7. Western Blot Analysis

The expression level of Beclin-1, LC3B-II, p62 and the pro-apoptotic Caspase-3 proteins expression level in left ventricular tissue was measured using Western blot analysis. Approximately 300 mg of heart tissues were homogenized by using a Polytron homogenizer in isolating buffer (25 mM Tris-HCL, 25 mM NaCl, 1 mM orthovanadate, 10 mM NaF, 10 mM pyrophosphate, 10 mM okadaic acid, 0.5 mM EDTA, 1 mM PMSF, and 1x protease inhibitor cocktail) and centrifuged at 2000 rpm at 4 °C for 10 min. The supernatant was transferred to a new tube and centrifuged at 10000 rpm at 4 °C for 20 min, and then the supernatant was used as a cytosolic fraction. Protein concentration was measured by a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Seventy five µg of protein from each sample was loaded and separated using electrophoresis on a polyacrylamide gel (TGX Stain-Free™ FastCast™ Acrylamide Kit, 12%, Bio-Rad, Hercules, CA, USA) and transferred on a PVDF membrane. Non-specific binding sites were blocked by using 7% skimmed milk in Tris-buffered saline solution with 0.5% Tween 20 for 1 hour at room temperature. Membranes were incubated overnight at 4 °C with primary antibodies (Cell

Signaling Technology, MA, USA). The following primer antibodies and 1:1000 dilution were used: anti- Beclin-1, anti-LC3B, anti-p62 and anti-Caspase-3 (Rabbit mAb). Antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). After extensive washing, membranes were incubated with corresponding horseradish-peroxidase-conjugated secondary antibody (HRP-linked anti-rabbit IgG and the dilution was 1:3000) for 1.5 h at room temperature, then signal intensities for each protein band were detected using Clarity Western ECL Substrate (Bio-Rad, California, USA). The optical density of bands was measured by using the ChemiDoc Touch Imaging System (Bio-Rad, California, USA). The level of the protein of interest was normalized against the total amount of protein in each lane with the Bio-Rad Image Lab 5.2.1 software [20] (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.8. Statistical Analysis

The data were presented as the mean ± standard error of the mean. The significance of differences among groups was evaluated with GraphPad Prism version 5 (GraphPad Prism Software, La Jolla, CA, USA). In case of survival rate, Chi-square and Fisher's exact tests were performed. Subsequently, One-way analysis of variance (ANOVA) test followed by Dunnett multiple comparison tests was performed, which identified the significant difference between control and treated groups. A probability value of $P<0.05$ was used as the criterion for statistical significance. When significant ($p<0.05$), *, **, and *** represent $p<0.05$, $p<0.01$, and $p<0.001$ at the Dunnett's post-test, respectively.

3. RESULTS

3.1. Survival Rate

The survival rate of animals (n = 24-41/group) was dose-dependently decreased (Fig. **1**) with the increase of intraperitoneal administration of ISO. Thus, at the highest doses of 0.5 mg/kg, 5 mg/kg and 50 mg/kg of ISO, the survival rate was significantly decreased in comparison with the control value, $p=0.0147$; $p=0.0016$ and $p<0.001$, respectively. ISO 50 reached the median lethal dose (LD_{50}) value.

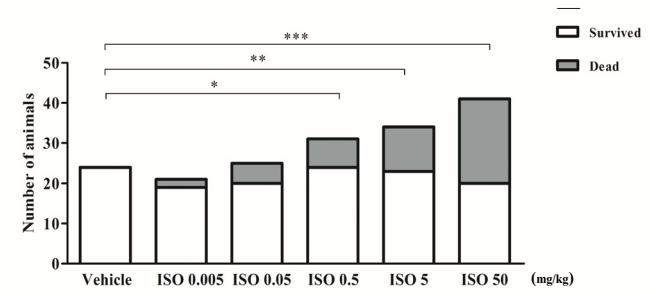

Fig. (1). Effect of ISO administration at different doses on the survival rate of animals.
(n=24-41/group). The significance of differences among groups was evaluated with Chi-square and Fisher's exact tests. *p* values of 0.05 or less were considered significant in each graph. *, **, and *** represent $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

