

Enteral Lactoferrin Administration Attenuates Myocardial Ischemia-Reperfusion Injury in an Isolated Rat Heart Model

Keisuke Omiya (✉ k.omiya0303@gmail.com)

University of Yamanashi <https://orcid.org/0000-0003-3882-5772>

Yosuke Nakadate

University of Yamanashi

Takeshi Oguchi

University of Yamanashi

Tamaki Sato

McGill University Health Centre Glen Site

Toru Matsuoka

University of Yamanashi

Masako Abe

University of Yamanashi

Akiko Kawakami

University of Yamanashi

Takashi Matsukawa

University of Yamanashi

Hiroaki Sato

McGill University Health Centre Glen Site

Research

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Abstract

Background

While lactoferrin, an iron-binding glycoprotein, has protective effects on intestinal and cerebral ischemia-reperfusion injuries, its cardioprotective effects against stunned myocardium are unknown. This study aimed to test the hypothesis that lactoferrin has cardioprotective effects against stunned myocardium.

Methods

Rat hearts were perfused using the Langendorff system, and two experiments were performed. In experiment 1, the hearts were divided into the enteral lactoferrin (E-LF) 7.5 m, 15 m, 30 m, and 60 m groups, where lactoferrin (1000 mg/kg) was administered enterally for 7.5, 15, 30, and 60 min, respectively, before perfusion; and a control group, where saline was administered 30 min before perfusion. In experiment 2, hearts were allocated to the perfusate lactoferrin (P-LF) 15 and 100 groups, where 15 mg/L and 100 mg/L lactoferrin were respectively added to the perfusate, and a control group. Each group was perfused for 20 min prior to 15 min of no-flow ischemia with pacing, followed by 20 min of reperfusion. The primary outcome was the maximum left ventricular derivative of pressure development (LV dP/dt max) 15 min after reperfusion. Myocardial phospho-protein kinase B (p-Akt) was assayed by western blotting.

Results

LV dP/dt max in the E-LF 15 m and 30 m groups was significantly higher than in the control group. In the second experiment, there were no significant differences in LV dP/dt max. Myocardial p-Akt was not significantly activated in any lactoferrin group.

Conclusion

Cardio-protection was observed with enteral but not parenteral lactoferrin administration, and myocardial p-Akt was not involved in this effect.

Background

Ischemia-reperfusion (IR) injury is a problem arising during cardiac surgery requiring cardiopulmonary bypass (CPB) [1–3]. Myocardial IR injury is induced by activation of the $2\text{Na}^+/\text{Ca}^{2+}$ exchanger and intracellular Ca^{2+} overload [2]. Some anesthetics provide cardioprotective effects against myocardial IR injury via activation of the myocardial phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt)

signaling pathway [4]. Further, insulin pre-conditioning has been shown to preserve cardiac contractility after ischemia via the PI3K/Akt signaling pathway [5, 6] in a stunned myocardium model.

Recently, the enhanced recovery after surgery (ERAS) program has been advocated [7, 8] to attenuate the stress response to surgery and promote rapid recovery. Although ERAS is relatively new to cardiac surgery, this cardiac program has been associated with significantly improved perioperative outcomes [9, 10]. Preoperative nutritional management, such as enteral carbohydrate supplementation, is one of the key factors in perioperative management. While preoperative inflammation leads to worse outcomes [11], preoperative enteral carbohydrate supplementation has been shown to improve inflammatory levels [12].

Breast milk, the major source of lactoferrin, exerts antiviral effects by adjusting the immune system [13, 14]. Lactoferrin is an 80 kDa iron-binding glycoprotein of the transferrin family [15] that contributes to the mammalian innate immune system [13] and anti-inflammatory system [16]. Enteral administration of lactoferrin attenuated intestinal and cerebral IR injury in rats in previous studies [17, 18]. However, while lactoferrin activates the PI3K/Akt signaling pathway [19, 20], the relationship between the cardioprotective effects of lactoferrin and the PI3K/Akt signaling pathway in isolated stunned rat hearts remains unknown.

In this study, we tested the hypothesis that enteral lactoferrin administration prior to ischemia provides cardioprotective effects against IR injury in isolated rat hearts. We also investigated the direct effect of parenteral lactoferrin on myocardial IR injury, because intravenous injection and cardioplegia are frequently employed in cardiac surgery. The primary outcome was maximum left ventricular pressure derivative (LV dP/dt max) 15 min after reperfusion. We also assessed the role of myocardial phosphoprotein kinase B (p-Akt) as a potential mediator.

Methods

The experimental protocol was approved by the Ethics Committee on Animal Research of our University.

Langendorff perfusion system

Male Wistar rats (weighing 300–320 g each) were anesthetized by intraperitoneal injection of pentobarbital sodium (80 mg/kg body weight). Hearts were excised and quickly immersed in cold modified Krebs-Henseleit (KH) buffer at 4 °C. The aorta was cannulated, and retrograde arterial perfusion was initiated at a constant pressure of 70 mmHg with modified KH buffer (NaCl, 118 mmol/L; NaHCO₃, 25 mmol/L; KCl, 4.7 mmol/L; KH₂PO₄, 1.2 mmol/L; MgSO₄, 1.2 mmol/L; CaCl₂, 2.0 mmol/L; di-NaEDTA, 0.5 mmol/L; and glucose, 11 mmol/L). The KH buffer was maintained at 37 °C and bubbled with 95% O₂ and 5% CO₂. The left ventricle was cannulated with a thin latex balloon via the pulmonary vein and connected to a pressure transducer (DTXPlus DT-12, Argon Critical Care Systems Singapore Pte. Ltd., Singapore) for continuous measurement of the left ventricular (LV) pressure. The balloon was inflated with water to adjust the LV end-diastolic pressure (LVEDP) to 5–10 mmHg. The pulmonary artery was

cannulated with a catheter to collect the coronary effluent for the measurement of coronary flow. Hearts with a heart rate (HR) < 200 bpm and frequent arrhythmias at baseline were excluded.

Experimental protocol

In the first experimental protocol, bovine lactoferrin (protein purity 95%) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). To assess the effect of enteral lactoferrin administration on myocardial IR injury, the rats were randomly divided into five groups (n = 8 per group): E-LF 7.5 m, E-LF 15 m, E-LF 30 m, E-LF 60 m, and control. Lactoferrin (1000 mg/kg in a volume of normal saline 4 mL/kg) was administered by gavage using a 2-mL syringe and a 15-gauge ball-tipped feeding needle 7.5 (E-LF 7.5 m), 15 (E-LF 15 m), 30 (E-LF 30 m), and 60 (E-LF 60 m) min before intraperitoneal pentobarbital injection. The control group was administered normal saline (4 mL/kg) by gavage 30 min before intraperitoneal injection. Following a stabilization period of 20 min, baseline hemodynamics were recorded. These groups received KH buffer for 20 min before the ischemic period (which lasted for 15 min) and during 20 min of reperfusion. The hearts were paced at 222 beats/min during no-flow ischemia with an electronic stimulator (SEN-3201, Nihon Kohden Corporation, Tokyo, Japan). The experimental protocol is shown in Fig. 1A.

In the second experimental protocol, human lactoferrin (protein purity > 90%) was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). To investigate the direct effect of lactoferrin on myocardial IR injury, lactoferrin was added to the perfusate. In this parenteral lactoferrin study, the hearts were randomly divided into three groups (n = 8 per group): P-LF 15, P-LF 100, and control. The P-LF 15 and P-LF 100 groups received 15 mg/L and 100 mg/L lactoferrin, respectively, in KH buffer for 20 min prior to 15 min no-flow ischemia, and during 20 min of reperfusion. The control group was perfused with KH buffer throughout. Following a stabilization period of 20 min, baseline hemodynamics were recorded. During the ischemic period, the hearts were paced at 222 beats/min. The experimental protocol is shown in Fig. 1B.

Measurements

LV dP/dt max (mmHg/s), HR, and LVEDP were continuously recorded. Coronary flow (CF) (mL/min) was measured by timed perfusate collections (baseline, just before ischemia, and after 5, 10, 15, and 20 min of reperfusion) from a catheter inserted into the pulmonary artery.

At the end of reperfusion, the whole rat's heart was promptly frozen in liquid nitrogen and freeze-dried for 6 days to measure p-Akt/total-Akt in the myocardial muscle.

Western blot analysis

The myocardium was suspended in RIPA lysis buffer (Sigma-Aldrich Corp., St. Louis, Missouri, USA) containing cOmplete protease inhibitor tablets (Roche, Basel, Switzerland) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich Corp.). The samples were then homogenized using the BioMasher II tissue homogenizer (Nippi Inc., Tokyo, Japan). Then, homogenates were centrifuged for 10 min at 12,000 × g at 4 °C, and the supernatants collected. These were diluted in 2 × Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, California, USA) containing 5% β-mercaptoethanol, and boiled at 95 °C for 5 min.

Proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions and then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). After blocking with 5% bovine serum albumin in 0.1% Tween-20 Tris-buffered saline, membranes were incubated with the primary antibodies in 5% bovine serum albumin in 0.1% Tween-20 Tris-buffered saline at 4 °C overnight. The primary antibodies were Akt and phospho-Akt (Ser473) antibodies (Cell Signaling Technology, Inc., Danvers, Massachusetts, USA), diluted 1:1000. The membranes were then incubated with the secondary antibody at room temperature for 1 h. The secondary antibody was anti-rabbit immunoglobulin G (Cell Signaling Technology, Inc.), diluted 1:1000. The bands were revealed using an enhanced chemiluminescence detection kit (GE Healthcare Japan Corporation, Tokyo, Japan).

Statistical analysis

Data are presented as mean \pm standard deviation (SD). Intra- and intergroup comparisons in hemodynamics were analyzed using two-way analysis of variance (ANOVA) followed by Dunnett’s test. Intragroup comparisons for baseline measurements and p-Akt/total-Akt ratios were analyzed with one-way ANOVA followed by Dunnett’s test. Two-sided P-values < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, California, USA). Sample size calculation was based on the expected difference in the LV dP/dt max at 15 min after reperfusion among groups using Power and Sample Size Calculation version 3.1.6 (available at <http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize>). The results of our pilot study showed 1000 ± 500 mmHg/s in the control group at 15 min after reperfusion, and 2000 ± 500 mmHg/s in the E-LF 30 m group at 15 min after reperfusion. In order to achieve a power level of 80%, with an alpha error of 5%, at least seven subjects were required in each group.

Results

There were no significant differences in baseline values among groups in either experiment (Table 1A, 1B).

Table 1: Baseline measurements

A: Enteral administration groups

	Control	E-LF 7.5 m	E-LF 15 m	E-LF 30 m	E-LF 60 m
Number (n)	8	8	8	8	8
LV dP/dt max (mmHg/s)	2500 \pm 213	2556 \pm 253	2608 \pm 271	2669 \pm 321	2391 \pm 147
Heart rate (bpm)	236 \pm 25	234 \pm 24	226 \pm 21	244 \pm 18	255 \pm 42
Coronary flow (mL/min)	14.1 \pm 1.3	13.3 \pm 0.7	14.4 \pm 0.9	14.0 \pm 1.7	13.0 \pm 1.5

B: Parenteral administration groups

	Control	P-LF 15	P-LF 100
Number (n)	8	8	8
LV dP/dt max (mmHg/s)	2583 ± 374	2546 ± 420	2569 ± 289
Heart rate (bpm)	270 ± 42	256 ± 52	246 ± 29
Coronary flow (mL/min)	15.7 ± 1.8	14.4 ± 0.9	14.7 ± 1.7
Data are presented as mean ± SD.			

LV dP/dt max: maximum left ventricular derivative of pressure development.

In the experiment with enteral lactoferrin, the LV dP/dt max of the E-LF 15 m and E-LF 30 m groups at 45 min and 50 min (10 and 15 min after reperfusion) was significantly higher than that of the control group (Fig. 2A). The E-LF 15 m and E-LF 30 m groups at 45 min (10 min after reperfusion) also showed significantly higher HR and lower LVEDP than those of the control group (Fig. 2B, 2C). However, there were no significant differences in CF compared to the control group (Fig. 2D). In western blotting, myocardial p-Akt was not significantly activated in the enteral lactoferrin groups compared with the control group (Fig. 3). The original source data sets in the enteral administration groups are available in supplementary Table S1.

In the experiment with parenteral lactoferrin, no significant differences between the lactoferrin and control groups in any hemodynamic parameters were found (Fig. 4). In western blotting, myocardial p-Akt was not significantly activated in the P-LF 15 or P-LF 100 groups compared to the control group (Fig. 5). The original source data sets in the parenteral administration groups are available in supplementary Table S2.

Discussion

In this study, enteral lactoferrin showed cardioprotective effects 15–30 min after ingestion, while parenteral lactoferrin did not show cardioprotective effects. Significant activation of p-Akt was not observed in any lactoferrin group. These results suggest that the lactoferrin-induced myocardial protection is an indirect effect which may be induced by some intermediate metabolites, and that the PI3K/Akt signaling pathway was not involved in the protective mechanism of lactoferrin.

