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Effect of lipid emulsion on vasoconstriction induced by epinephrine or norepinephrine in isolated rat aorta

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Running title: Lipid emulsion and epinephrine

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1   **Effect of lipid emulsion on vasoconstriction induced by epinephrine or norepinephrine in**

2   **isolated rat aorta**

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4

1 **Abstract**

2 **Background**: Epinephrine (EPI) or norepinephrine (NOR) is widely used to treat cardiovascular

3 collapse during lipid emulsion (LE) resuscitation for drug toxicity. However, the effect of LE on the

4 vasoconstriction caused by EPI or NOR remains unknown. The purpose of this study was to examine

5 the effect of an LE (Intralipid) on the vasoconstriction caused by EPI and NOR in isolated rat aorta.

6 **Methods:** The effect of LE on the vasoconstriction caused by EPI or NOR in isolated rat aorta was

7 examined. Additionally, the effect of LE on the calcium increase caused by EPI or NOR was

8 investigated. The distribution constant (K_D: lipid to aqueous phase) of EPI or NOR between a LE

9 (1%) and an aqueous phase was determined.

10 **Results:** LE (1 and 2%) did not significantly alter vasoconstriction caused by EPI or NOR in isolated

11 endothelium-intact aorta. Moreover, the LE did not significantly alter the increased calcium level

12 caused by EPI or NOR. The log K_D of EPI in the LE (1%) was -0.71, -0.99, and -1.00 at 20, 50, and

13 100 mM ionic strength, respectively. The log K_D of NOR in the LE (1%) was -1.22, -1.25, and -0.96

14 at 20, 50, and 100 mM ionic strength, respectively.

15 **Conclusions**: Taken together, the Intralipid emulsion did not alter vasoconstriction induced by EPI

16 or NOR that seems to be due to the hydrophilicity of EPI or NOR, leading to sustained hemodynamic

17 support produced by EPI or NOR used during LE resuscitation.

18 **Keywords:** Distribution constant; Epinephrine; Intralipid; Lipid emulsion; Norepinephrine;

19 Vasoconstriction.

Introduction

Lipid emulsion (LE) is extensively utilized in the management of systemic toxicity resulting from local anesthetics [1]. Furthermore, it has been documented that LE, when used as an adjunctive medication, proves effective in addressing refractory cardiovascular collapse induced by toxic levels of non-local anesthetic drugs such as calcium channel blocker, anti-depressant, and anti-psychotic drug [2-4]. The widely accepted underlying mechanism of LE resuscitation is lipid shuttle that is basically associated with the lipophilicity of the compound [1]. Lipid shuttle states that the lipid phase of the LE absorbs highly lipophilic drugs (for example bupivacaine) from the brain and heart, and subsequently the LE with the lipophilic drugs, is transported to the liver, muscle, and adipose tissue for detoxification and storage [1]. In addition, LE directly produces the following effects: enhancement of cardiac contractility, provision of fatty acids, reduction of nitric oxide release, phosphorylation of glycogen synthase kinase-3β, and mitigation of mitochondrial dysfunction [1].

Vasopressors that include epinephrine (EPI) and norepinephrine (NOR) are widely used to treat cardiovascular collapse caused by a toxic dose of local anesthetics or non-local anesthetics [5-7]. Thus, these vasopressors (EPI or NOR) and LE are concurrently used to address severe cardiovascular collapse resulting from toxic levels of drugs that are difficult to manage. A recent study suggests that LE (Intralipid) may reduce plasma concentration and half-life of lipid soluble non-offending drug used for hemodynamic support during LE resuscitation for cardiovascular collapse caused by drug toxicity that may lead to a reduction in the therapeutic concentration of that drug and a subsequent attenuation of the hemodynamic support [8]. Moreover, some emergency physicians express concerns about whether LE may absorb beneficial vasopressors used to treat cardiovascular collapse during LE resuscitation for drug toxicity induced by local anesthetic or non-local anesthetic drugs. This absorption could potentially lead to a decrease in the vasopressor's overall effectiveness. The extent to which LE reduces drug concentration is strongly positively correlated with the log P (or log $P_{o/w}$)

1   value (octanol/water partition coefficient) [9]. Log P is only one of several surrogate indicators that

2   can be used to predict LE-induced reversal of cardiovascular collapse caused by a toxic dose of some

3   drug, or the extent of LE-mediated absorption of offending drug to cause drug toxicity due to local

4   anesthetic or non-local anesthetic drug [10]. However, it is important to note that the log P primarily

5   reflects the distribution ratio of the non-ionized form of the compound in lipid (octanol) and aqueous

6   phases [11]. Therefore, considering that the negative logarithm of the acid dissociation constants (pK_a)

7   of EPI and NOR is 8.59 and 8.58, respectively (Table 1), the calculated non-ionized fractions of total

8   EPI and NOR using the Henderson-Hasselbalch equation are 6.06% and 6.20% at pH 7.4, respectively,

9   and 3.13% and 3.21% at pH 7.1, respectively (Table 1). These percentages appear relatively small.