3.2. Serum Biomarkers

Fig. **2** depicts that the 0.005 mg/kg, 0.05 mg/kg and 0.5 mg/kg of ISO administration resulted in a gradual slight increase in serum enzyme activities of Troponin T (Fig. **2A**), creatine kinase isoenzyme MB (CK-MB) (Fig. **2B**) and lactate dehydrogenase (LDH) (Fig. **2C**) in comparison with the vehicle-treated control following 24 hours of ISO administration. In addition, we have found a significantly higher ($p<0.05$) Troponin T and CK-MB level at 5 mg/kg of ISO and 50 mg/kg of ISO groups, and a significantly ($p<0.05$) elevated LDH level at 50 mg/kg of ISO group compared to the vehicle treatment, indicating the presence of acute myocardial damage.

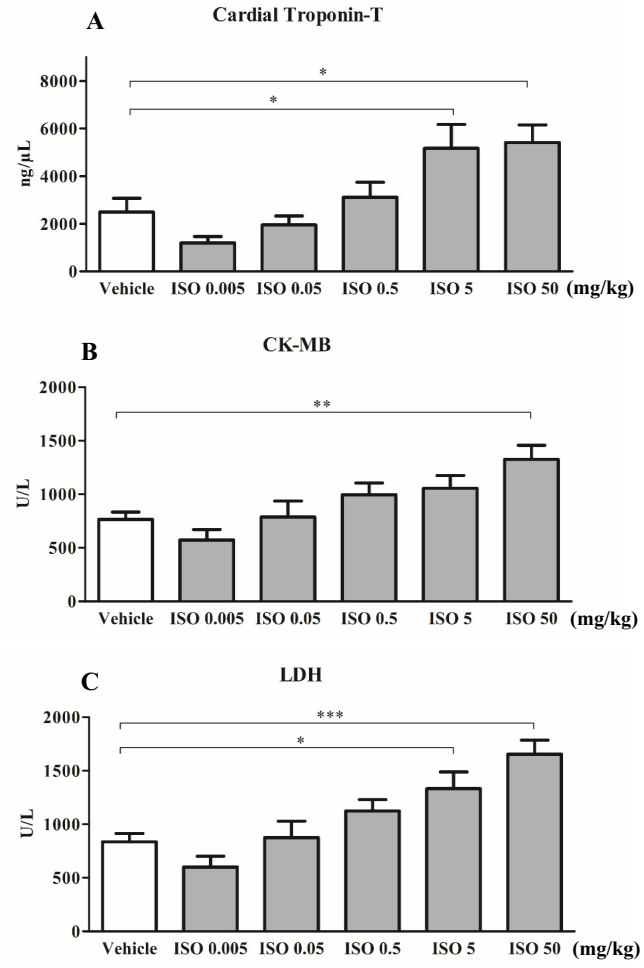

Fig. (2). Effect of ISO administration at different doses on serum biomarkers.
(**A**) Cardial Troponin T, (**B**) creatine-kinase iso-enzyme MB (CK-MB), (**C**) lactate-dehydrogenase levels were obtained from blood samples taken from the left jugular vein. Results are expressed as the average magnitude of each value within a group of animals ± SEM (n=17-22/groups). The significance of differences among groups was evaluated with one-way analysis of variance (ANOVA) followed by the Dunnett multiple comparison tests. *p* values of 0.05 or less were considered significant in each graph. *, **, and *** represent $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

3.3. TTC Staining (necrosis)

To further confirm myocardial damage, TTC staining was carried out to measure infarct size. In line with the serum biomarkers, as it is depicted in Fig. **3**, in hearts originated from animals challenged by smaller doses of ISO (0.005 mg/kg; 0.05 mg/kg) no alterations were observed in comparison with hearts obtained from the drug-free control animals. However, starting from a dose of 0.5 mg/kg of ISO, a gradually increasing infarct size was observed. Infarct size at higher doses including ISO 5 and ISO 50 was significantly enlarged compared to the vehicle-treated group.