In this study, a reversible rat model of stunned myocardium was used to mimic the context of CPB. The use of specific pharmacological agents as myocardial preconditioning can mitigate the adverse effects of CPB or ischemia [6]. Pharmacological options include pre-emptive administration of volatile anesthetics, such as nicorandil and insulin [4–6, 21], to protect cardiomyocytes by exerting anti-inflammatory effects. However, these agents are mainly intraoperative and dosage-limited. Therefore,

preoperative nutritional treatment can be important for anti-inflammatory therapy, as emphasized in the ERAS program.

We observed that enteral lactoferrin yielded cardioprotective effects 15–30 min after ingestion. The dose of enteral lactoferrin used in our study was in accordance with previous reports by Ono et al. [22], Cerven et al. [23], and Takeuchi et al. [24], and this 15–30 min time is consistent with that at which the highest concentration of lactoferrin is detectable in mesenteric fat tissue after gavage administration [22].

On the other hand, in the second experiment, parenteral lactoferrin did not yield cardioprotective effects, despite administration at concentrations 75 and 500 times higher than the normal plasma concentration (approximately 0.2 µg/mL) [13]. Considering our finding that parenteral lactoferrin had no cardioprotective effects and the reports that lactoferrin administered enterally is not transported into the blood [25] but to the mesenteric fat tissue [22], lactoferrin itself may not have cardioprotective effects. Rather, some intermediate metabolite production or regulation induced after lactoferrin reaches the mesenteric fat tissue allows it to provide the best cardioprotective effects. This suggests that patients undergoing cardiac surgery should receive enteral lactoferrin, and that intraoperative intravenous or cardioplegic administration is not recommended.

The cardioprotective effects of volatile anesthetics and insulin are associated with activation of the PI3K/Akt signaling pathway [4–6]. In immature hypoxic ischemic rat brains, lactoferrin supplementation through lactation decreased brain TNF-α and IL-6 gene transcription via p-Akt activation [18]. In C57BL/6J mouse vessels, after unilateral hindlimb surgery, lactoferrin also promoted vascular endothelial cell function via the Src/Akt/eNOS-dependent pathway on angiogenesis, thereby contributing to revascularization after ischemia [26]. However, in our experiments, the cardioprotective effects of lactoferrin were not associated with the PI3K/Akt signaling pathway, because the p-Akt in the heart of rats from the lactoferrin groups was not activated, regardless of administration route, as shown by western blotting. These findings also support the hypothesis that the protective effects against IR injury may not be induced by lactoferrin itself but by some intermediate metabolites.

Several substances could be intermediate lactoferrin metabolites, including glucagon-like peptide-1 (GLP-1) and adipocytokines. GLP-1, a hormone secreted from intestinal endocrine L cells, has cardioprotective effects via the cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) and protein kinase C (PKC) signaling pathways [27–29]. Maekawa et al. [30] reported that lactoferrin administered to rats by intraperitoneal injection increased GLP-1 in plasma. Adipocytokines such as omentin, apelin, and adiponectin could also be intermediate metabolites, considering that lactoferrin is associated with lipid metabolism [31, 32]. Adipocytokines, hormones secreted from fat tissues, are involved in the innate immune mechanism [33], and have anti-inflammatory and cardioprotective effects [34]. In particular, adiponectin can suppress inflammation and attenuate myocardial inflammation and injury because it activates the cAMP-PKA signaling pathway [35] and inhibits the toll-like receptor 4 signaling pathway [36].

Clinically, with regard to preoperative oral injection, carbohydrate loading two hours before surgery is the only nutritional recommendation in the ERAS program for reducing the inflammatory response [7, 8]. This can mitigate anxiety and discomfort, sustain muscle strength and lean body mass, facilitate the return of bowel function, and decrease insulin resistance [37–39]; however, the evidence is limited in cardiac surgery, especially concerning cardio-protection. Hence, enteral lactoferrin may be effective as a preoperative enteral nutritional treatment in cardiac surgery. In addition, it may be useful in emergency surgery, judging from the early onset time of enteral lactoferrin in the present study and the rapid gastric emptying (30 min) in healthy volunteers [40].

We acknowledge some limitations of this study. First, the type of lactoferrin employed between in the enteral and parenteral groups differed because of economic cost. Second, we did not measure other signaling pathways which may also be involved in the investigated process; therefore, further studies are needed to clarify the mechanism underlying lactoferrin's cardioprotective effects.

Conclusions

In summary, lactoferrin administered enterally protected cardiac contractility in isolated stunned rat hearts, but no beneficial effects were observed for parenteral administration. Enteral lactoferrin took ~ 15–30 min to exert its effects on the heart muscle. According to our results, the PI3K/Akt signaling pathway, which is activated by other preconditioning techniques, was not involved in the protective mechanism of lactoferrin. The present study suggests that enteral lactoferrin-induced myocardial protection might be mediated by other intermediate metabolic pathways.

Abbreviations

ANOVA, analysis of variance; CF, coronary flow; CPB, cardiopulmonary bypass; E-LF, enteral lactoferrin; ERAS, enhanced recovery after surgery; GLP-1, glucagon-like peptide-1; HR, heart rate; IR, ischemia-reperfusion; LV dP/dt max, maximum left ventricular derivative of pressure development; LV, left ventricular; LVEDP, LV end-diastolic pressure; P-LF, parenteral lactoferrin; p-Akt, phospho-protein kinase B; PI3K/Akt, phosphatidylinositol 3-kinase/protein kinase B; PKA, cyclic adenosine monophosphate protein kinase A; PKC, protein kinase C; P-LF, perfusate lactoferrin; SD, standard deviation

Declarations

Ethics approval

This study was approved by the Ethics Committee on Animal Research of the University of Yamanashi (Protocol number A 2-8, 2020).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Keisuke Omiya: Conceptualization, Methodology, Investigation, Formal analysis, Writing-Original draft, Writing-Review & Editing. Yosuke Nakadate and Takeshi Oguchi: Investigation, Writing-Original draft and Writing-Review & Editing. Tamaki Sato and Toru Matsuoka: Writing-Original draft and Writing-Review & Editing. Masako Abe and Akiko Kawakami: Investigation (western blotting). Takashi Matsukawa and Hiroaki Sato: Conceptualization and Writing-Review & Editing. All authors have read and approved the final manuscript.

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Figures

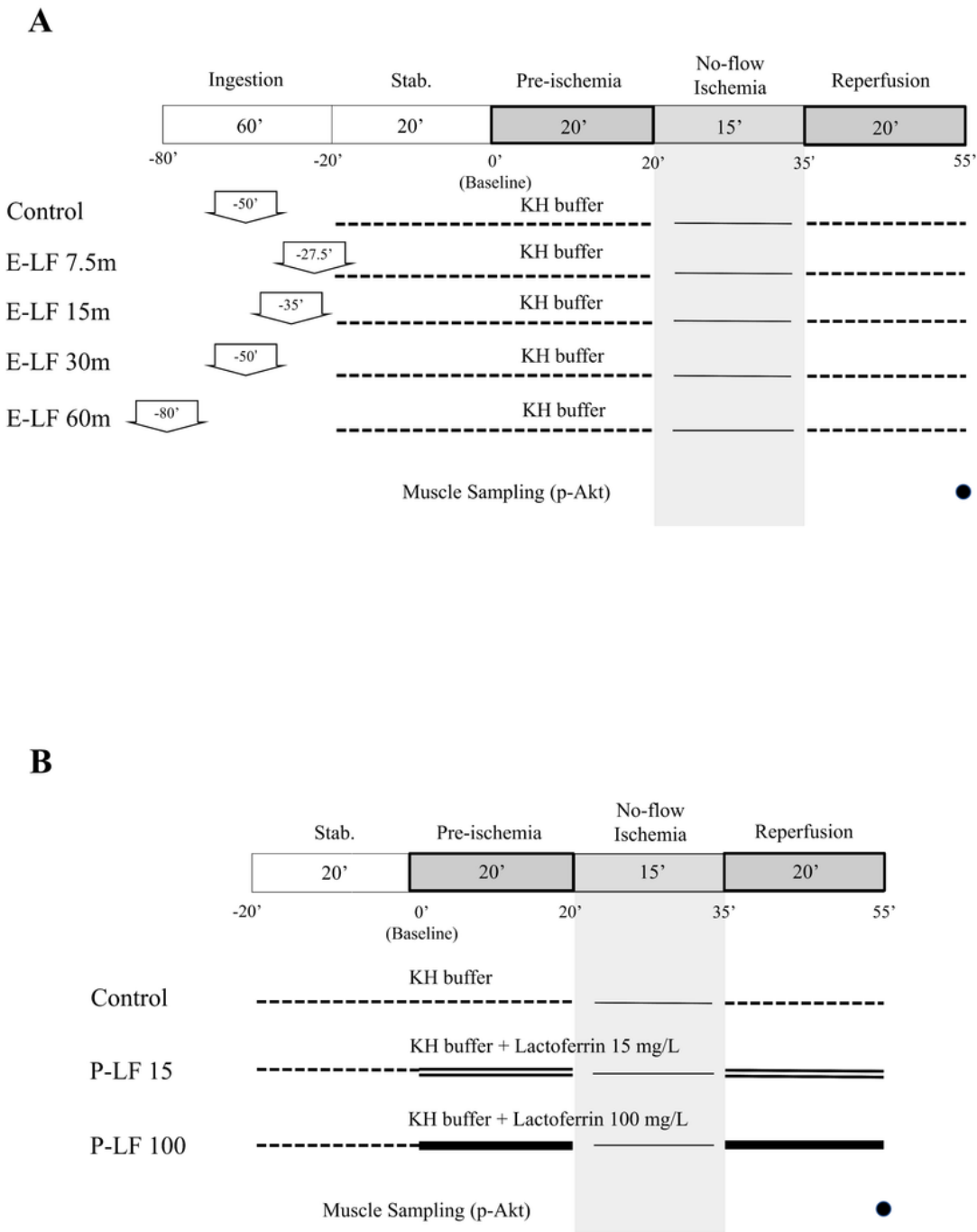


Figure 1

Experimental protocols. A: Enteral administration groups. The E-LF 7.5m, 15m, 30m, and 60m groups received lactoferrin by gavage 7.5, 15, 30, and 60 min, respectively, before intraperitoneal pentobarbital injection. The control group received normal saline by gavage 30 min before intraperitoneal injection. All groups were perfused with KH buffer throughout. B: Parenteral administration groups: The P-LF 15 and P-LF100 groups received 15 mg/L and 100 mg/L lactoferrin, respectively, in KH buffer. The control group was perfused with KH buffer throughout.