10   Taking into consideration these calculated non-ionized fractions, it is worth noting that although EPI

11   and NOR are water soluble (log P: EPI = -1.37, NOR = -1.24), these low log P values only represent

12   the distribution of a small portion (non-ionized portion) of the total amount of EPI and NOR between

13   the lipid and the aqueous phase (Table 1) [11-13]. However, since all compounds, including local

14   anesthetics, exist in both the ionized and non-ionized forms *in vivo*, the distribution coefficient that

15   indicates the distribution ratio of both forms (ionized and non-ionized) in the lipid and aqueous phases

16   holds more clinical relevance than log P [11]. An *in vivo* experiment using a rat model found that LE

17   does not significantly change the peak increase in mean arterial pressure induced by EPI, but it does

18   extend the time taken to reach this peak mean arterial pressure [14]. In addition, the effect of LE on

19   the vasoconstriction caused by EPI and NOR, and the distribution coefficient of EPI and NOR is

20   unknown. Moreover, due to ethical reasons, it is very difficult to examine the effect of LE on

21   cardiovascular support induced by EPI and NOR during cardiopulmonary resuscitation using LE as

22   an adjuvant drug. Thus, the goal of this study was to examine the effect of a LE (Intralipid) on the

23   vasoconstriction caused by EPI and NOR in isolated rat aorta, and to get a better understanding of the

24   underlying mechanism with particular focus on distribution constant. Based on the water-soluble

1 properties of EPI and NOR, we tested the biological hypothesis that LE (1 and 2% Intralipid) does

2 not alter vasoconstriction induced by EPI and NOR. If it is proven through this experiment that LE

3 has a negligible effect on the vasoconstrictive support induced by EPI or NOR, this research may help

4 alleviate some physicians' concerns regarding LE-mediated sequestration of these drugs used for

5 hemodynamic support.

Material and methods

The experimental procedure (GNU-211217-R0106, 22nd December, 2021) was approved by the Institutional Animal Care and Use Committee at Gyeongsang National University. All experimental methods followed the Guidelines for the Institutional Animal Care and Use Committee at Gyeongsang National University.

Materials

The commercially available compounds with highest purity were used. EPI, NOR, phenylephrine, acetylcholine, N^{ω}-nitro-L-arginine methyl ester (L-NAME), and potassium chloride (KCl) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Intralipid 200 mg/ml (20% weight/volume) was acquired from Fresenius Kabi AB (Sweden). The buffer reagents disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4*12\ H_2O$) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4*H_2O$) were from J.T. Baker (Deventer, the Netherlands) and Merck (Darmstadt, Germany), respectively. High performance liquid chromatography (HPLC) grade 99.5% dimethyl sulfoxide was from Labscan (Dublin, Ireland). LC-MS grade ≥ 99.9% methanol from Fisher Chemical (Geel, Belgium) was used as solvent for the analyte stock solutions. The standard pH solutions (4.0, 7.0, and 10.0) used for pH meter calibration were purchased from Merck (Darmstadt, Germany).

Preparation of isolated rat aorta and isometric tension measurement

Male Sprague–Dawley rats weighing between 250 and 280 g obtained from Koatech (Pyeongtaek, Gyeonggi-do, Republic of Korea) underwent anesthesia with 100% carbon dioxide administered through a small opening in their cages. Subsequent to anesthesia, the isolation of rat aortas and isometric tension measurements were conducted following established procedures [15]. The thoracic cavity was surgically opened to retrieve the descending thoracic aorta that was subsequently immersed in Krebs solution. This solution contains the following concentrations: 118 mM of sodium

1 chloride, 25 mM of sodium bicarbonate, 11 mM of glucose, 4.7 mM of KCl, 2.4 mM of calcium

2 chloride, 1.2 mM of magnesium sulfate, and 1.2 mM of monopotassium phosphate. Removal of

3 surrounding fat and connective tissues was performed under a microscope, and the isolated rat

4 thoracic aortas were sliced into 2.5 mm-length segments. To eliminate the endothelium from some

5 thoracic aortas, rolling movements were applied using two 25-gauge needles inserted into the aorta's

6 lumen. The isolated descending thoracic aortas were mounted on a Grass isometric transducer (model