3.4. TUNEL Assay and Caspase-3 (apoptosis)

To evaluate the effect of ISO on cardiomyocyte apoptosis, we first analyzed DNA fragmentation by TUNEL assay, which is the hallmark of the early stage of apoptosis. We found a significantly elevated level of TdT+ cells at 0.05 mg/kg of ISO; 0.5 mg/kg of

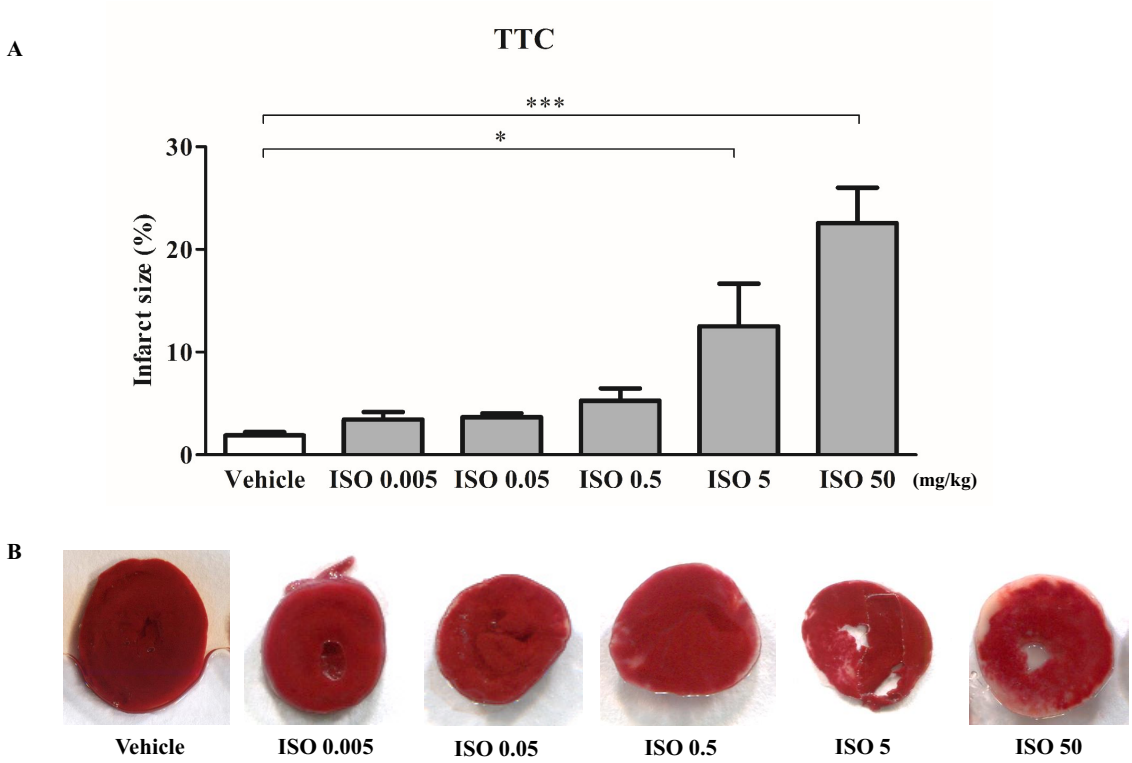

Fig. (3). Effect of ISO administration at different doses on necrosis.
(**A**) TTC staining was performed to measure the level of necrosis. Results are expressed as the average magnitude of each value within a group of animals ± SEM (n=4-6/group). The significance of differences among groups was evaluated with one-way analysis of variance (ANOVA) followed by the Dunnett comparison test. *p* values of 0.05 or less were considered significant in each graph. * and *** represent $p<0.05$ and $p<0.001$, respectively. (**B**) Representative pictures of infarct size by TTC staining.