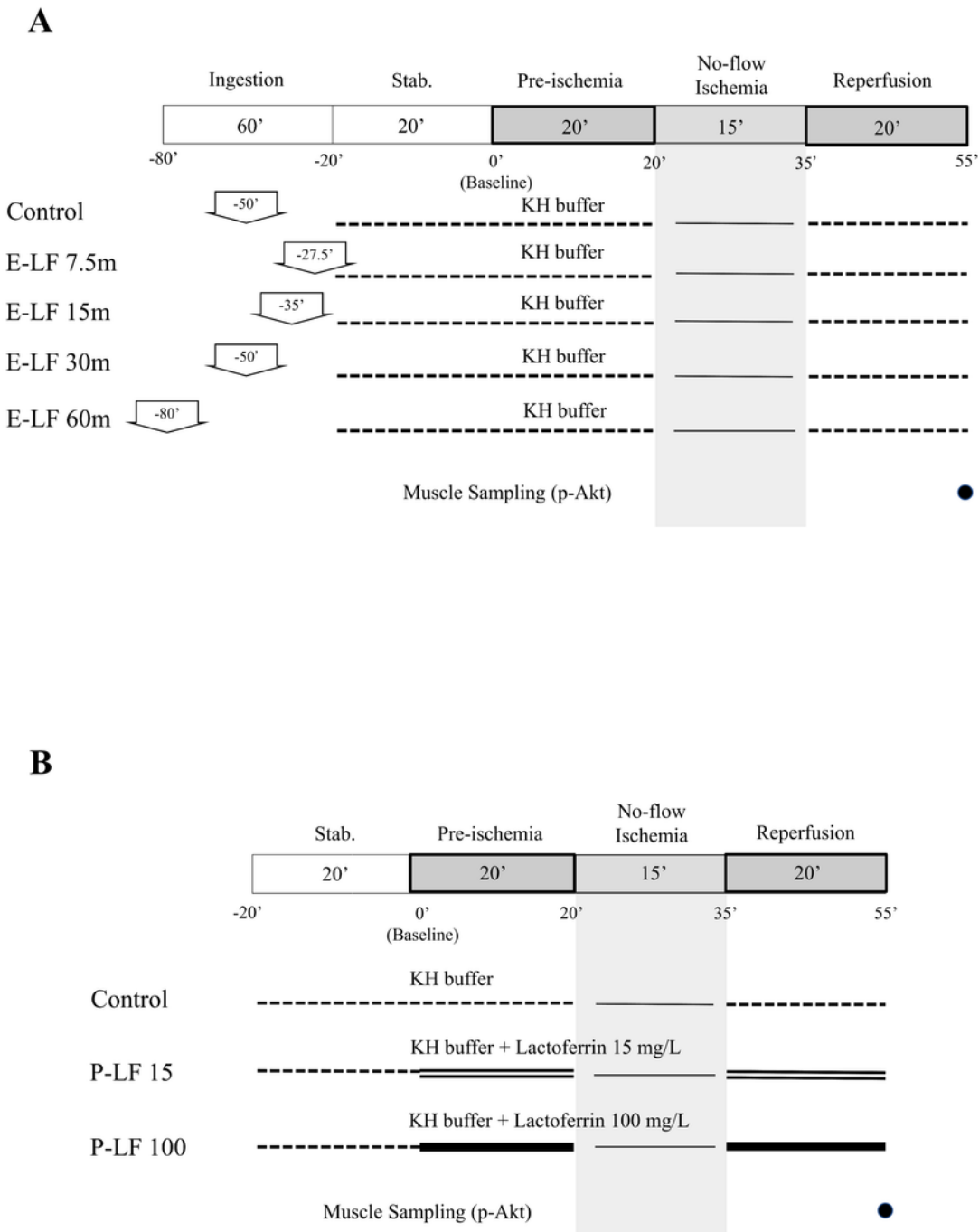


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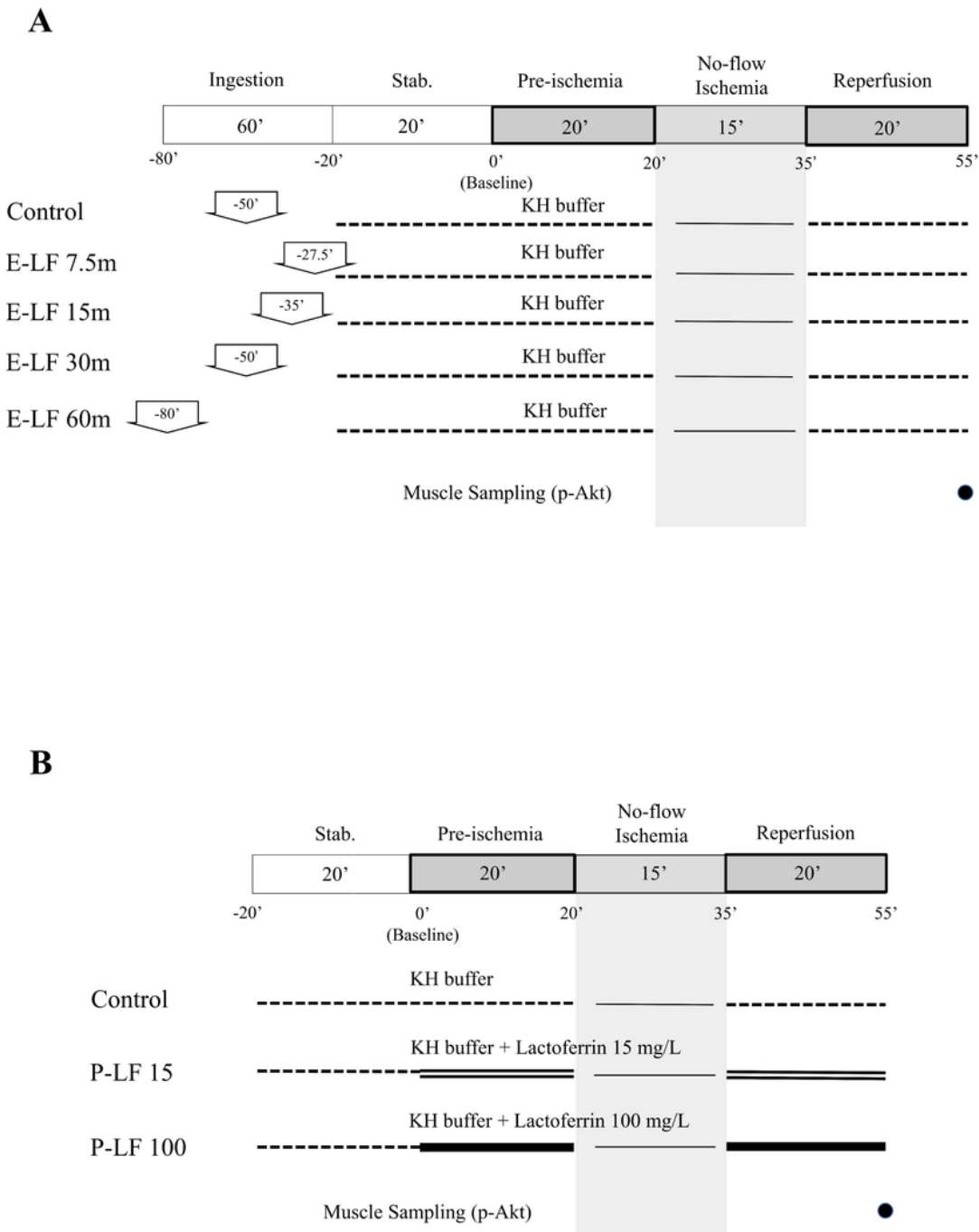


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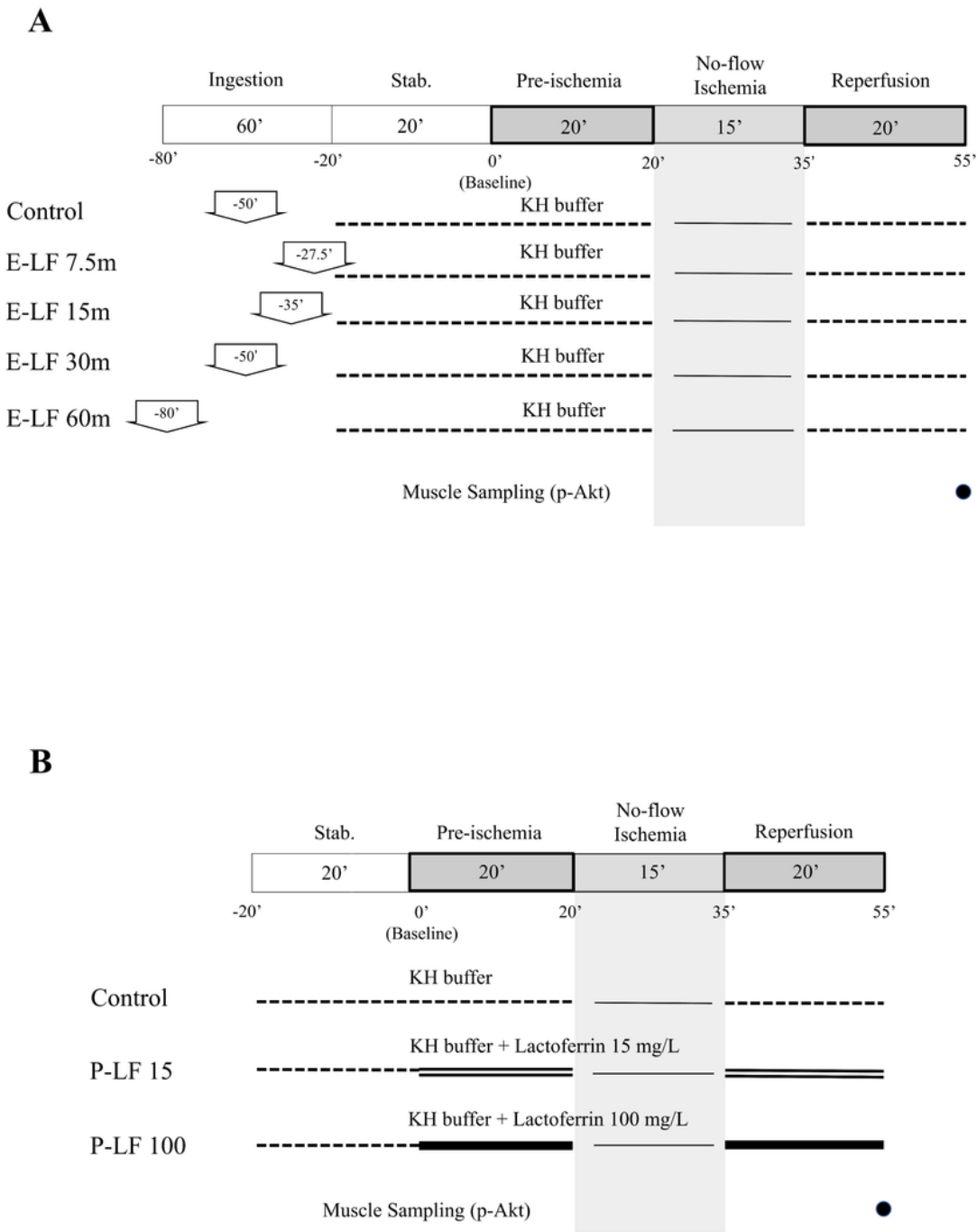


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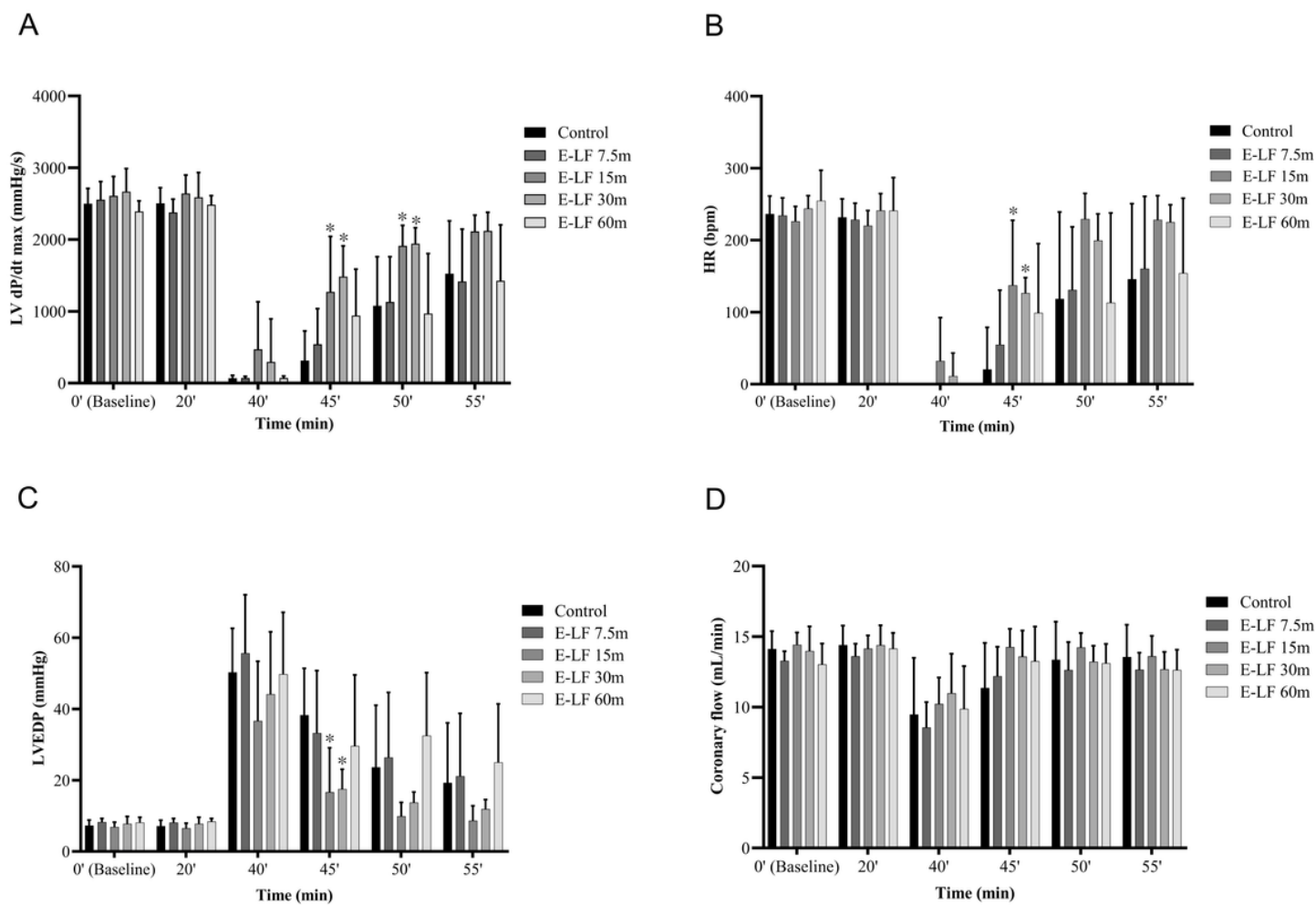


Figure 2

Changes over time, before and after ischemia, in the enteral administration groups The following variables were measured for each group (n=8). A: LV dP/dt max (mmHg/s): maximum of left ventricular derivative of pressure development. B: HR (bpm): heart rate. C: LVEDP (mmHg): left ventricular end-diastolic pressure. D: Coronary flow (mL/min). The bars represent mean and the error bars SD. *: P<0.05 vs control.