7 FT-03™, Grass Instrument, Quincy, MA, USA) within an organ bath maintained at 37°C. While a

8 90-min equilibrium period with a baseline resting tension of 24.5 mN was maintained, every 30 min,

9 the Krebs solution was refreshed with freshly aerated Krebs solution (containing 95% oxygen and 5%

10 carbon dioxide) to sustain a pH level of 7.4, as per previous protocols [15,16]. Endothelial integrity

11 was confirmed by inducing sustained contractions with 10^{-7} M (0.1 μM) phenylephrine in the

12 endothelium-intact thoracic aorta, followed by the addition of 10^{-5} M (10 μM) acetylcholine. An

13 endothelium-intact thoracic aorta was defined as having more than 80% relaxation from

14 phenylephrine-induced contraction. Endothelial denudation was verified by introducing acetylcholine

15 (10^{-5} M; 10 μM) after stable contractions induced by 10^{-8} M (0.01 μM) phenylephrine, with less than

16 15% relaxation indicating an endothelium-denuded thoracic aorta. Aortas displaying acetylcholine-

17 induced relaxation in both intact and denuded states were thoroughly washed with fresh Krebs

18 solution to return to baseline resting tension. Subsequently, a contraction caused by isotonic 60 mM

19 KCl solution was initiated to gauge the extent of vasoconstriction prompted by EPI or NOR. Once a

20 sustained contraction was achieved through the addition of isotonic 60 mM KCl solution, the isolated

21 aorta that displayed this contraction was thoroughly washed with fresh Krebs solution to return to its

22 initial baseline tension. The subsequent experiment was then conducted.

23 ***Experimental protocols***

24 The effect of LE (Intralipid) on the vasoconstriction caused by EPI or NOR was examined in isolated

1   rat aorta. After pretreating rat aortas with intact or denuded endothelium with LE (1% and 2%) for 20

2   min, EPI (at concentrations ranging from 10^{-9} to 10^{-5} M) or NOR (at concentrations ranging from 10^{-8}

3   to 10^{-5} M) was incrementally added to the organ bath to induce vasoconstriction, with or without

4   the presence of LE. To mitigate the influence of residual endothelium on the contraction induced by

5   EPI or NOR following endothelial denudation, all endothelium-denuded rat aortas were pre-treated

6   with L-NAME (10^{-4} M) for 10 min prior to the addition of LE [17]

7 *Determination of intracellular calcium concentration*

8   To examine the effect of EPI and NOR treatment on intracellular calcium levels, we assessed the

9   calcium concentration using a confocal laser microscope (IX70 Fluoview™, Olympus, Tokyo, Japan)

10   [18]. Vascular smooth muscle cells were seeded onto a confocal cell culture dish (SPL, Pocheon,

11   Korea) and allowed to incubate overnight in media devoid of serum. To measure intracellular calcium

12   concentrations, vascular smooth muscle cells were treated with 2.5 µM Fluo-4 AM (Invitrogen,

13   Waltham, MA, USA) in Hanks Balanced Salt Solution for 30 min. Subsequently, they were washed

14   with phosphate-buffered saline solution and the Hanks Balanced Salt Solution was then replaced with

15   fresh solution. Cells were treated with EPI (10^{-5} M; 10 µM) and NOR (10^{-5} M; 10 µM) alone, as well

16   as with a mixture of EPI (10^{-5} M; 10 µM) with LE (Intralipid: 1 or 2%), and a mixture of LE (1 or

17   2%) with NOR (10^{-5} M; 10 µM). For 5 min, intracellular calcium concentrations were assessed every

18   2.5 s at wavelengths of 485 nm (excitation) and 520 nm (emission), respectively. Intracellular calcium

19   levels were evaluated using images expressed by Fluo-4 AM, and calculated from the Flou-4 AM-

20   induced fluorescence intensity (F) after EPI and NOR treatment divided by the fluorescence intensity

21   without treatment (F0). The net alteration in calcium ions is expressed as (Fmax-F0) / F0, where Fmax

22   denotes the fluorescence intensity at the peak calcium level after exposure to either EPI (10^{-5} M) or

23   NOR (10^{-5} M) alone, or in combination with LE (1% or 2%) and EPI (10^{-5} M) or NOR (10^{-5} M).

1 *Capillary electrophoresis (CE)*

2 An Agilent Technologies 7100 CE system equipped with an UV/DAD absorbance detector was used.

3 The capillary was a 68.5 cm fused coated silica capillary (Polymicro Technologies) with 60 cm to the

4 detector and inner/outer diameters of 50/360 μm. The software was Agilent OpenLAB CDS