ISO; 5 mg/kg of ISO and 50 mg/kg of ISO groups compared to vehicle (Figs. **4A** and **4B**). In agreement with Western blot analyses of Caspase-3 (Fig. **4C**), results demonstrated that the level of apoptosis was increased depending on ISO concentration. It reached the highest level in ISO 5 group and it slightly decreased in ISO 50 group.

3.5. Autophagic Markers

In order to monitor autophagic pathway, the levels of Beclin-1, LC3B-II and p62 were measured by Western blot. Left ventricular tissue levels of Beclin-1, which is an initiator in the macroautophagic pathway, were significantly ($p<0.05$) elevated at 50 mg/kg of ISO group in comparison with vehicle (Fig. **5A**). A significantly increased LC3B-II and p62 expression levels were detected in ISO 5 and ISO 50 groups compared to the vehicle-treated group (Figs. **5B**, **5C**), indicating that ISO treatment impaired the autophagic process in a dose-dependent manner.

4. DISCUSSION

The major clinical limitation of ISO therapy, in a long term period, is cardiac toxicity, which consequently leads to irreversible injuries in the myocardium. The underlying mechanisms are multifactorial and still not well-understood [21]. The injection of ISO in animals provides a rapid and simple method, developing myocardial damages similar to that seen in acute myocardial infarction in patients [22]. In the present study, we investigated the role of autophagy, and two different cell death pathways; apoptosis and necrosis following the administration of ISO at different doses.

In line with the literature, the dose-dependent increase was seen in the serum level of cardiac- specific Troponin T, CK-MB and LDH [23, 24]. Furthermore, enlarged infarct size at the higher dose indicates the infarct-like cardiac damage.

Autophagy and apoptosis often appear in the same cell and control the turnover of damaged or long-lived organelles in cells [15]. In most cases autophagy precedes apoptosis and the autophagy could block the apoptosis, although in some cases autophagy may help to induce apoptosis together with necrosis, however, it has important consequences when the interruption occurs between autophagy and apoptosis [25]. To analyse the autophagic and apoptotic pathways after ISO injection, Beclin-1, LC3B-II, p62 and Caspase-3 expression levels were measured in left ventricular tissue. LC3B-II and p62 are considered as major markers of autophagy processes. Enhanced level of LC3B-II indicates enhanced autophagy in a dose-dependent manner by ISO treatment. p62 is an autophagy substrate that is used as a reporter of autophagy activity, and p62 is widely used as a predictor of autophagic flux [26, 27]. The administration of 5 mg/kg and 50 mg/kg ISO resulted in a significant p62 protein levels indicating the accumulation of damaged macromolecules and suggests that the autophagy process is malfunctioning [28]. Based on these findings we may assume that after the administration of ISO at lower doses the autophagy may serve as a survival pathway, however treating with a high dose of ISO the cells fail to adapt and cope with the increasing stress. DNA fragmentation represents a characteristic hallmark of apoptotic cell death, and TUNEL is a well-established method for its detection. Treatment of ISO 0.5; ISO 5 and ISO 50 enhanced the level of TdT+ nuclei, which was expertly correlated with the expression level of Caspase-3. These data suggest that autophagy is malfunctioning, which leads to the accumulation of removal of damaged proteins and organelles. This impairment of the autophagic pathway was detrimental for the cardiomyocytes, which eventually induced cell death and triggered the apoptotic pathway. During the administration of the highest doses of isoproterenol, autophagy could have been a survival mechanism for cells, however, it failed to be completed and promoted the interplay of autophagy and apoptosis.