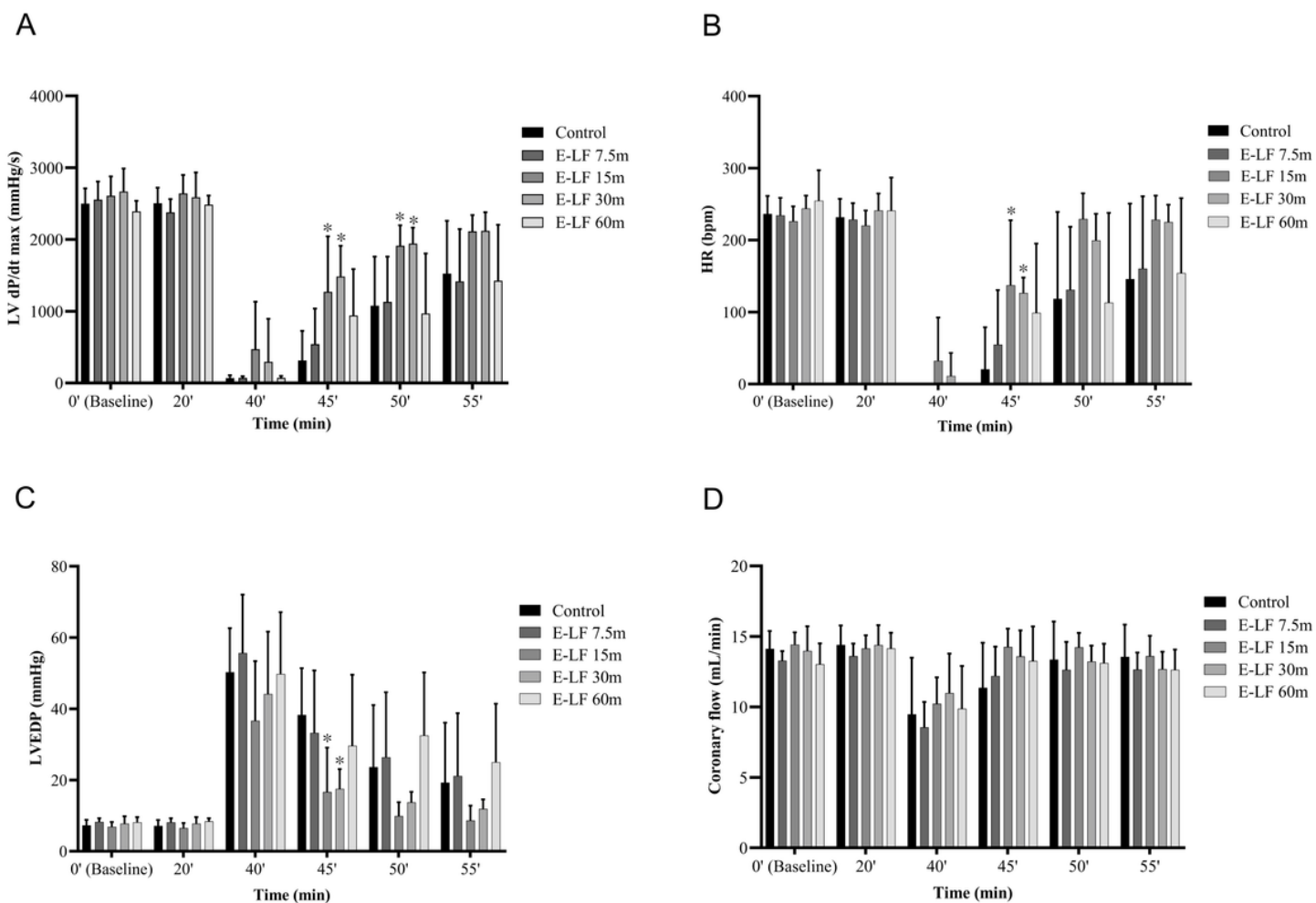


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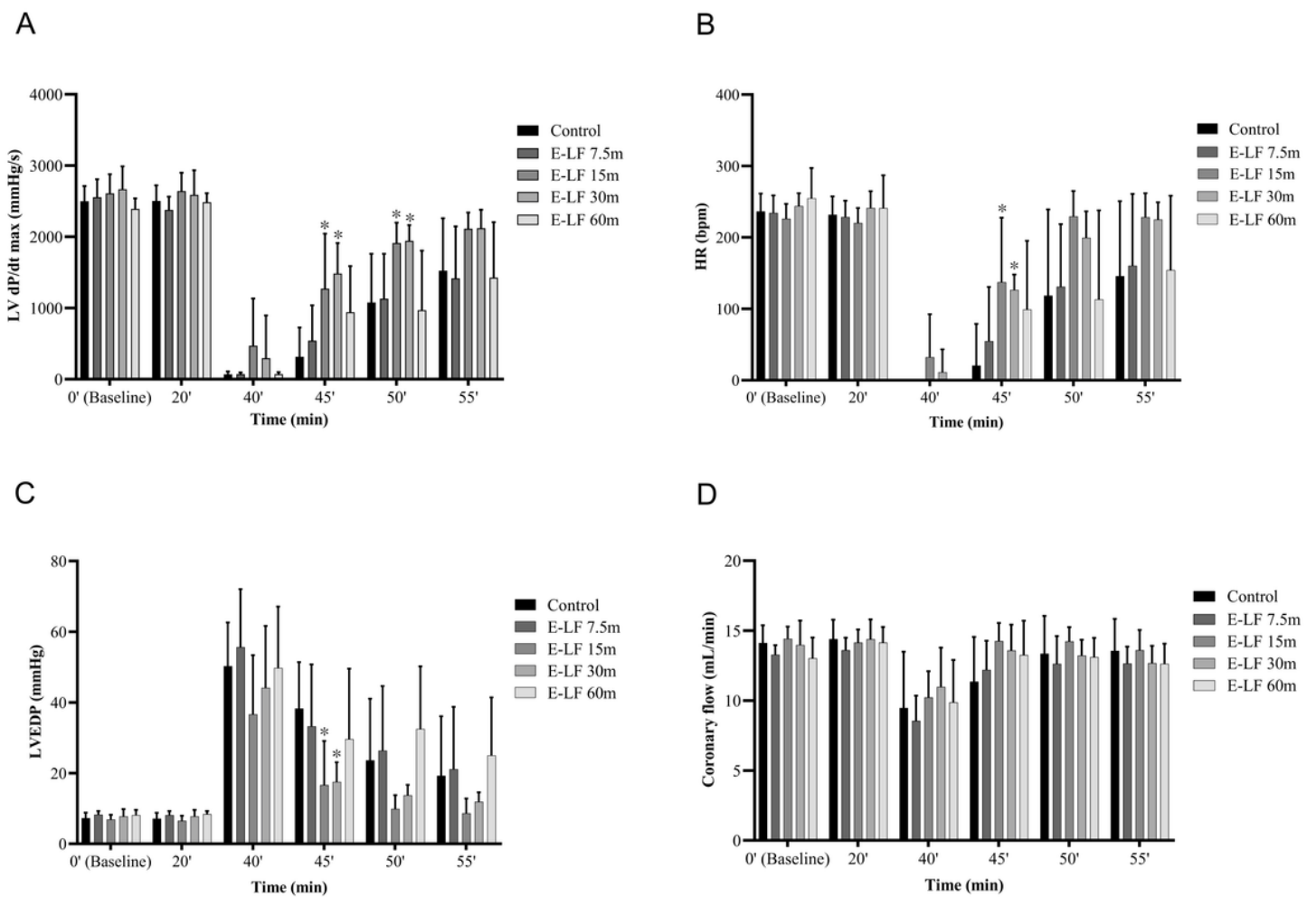


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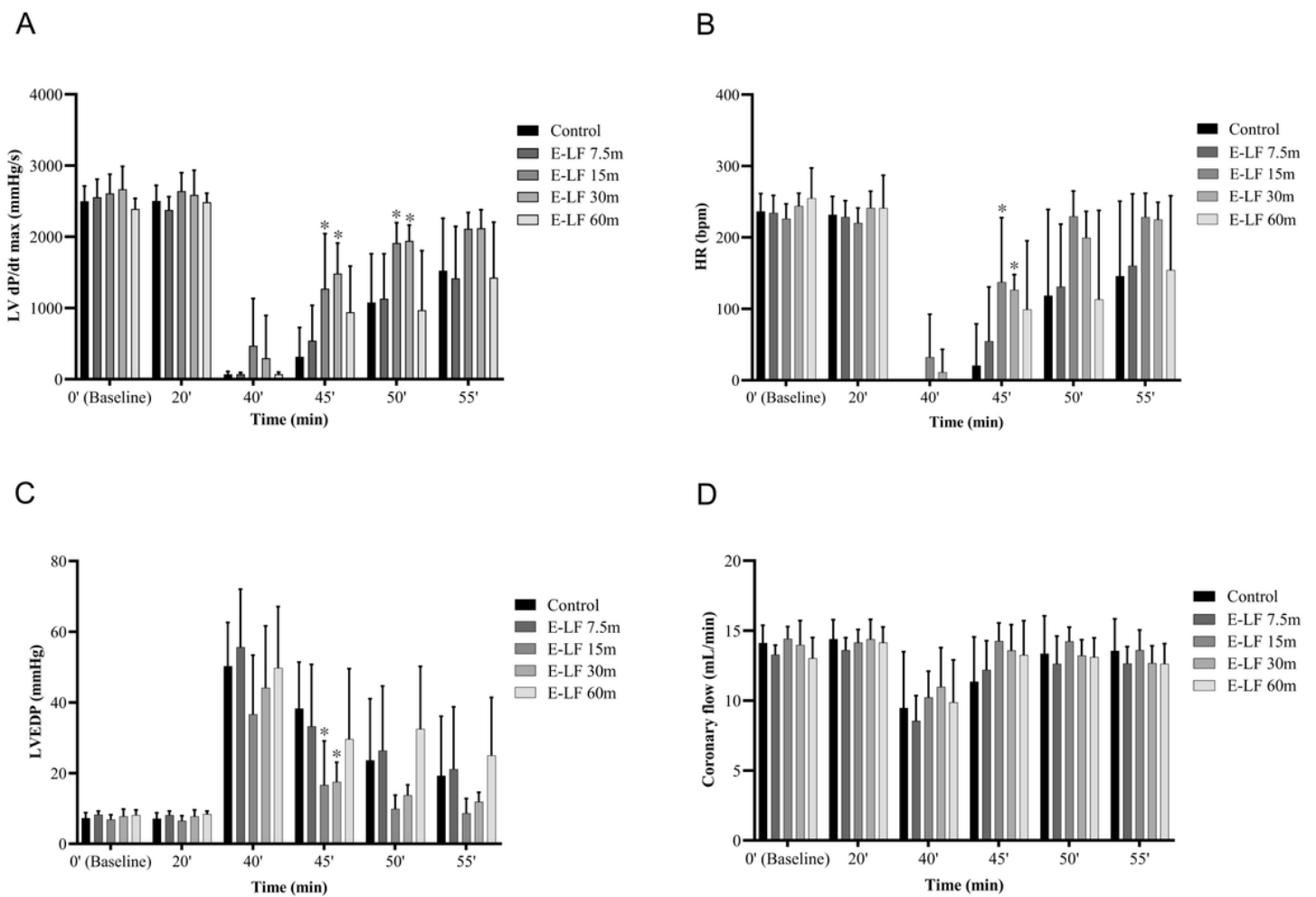


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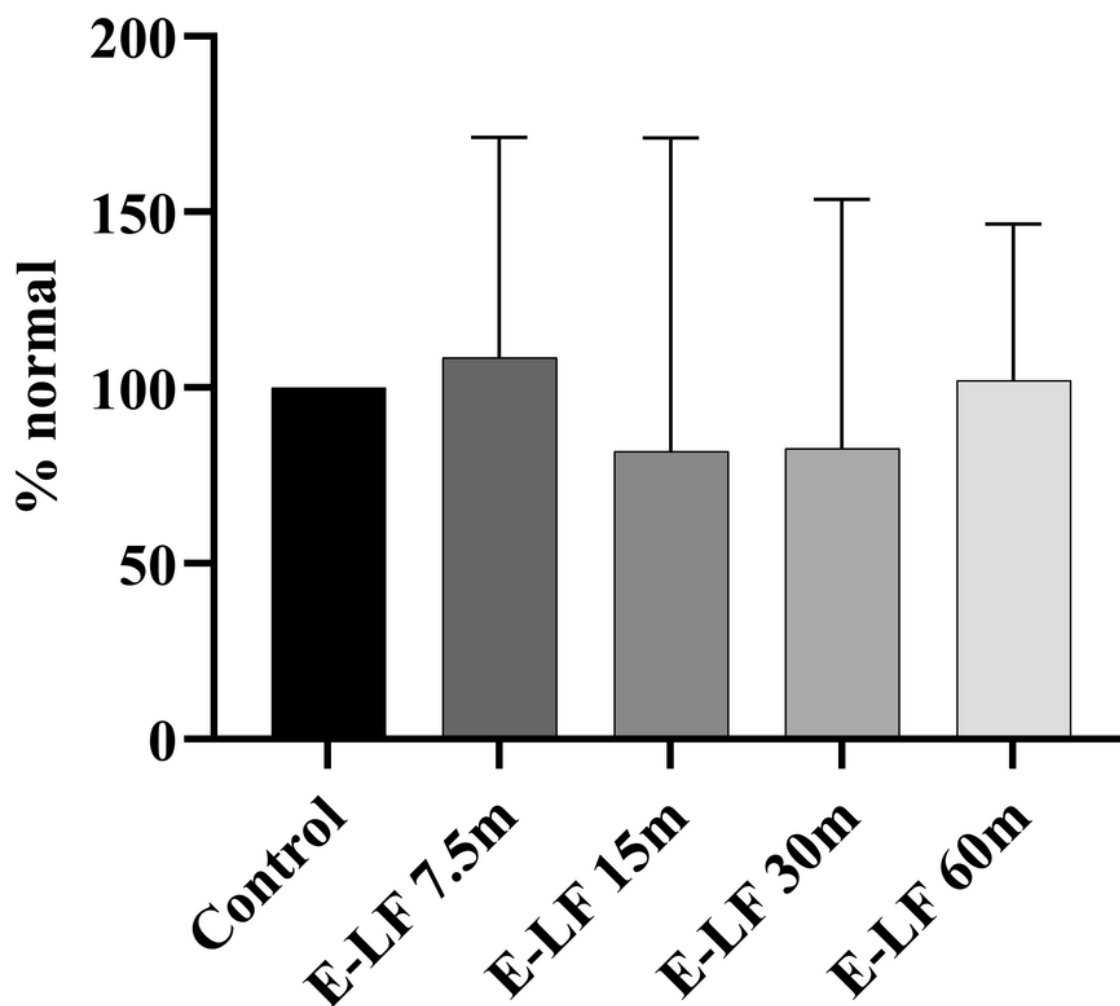
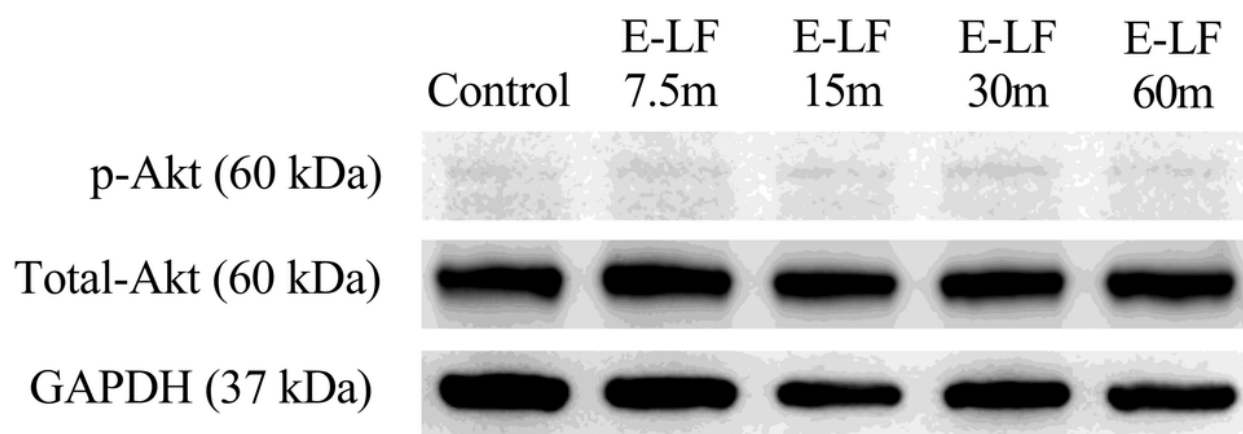


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Percent p-Akt over total-Akt 20 min after reperfusion in the enteral administration groups. The bars represent mean and the error bars SD (n=3 hearts from eight rats in each group). % normal is a percentage against the control group. The mean of p-Akt over total-Akt in the control group was normalized to 100%.

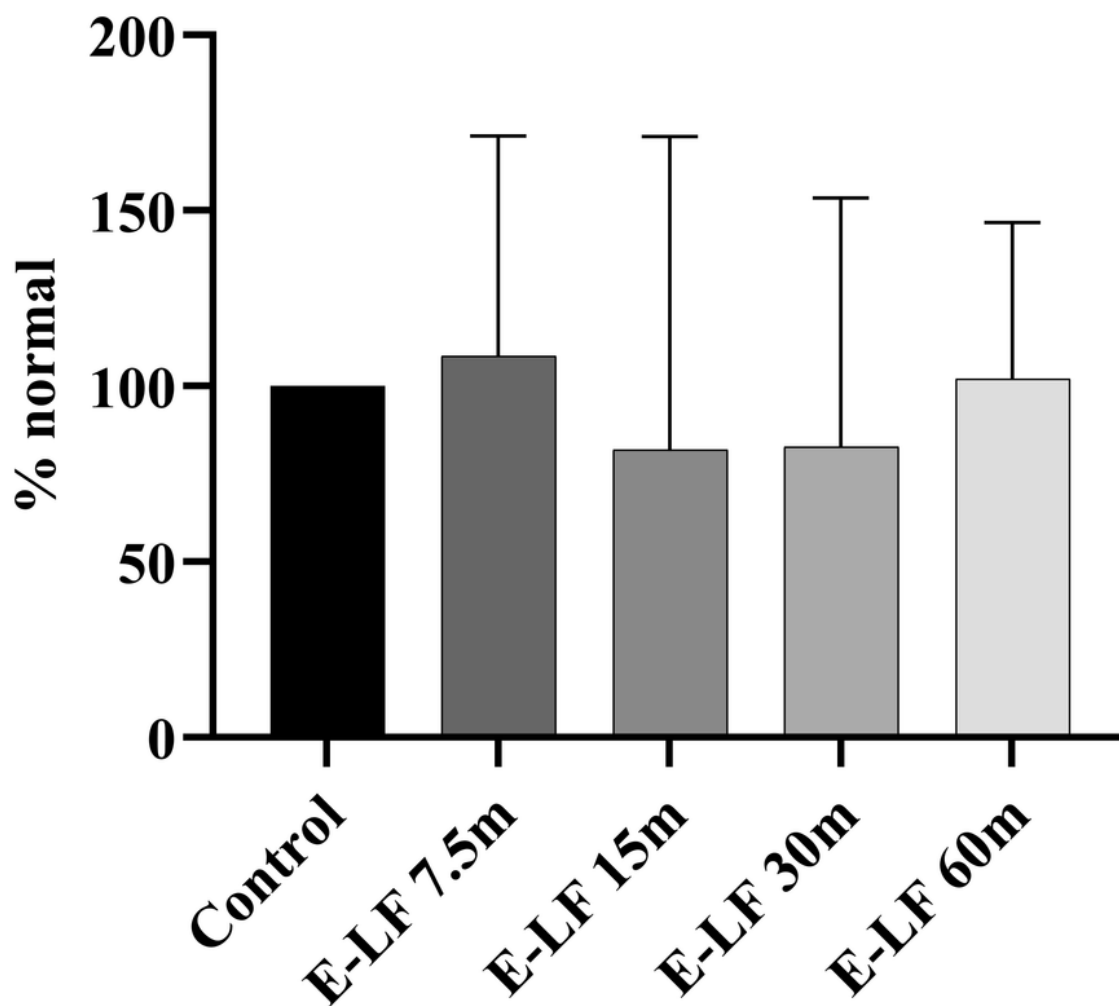
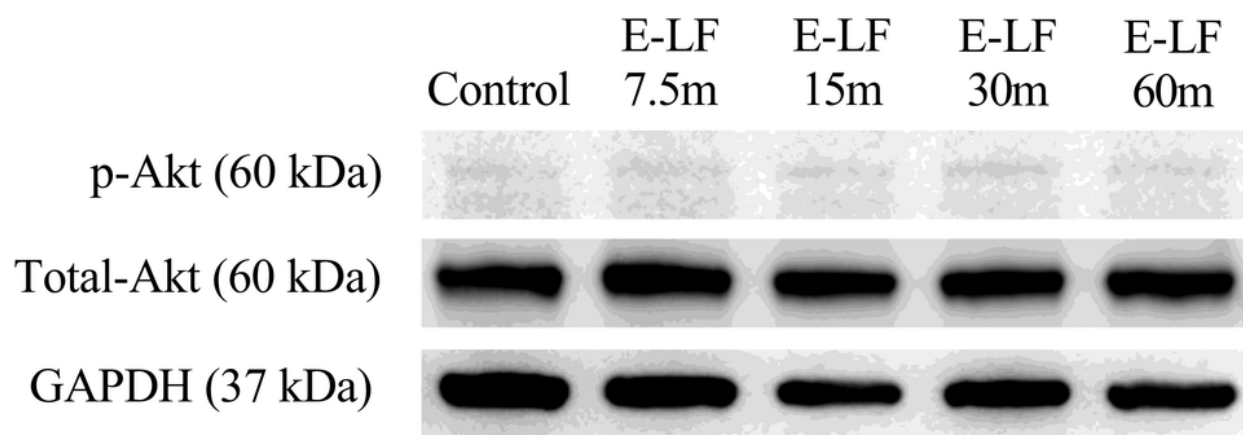


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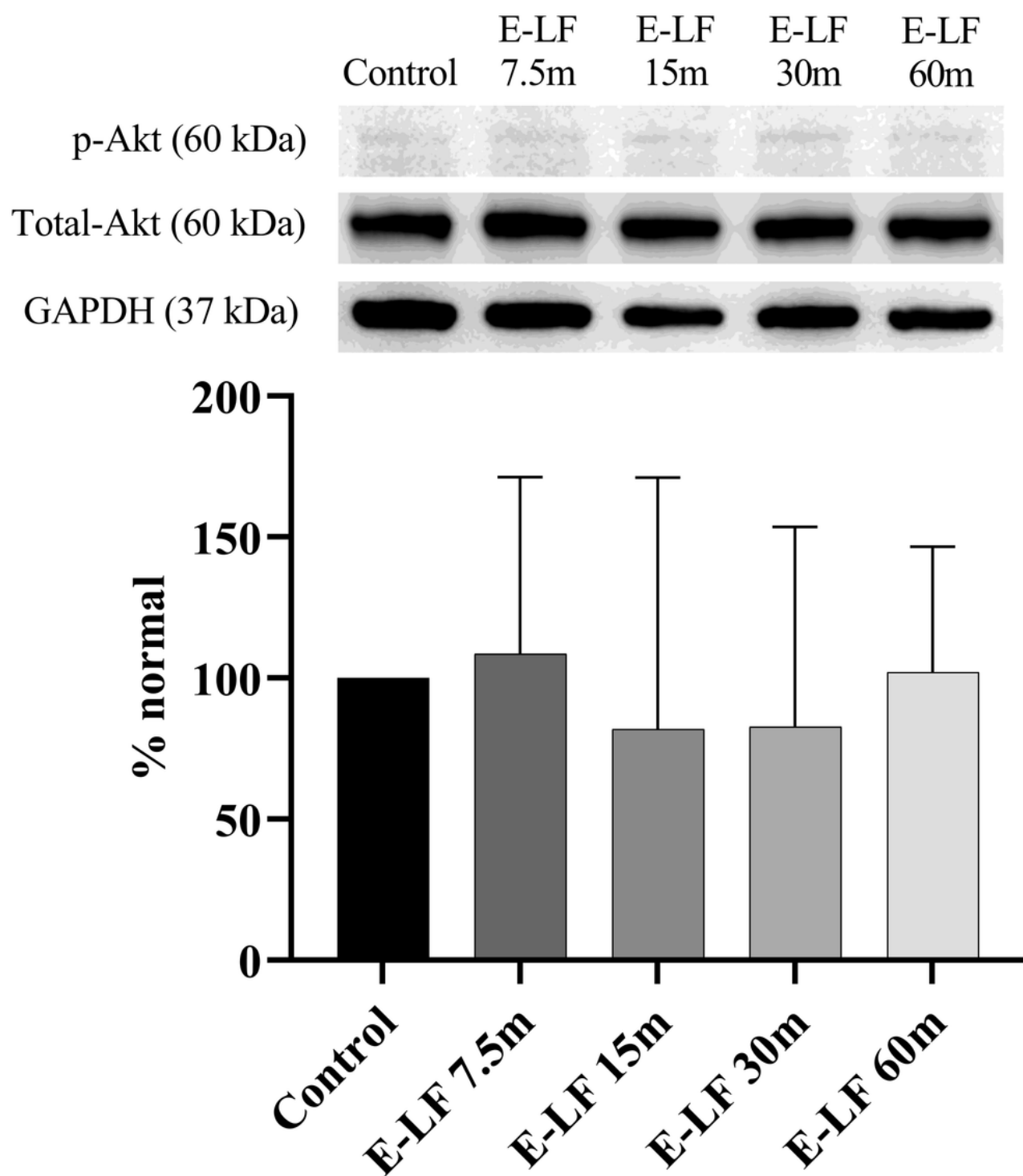