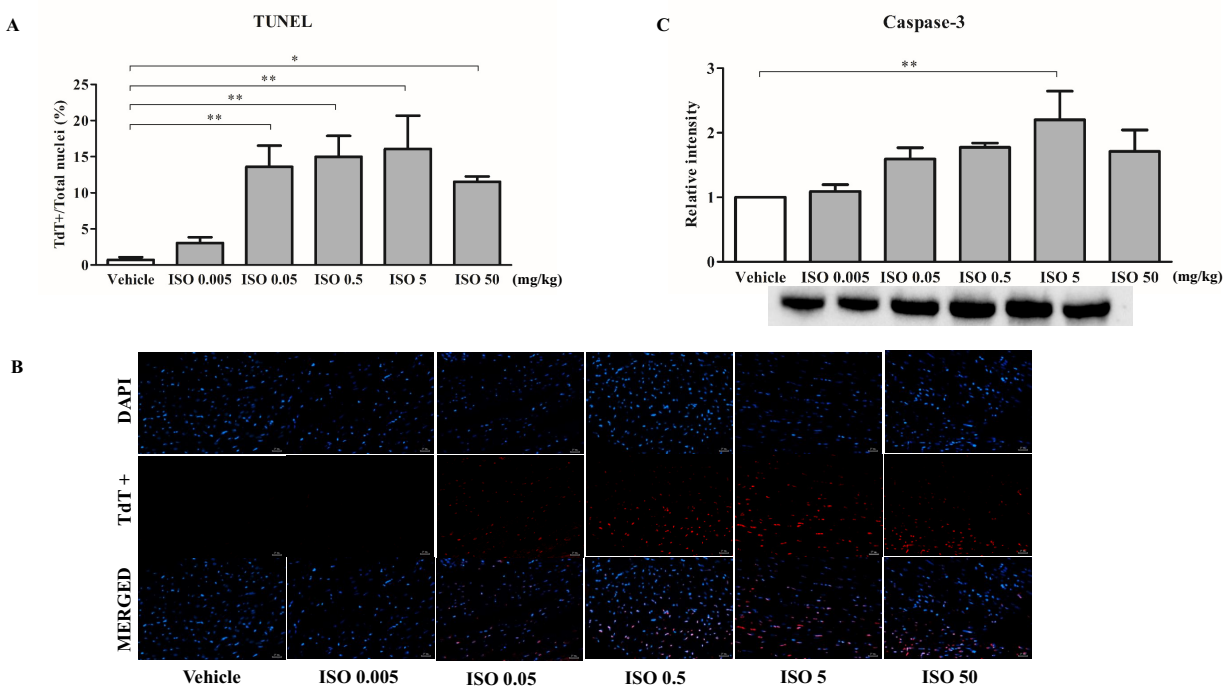

Fig. (4). Effect of ISO administration at different doses on the apoptotic marker.
(**A**) TUNEL assay was carried out to study the expression level of DNA fragmentation. Results are presented as a percent of the ratio of TdT+ to total nuclei (n = 4/ groups). (**B**) Representative immunofluorescence pictures obtained in heart tissue samples from all groups. Blue: total nuclei by DAPI staining, Red: TdT+ nuclei. Scale bar: 20 µm. (**C**) Caspase-3 was evaluated using Western blot analysis. Results are expressed as the average magnitude of each value within a group of animals ± SEM (n=7). The significance of differences among groups was evaluated with one-way analysis of variance (ANOVA) followed by the Dunnett comparison test. *p* values of 0.05 or less were considered significant in each graph. *, and ** represent p<0.05 and p<0.01, respectively. (*The color version of the figure is available in the electronic copy of the article*).

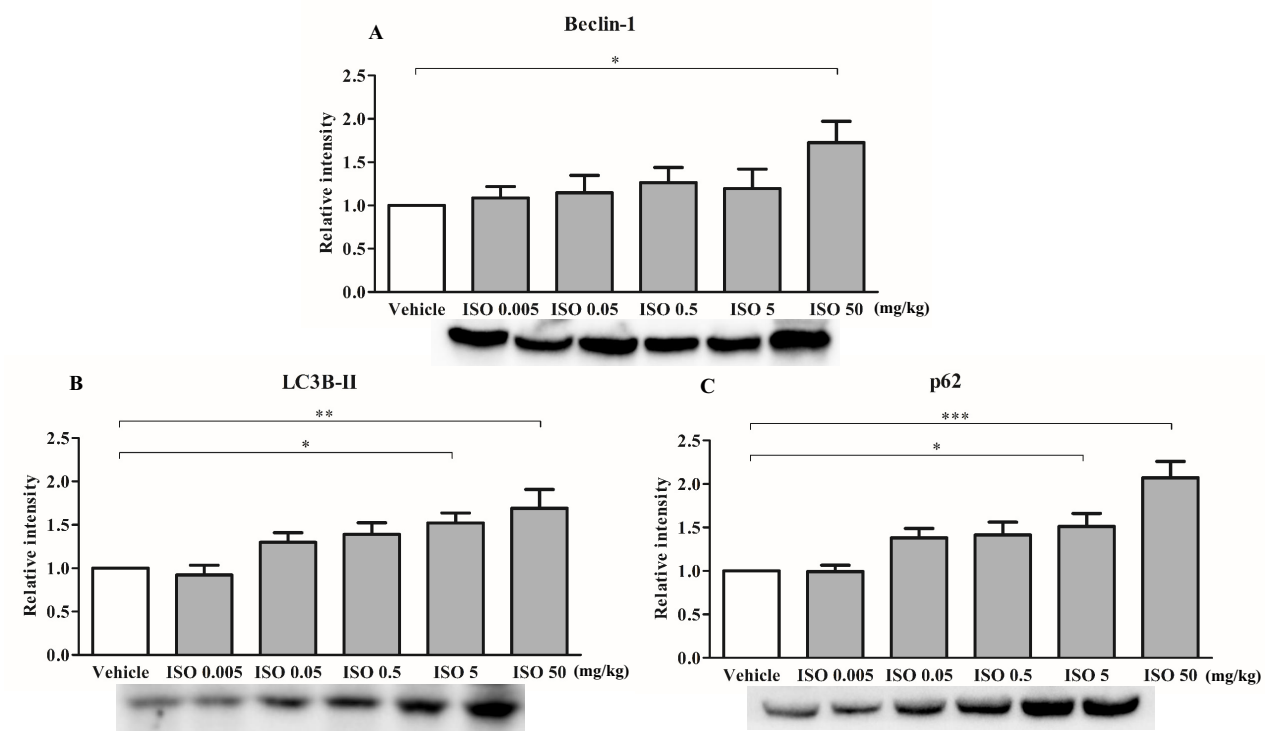

Fig. (5). Effect of ISO administration at different doses on autophagic markers.
The expression levels of (**A**) Beclin-1, (**B**) LC3B-II and (**C**) p62 in left ventricular tissues were evaluated using Western blot analysis. Results are expressed as the average magnitude of each value within a group of animals ± SEM (n=7-11). The significance of differences among groups was evaluated with one-way analysis of variance (ANOVA) followed by the Dunnett comparison test. *p* values of 0.05 or less were considered significant in each graph. *, **, and *** represent p<0.05, p<0.01, and p<0.001, respectively.