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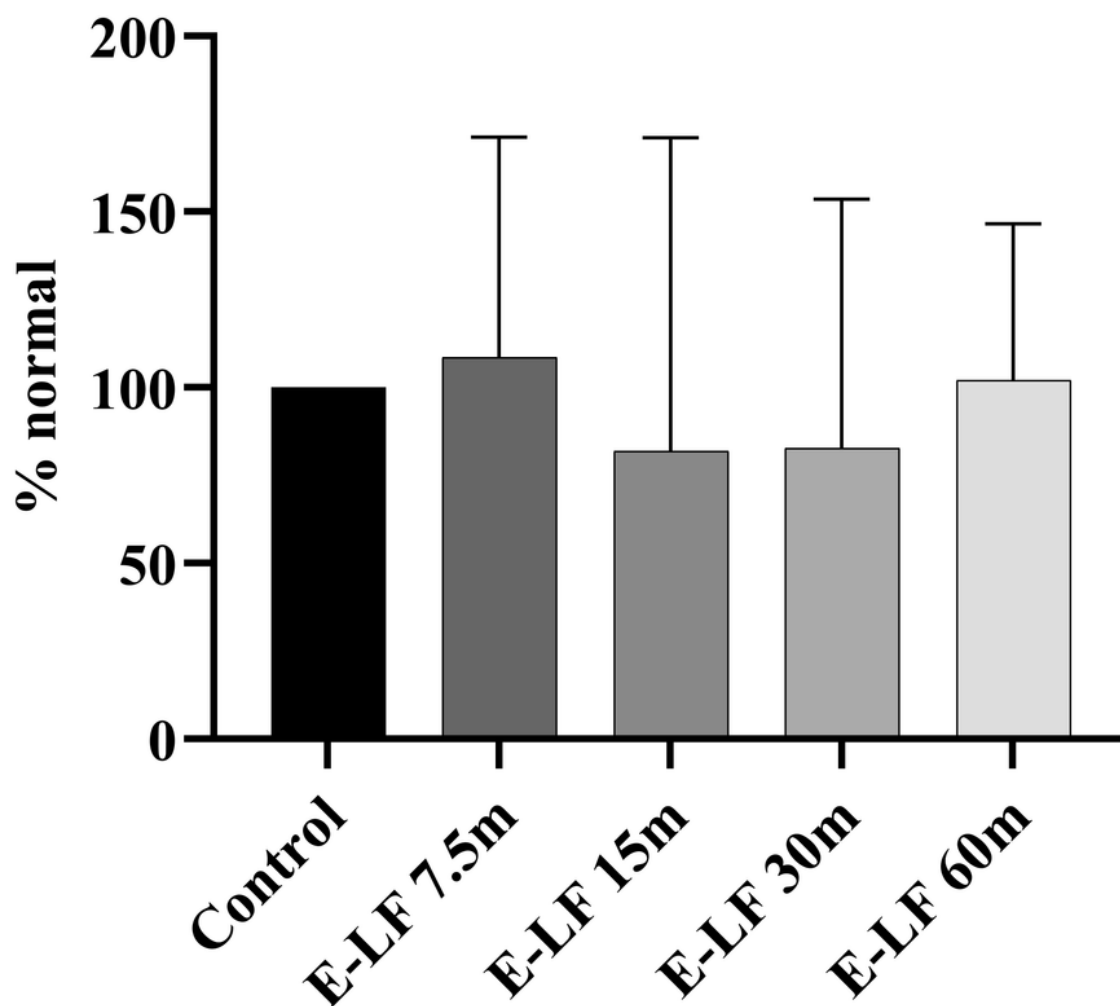
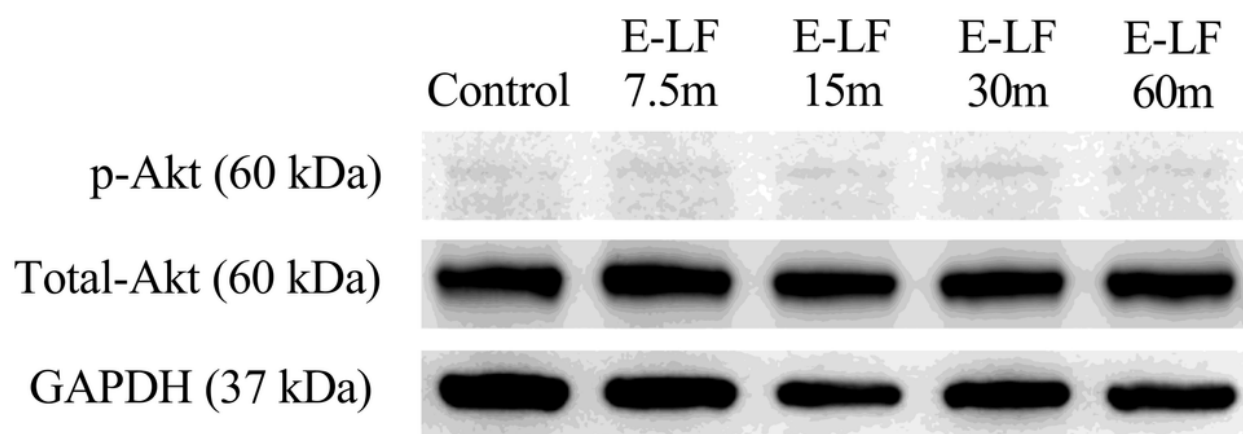


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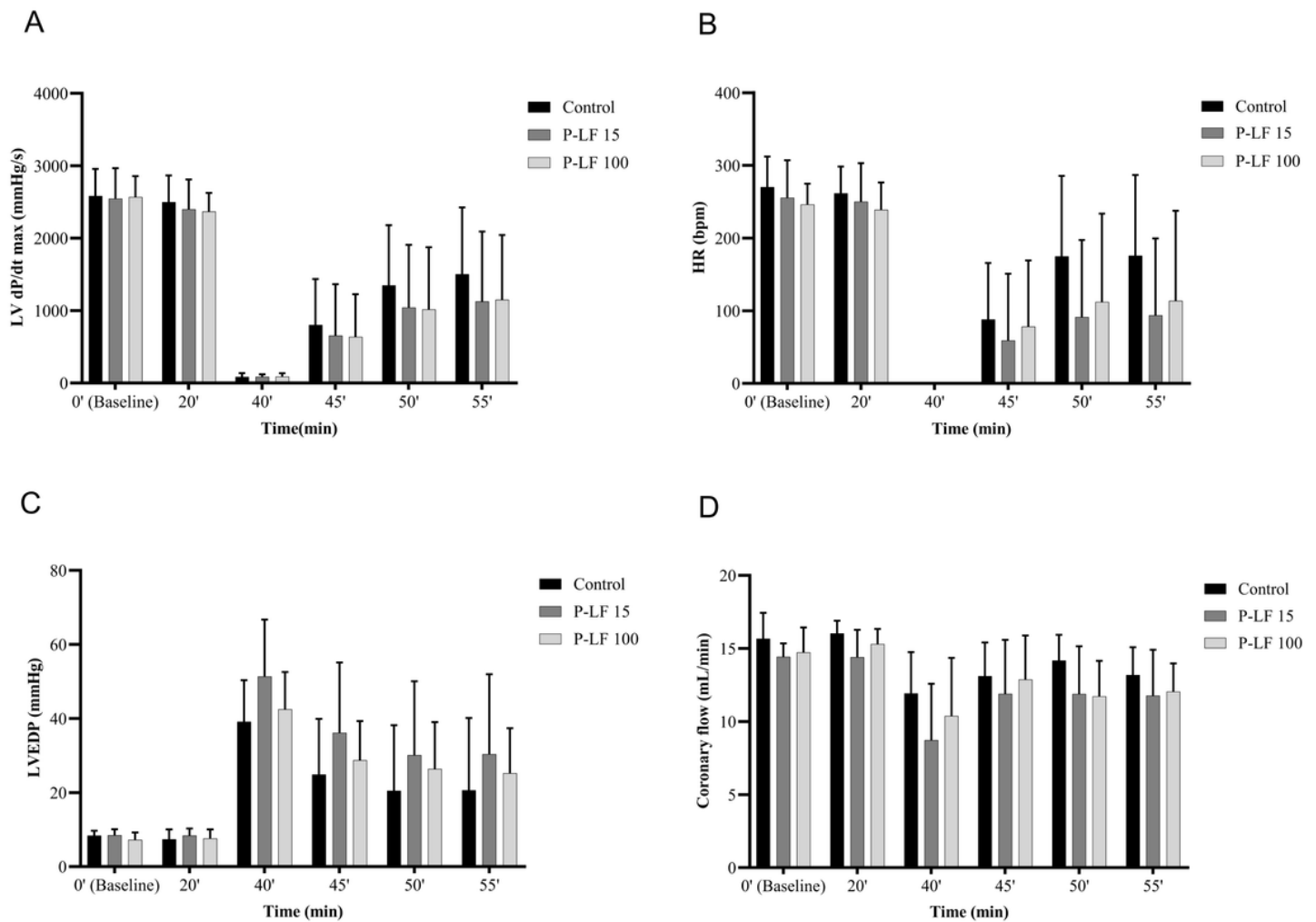


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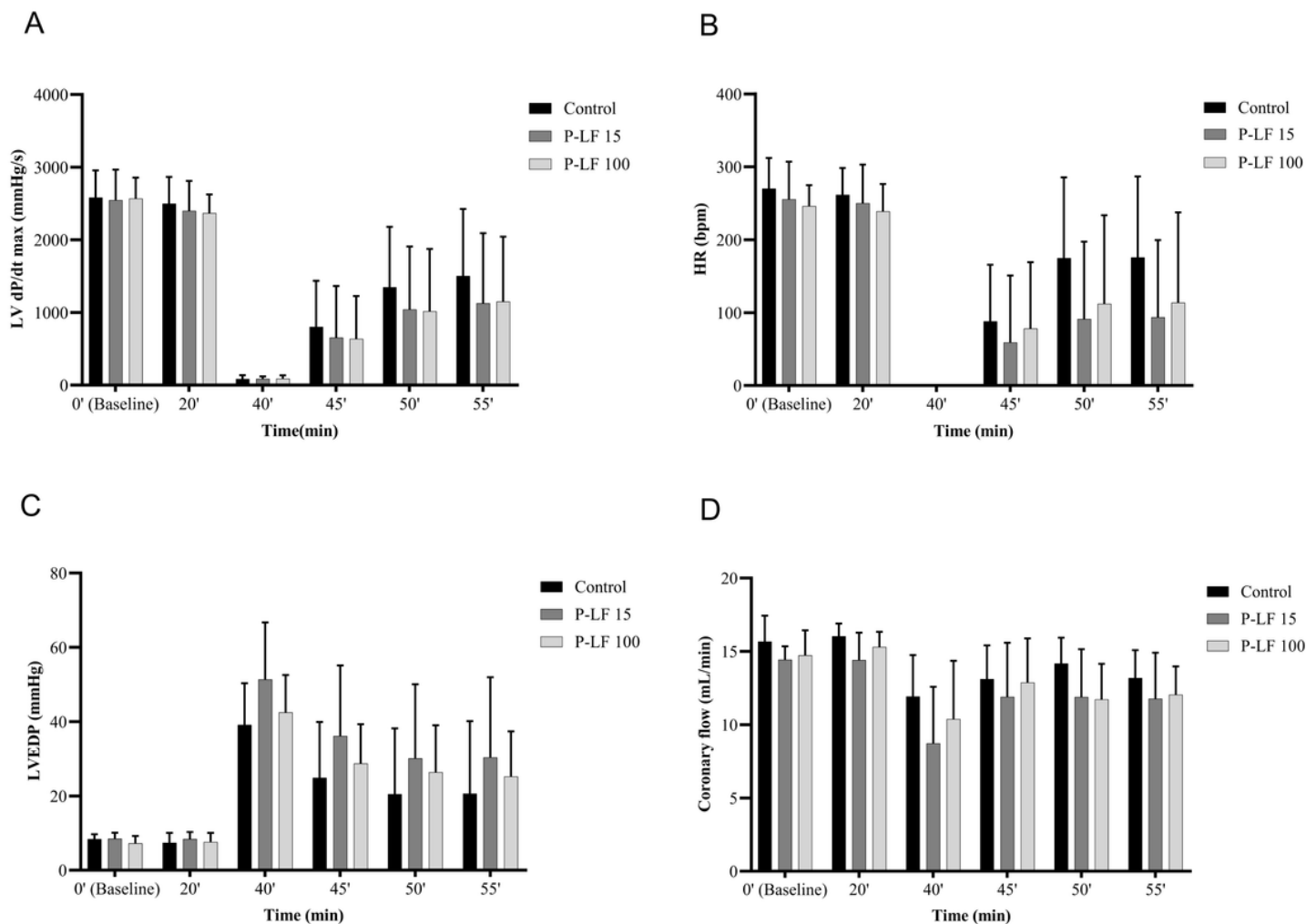