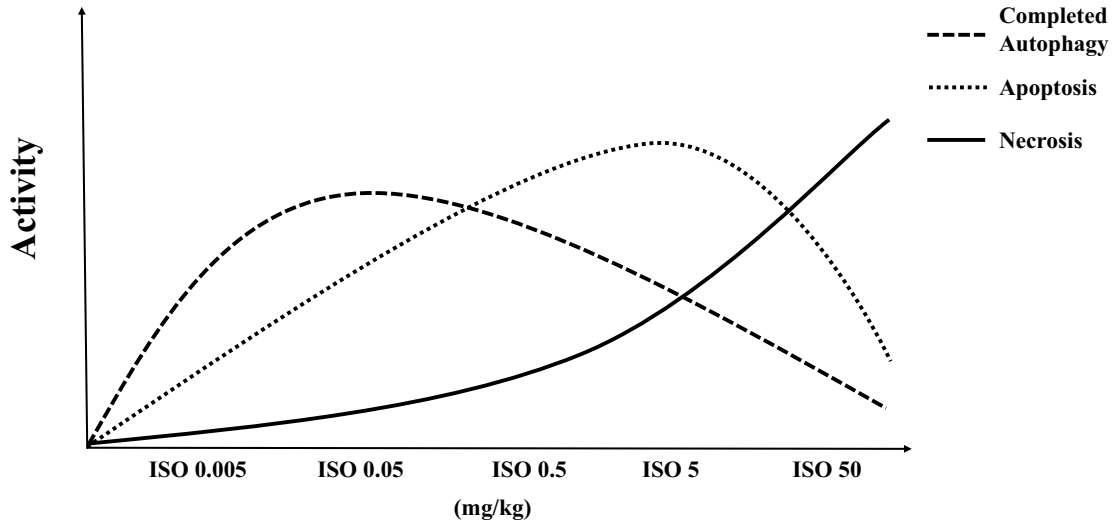

Fig. (6). Effect of ISO administration at different doses on the investigated pathways.
Based on the experiments and results, theoretically, isoproterenol treatment initiates first autophagy, and then apoptosis, and finally necrosis-induced cell death, suggesting that necrosis masks the processes of autophagy and apoptosis.

Interestingly, we observed a diminished level of apoptosis in the ISO 50 group, moreover, in this group, the level of lysosomal degradation during the autophagic process was extremely damaged. Our hypothesis is further supported by the results of survival rate and serum biomarkers, which were significantly evaluated in ISO 50 group. In conclusion, our results indicate that ISO treatment stimulated autophagy in a dose-dependent manner, although ISO treatment at high doses including ISO 5 and ISO 50, this pathway was impaired, and apoptosis and necrosis take place in the heart tissue (Fig. **6**). Thus, in such cases, understanding the reason for the incomplete degradation and the excessive cell death, and then specific stimulation and boosting of the autophagic pathway might be a novel strategy to enhance the survival rate.

5. LIMITATIONS OF THE STUDY

One limitation of the study is that ISO was administered intraperitoneally, whereas the protocol for patients involves intravenous administration. Furthermore, in humans, ISO infusion is between 0.5 micrograms/min and 5.0 micrograms/min [29], which is significantly lower in comparison with animal studies. Under experimental conditions, especially in rats, the doses of ISO or other drugs are about 50 and 100 times or even higher compared to human beings. However, it has to be noted that the doses used in our experiments are in line with the literature [30, 31], and our aim was to analyze the role of macroautophagy and the two death-pathways, including apoptosis and necrosis, after an acute ISO administration in a dose-dependent manner.

CONCLUSION

Taken together, based on our results, we may assume that during high dose of ISO treatment there was a disruption of the cross-talk between autophagy and apoptosis, and the extremely high unsupplied oxygen demand lead to an acute and significant increment of myocardial necrosis. Under conditions, such as tachycardia and/or myocardial injury induced by ISO, the restoreation of the autophagy could be a protective mechanism against the extreme activation of death pathways and the development of cardiac toxicity.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experiments involving animals were approved by the Institutional Animal Care and Use Committee of the University of Debrecen, Debrecen, Hungary. Approval number: DEMAB/64-1/2015.

HUMAN AND ANIMAL RIGHTS

No humans were used in this research. All the experiments on animals were according to the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals”.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available from the corresponding author (Dr. Istvan Lekli) upon reasonable request.

FUNDING

This research was supported by grants from OTKA-PD-111794 (I.L.), NKFIH-K-124719 (A. T.) and also supported by the European Social Fund in the framework of TÁMOP 4.2.4. A/2-11-1-2012-0001 (A.G., A.T. and I.L.), the ÚNKP-17-4-III-DE-219 (I.L.) New National Excellence Program of the Ministry of Human Capacities, GINOP-2.3.2-15-2016-00043 (A.G., R.Z, A.T. and I. L.), EFOP- 3.6.3-VEKOP-16-2017-00009 (R.Z.) and EFOP-3.6.1-16-2016-00022 (R.Z.).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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