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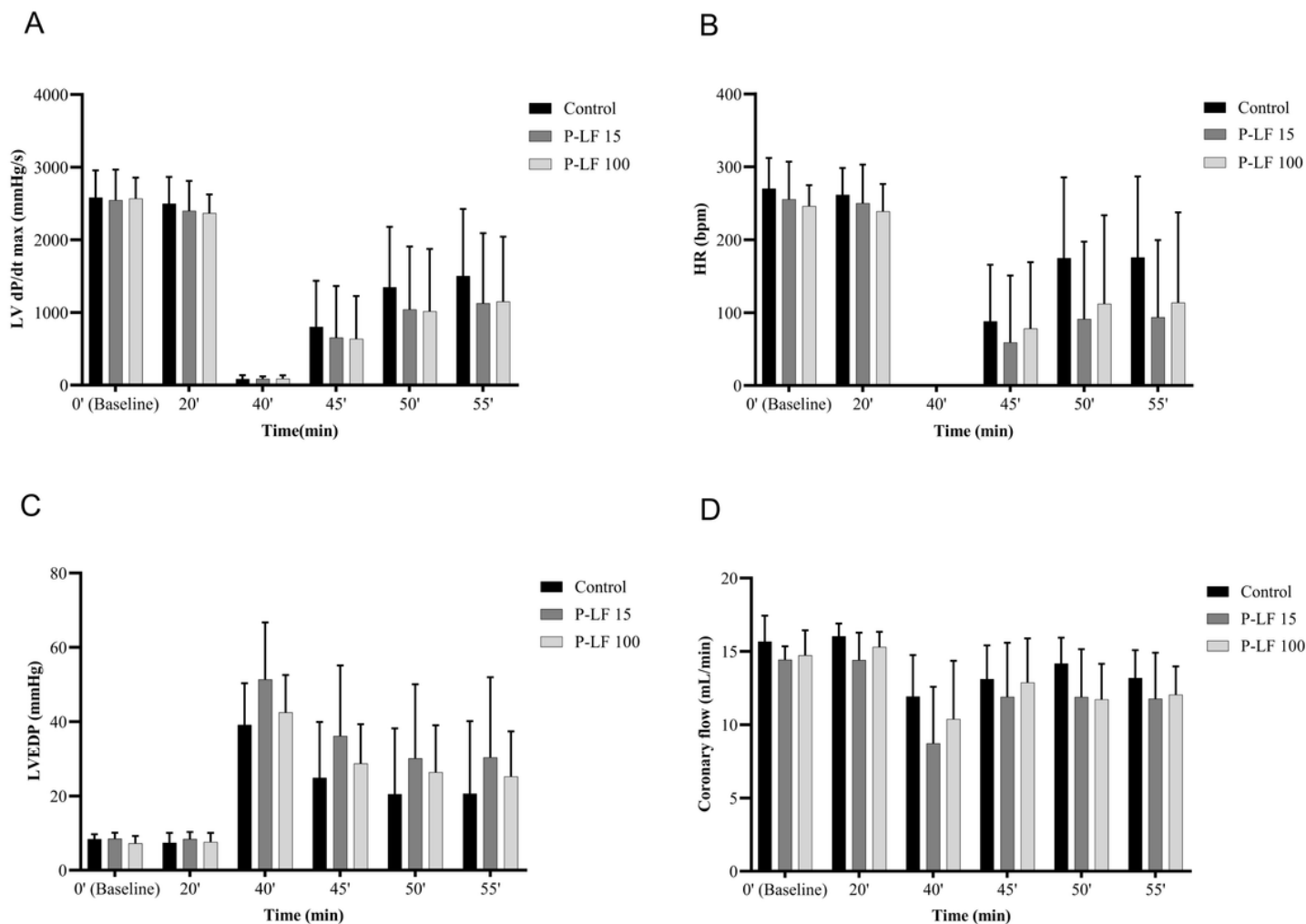


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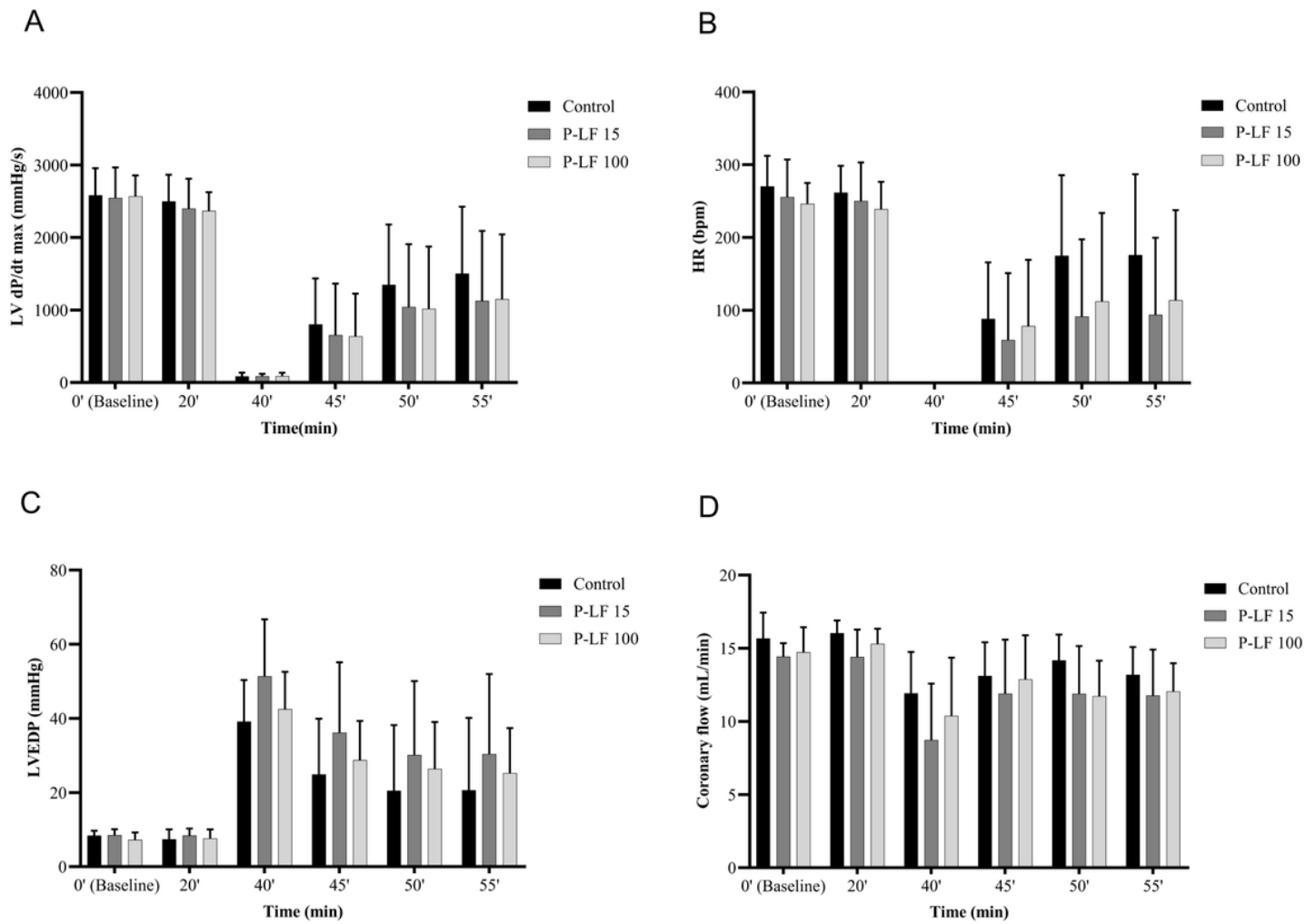


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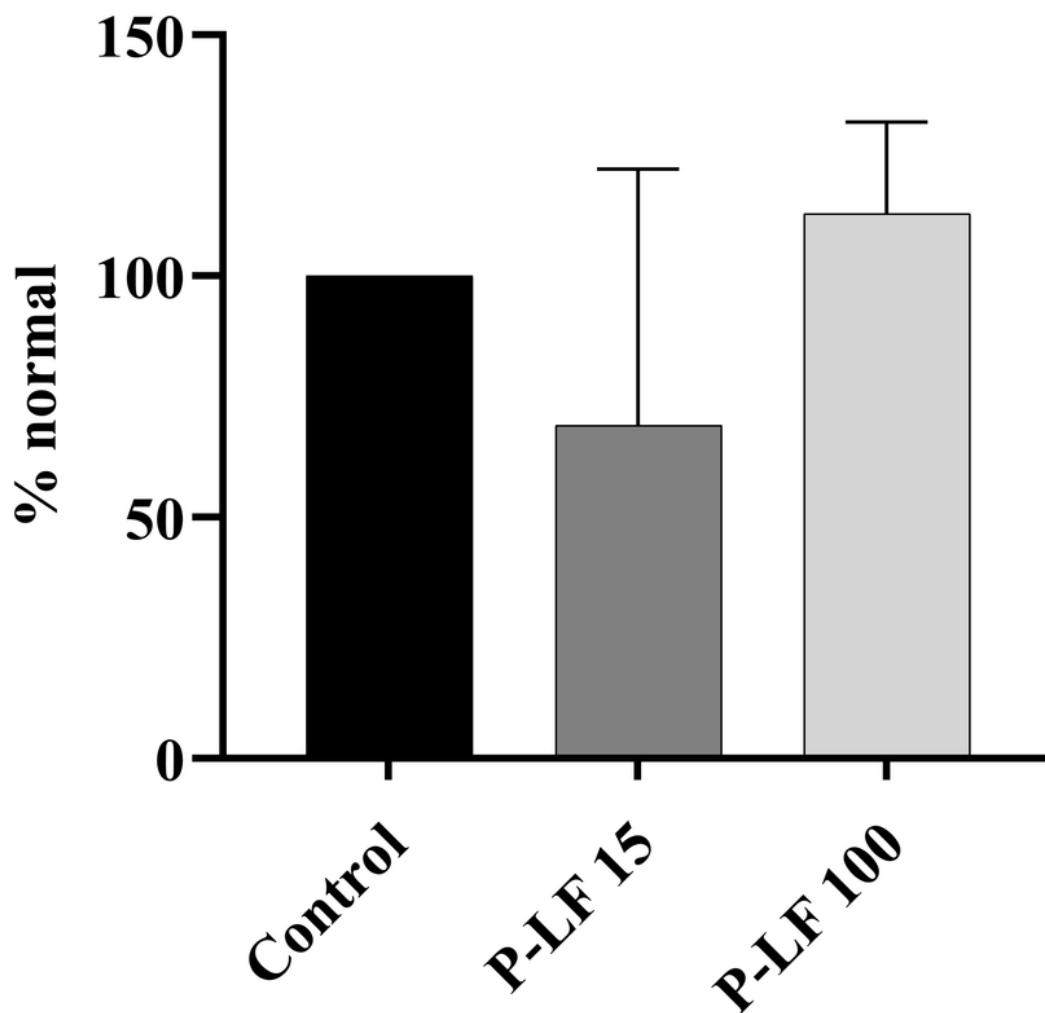
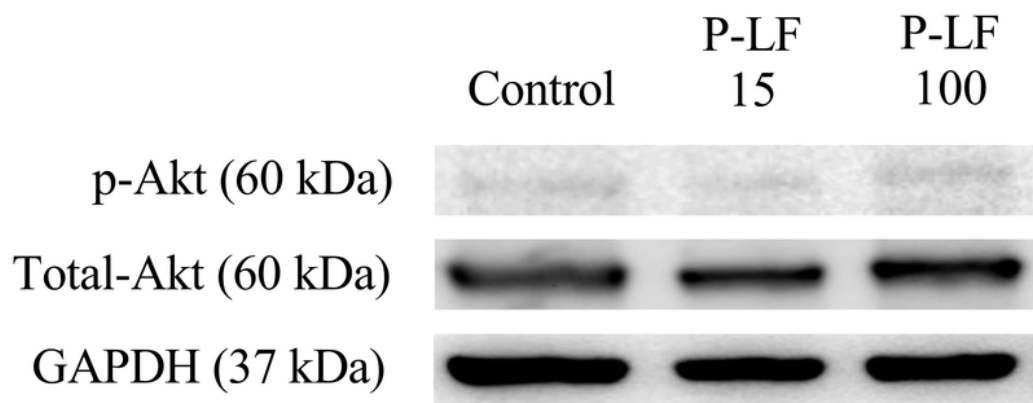


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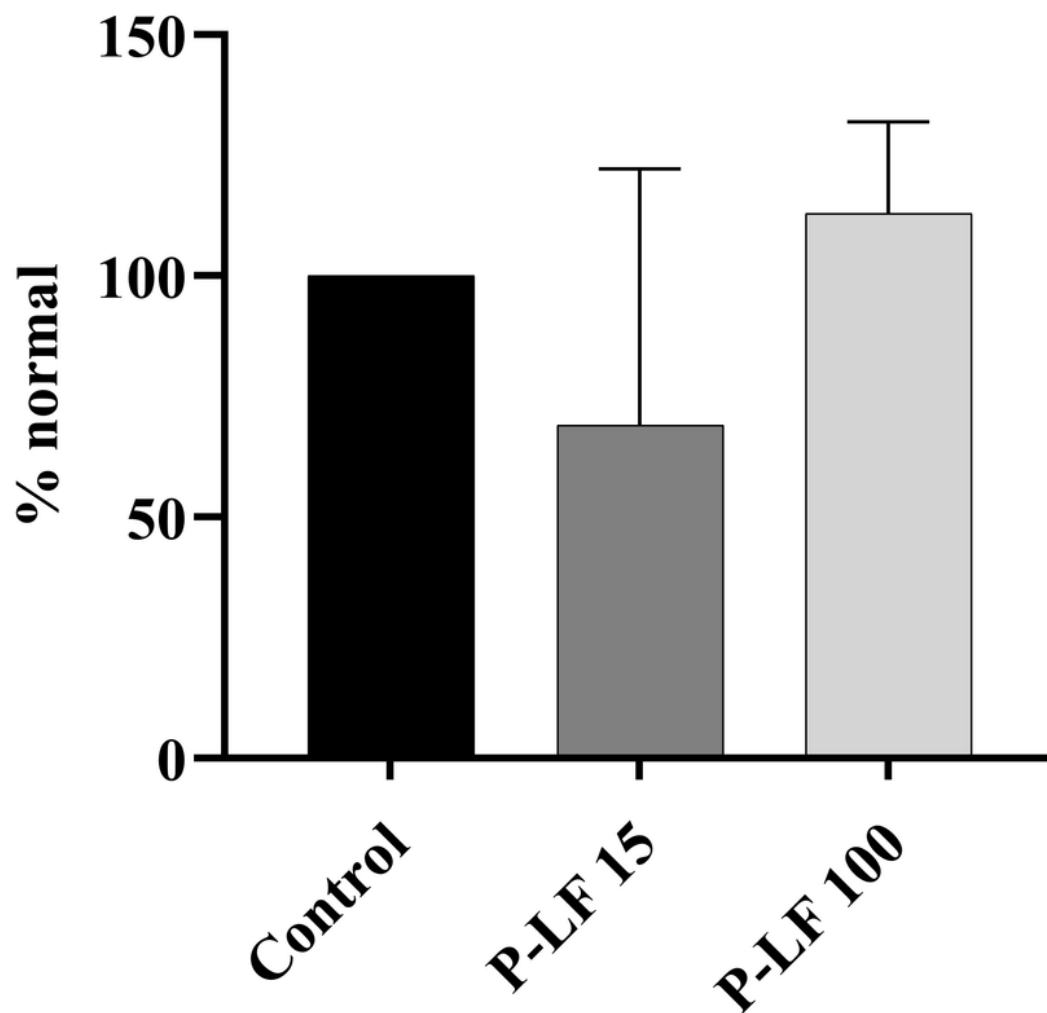
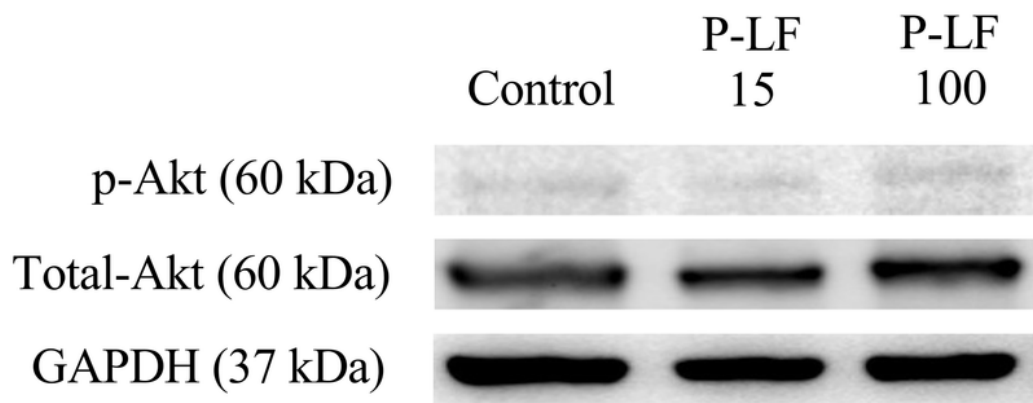


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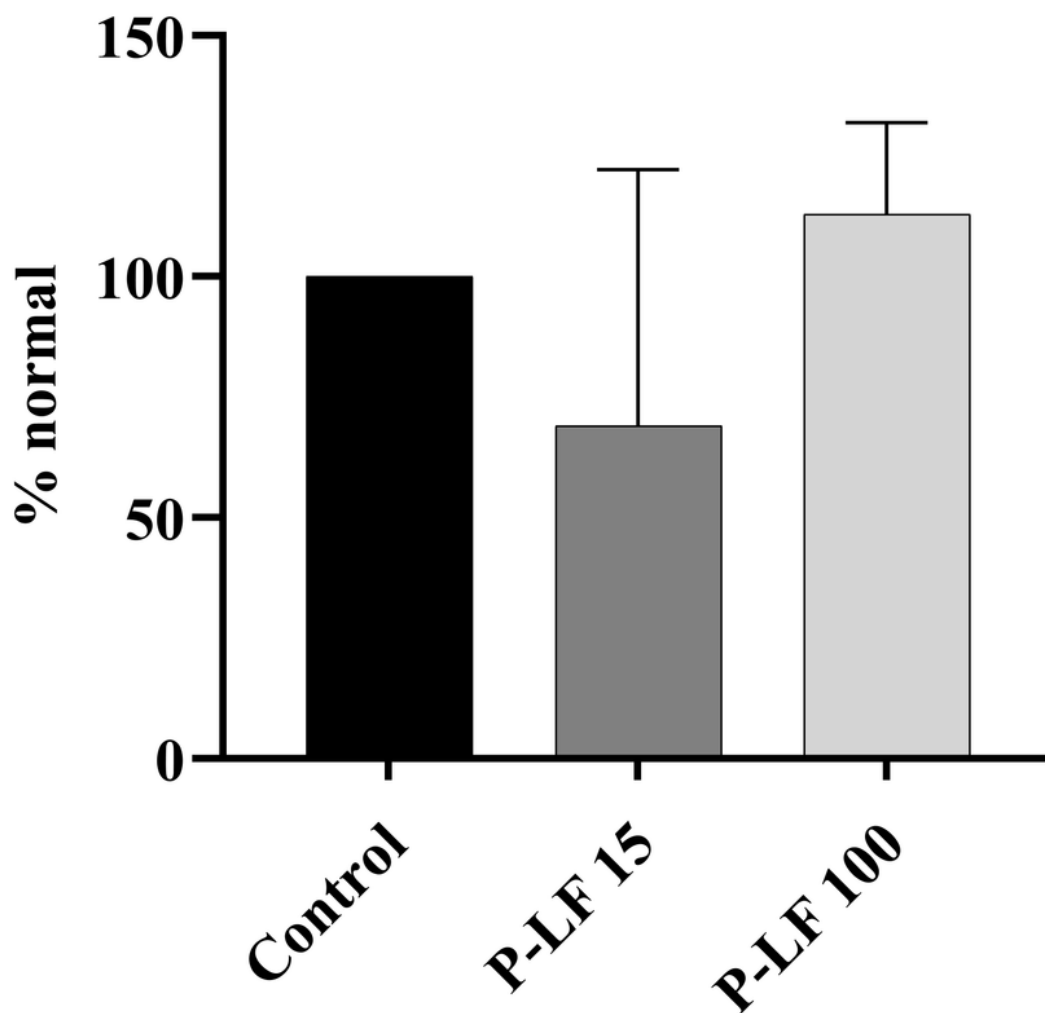
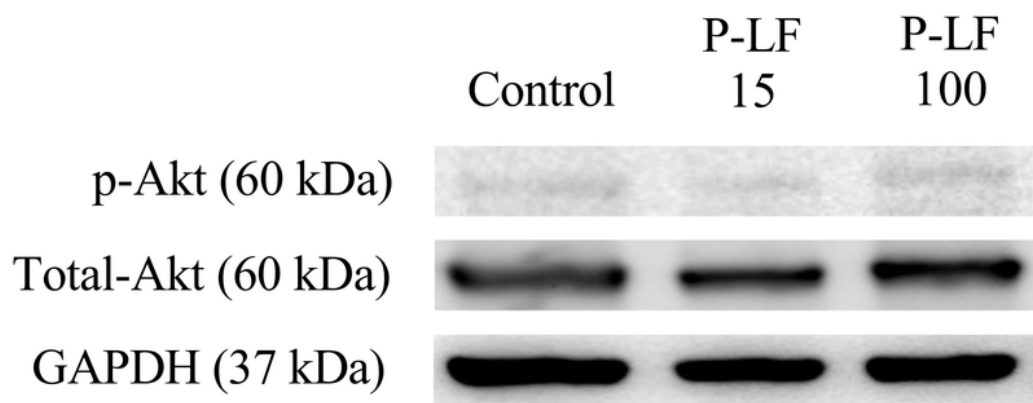


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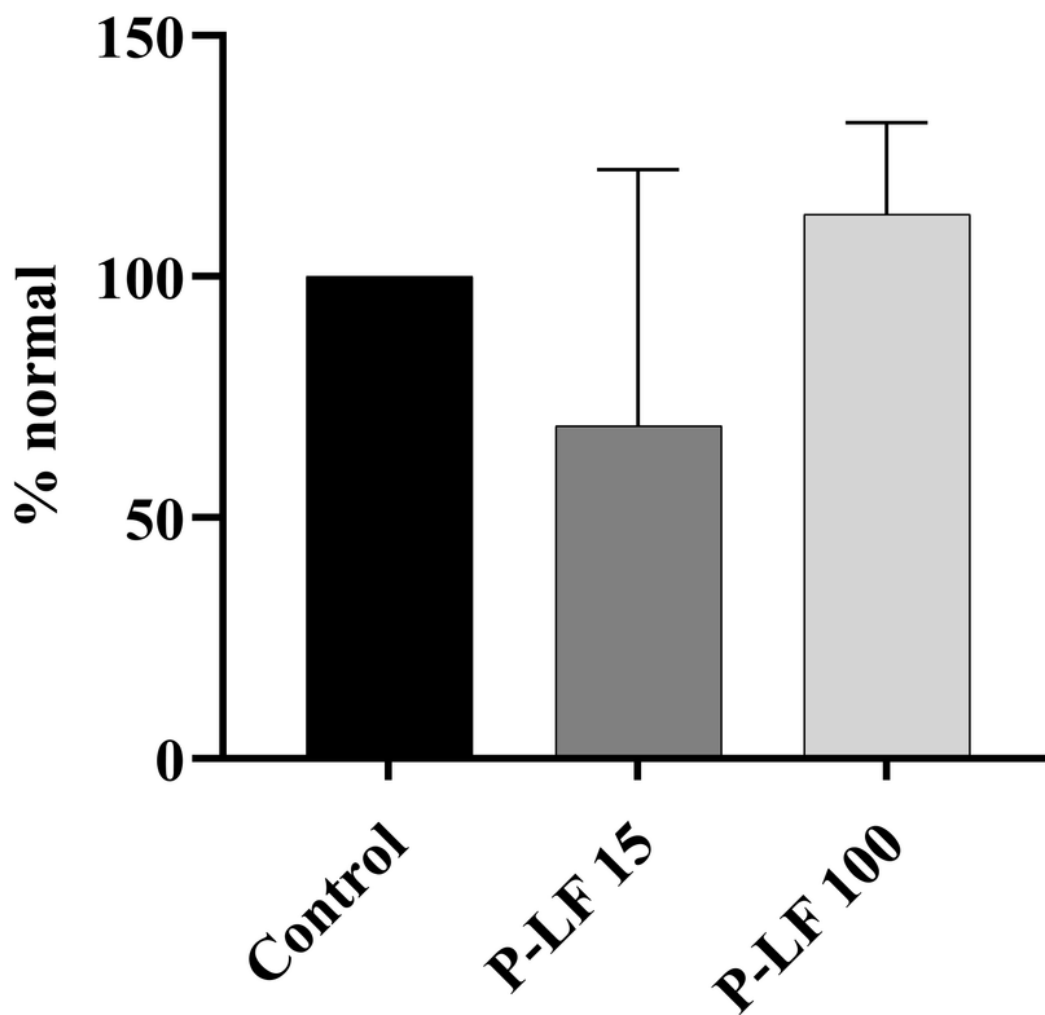
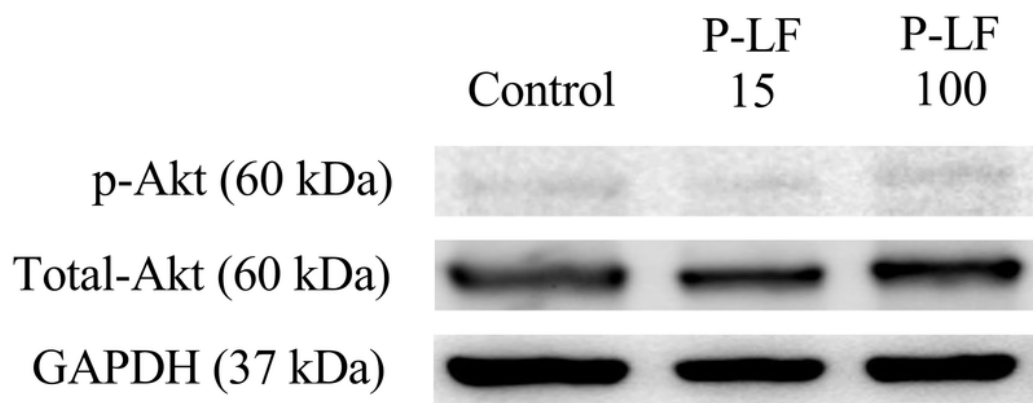


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