5 ChemStation Edition c.01.05. The pH of the buffer solutions was checked with a WTW inoLab pH

6 7110 pH meter. A Sartorius BP301S analytical balance was used for weighing the analytes. Purified

7 water was received from a water purification system Milli-Q Direct-Q 3 UV. The centrifuge used was

8 a Micromax ThermoIEC with a Thermo IEC 851 rotor. In the CE runs, the capillary was flushed with

9 0.1 M sodium hydroxide for 3 min, with water for 2 min, and with phosphate buffer (PHB) for 5 min

10 at high pressure (ca 940 mbar) before each sequence. Before each injection, the capillary was flushed

11 with 0.1 M sodium hydroxide, water, and PHB solution for 1 min each. The applied separation voltage

12 was 30 kV and the temperature was 37°C. All the samples were injected at 100 mbar pressure at 10 s

13 injection time. UV spectra of the runs was recorded at wavelengths 190–400 nm with 2 nm steps and

14 the spectra of the analytes were saved in the UV spectrum library. Electropherograms were extracted

15 at wavelengths 200 nm, 230 nm, 214 nm, and 254 nm. CE runs were conducted for both EPI and

16 NOR samples at three different ionic strengths of PHB at pH 7.4 that were 20 mM, 50 mM, and 100

17 mM. EPI and NOR samples were prepared at concentrations of 100 μg/ml, dissolved in the respective

18 PHB for each ionic strength. Dimethyl sulfoxide was used as the electroosmotic flow marker. The

19 migration times and peak areas of all analyzed samples were recorded.

20 *Liquid-liquid extraction*

21 The commercial 20% Intralipid solution was diluted to 1% solution using the PHB with ionic

22 strengths of 20 mM, 50 mM, and 100 mM. EPI and NOR were added to the Intralipid solutions

23 separately, resulting in 1 ml solutions of 100 μg/ml concentration. This concentration corresponds to

24 5.46×10^{-4} M and 5.91×10^{-4} M of EPI and NOR, respectively. For both compounds, three separate

1   samples were prepared for each ionic strength. The samples were shaken for 30 min, followed by 5

2   min centrifugation at 15,000 rpm (21,000 rcf). Part of the separated Intralipid layer was carefully

3   removed so that the bottom aqueous layer could be obtained without contamination from the Intralipid

4   layer.

5   The separated aqueous layers (bottom layers) of the samples were analyzed by CE with PHB at

6   respective ionic strengths. The peak areas were recorded from three injections per sample, the

7   migration time-corrected peak areas were calculated, and the compound concentrations were

8   calculated using a calibration curve (six-point calibration curve at 5–200 µg/ml; each concentration

9   was injected five times). The calibration curves were constructed by spiking the extracted aqueous

10 phase (no drugs) with the studied compounds. The initial concentration and the EPI or NOR

11 concentration in the separated aqueous layers were used to calculate the distribution. The distribution

12 constant (K_D), being the equilibrium constant for the distribution of a compound in two immiscible

13 solvents, was calculated by equation (1),

14 $$K_D = \frac{C_{upper\ layer\ (lipid\ layer)}}{C_{lower\ layer\ (aqueous\ layer)}} = \frac{C_{initial} - C_{aqueous\ layer}}{C_{aqueous\ layer}} \qquad (1)$$

15 where $c_{Initial}$ is the initial concentration of the compound and $c_{aqueous\ Layer}$ is the concentration of the

16 compound in the separated (lower) aqueous layer. The percentual concentration ($X\%_{c(lipid\ layer)}$) of the

17 compound in the lipid layer can be calculated from the K_D using equation (2)

18 $$X\%_{c(lipid\ layer)} = 100 \frac{K_D}{1 + K_D} \qquad (2)$$

19 ***Statistical analysis***

20 The primary endpoint of this study was to examine the effect of LE on the vasoconstriction caused

21 by EPI or NOR. Sample size in each group was calculated using the resource equation method [19].

22 Normality test was performed using the Shapiro-Wilk test. The impact of LE on the vasoconstriction

23 triggered by EPI and NOR was examined utilizing a linear mixed-effect model (Stata[®] version 14.2,

24 StataCorp LLC, Lakeway Drive, TX, USA) [20]. The impact of EPI, NOR, or a combination of LE

1 and EPI, or LE and NOR on intracellular calcium levels was assessed using the Kruskal-Wallis test,

2 followed by Dunn's multiple comparison test, utilizing Prism® 5.0 software (GraphPad Inc., San

3 Diego, CA, USA). A P value of less than 0.05 was considered statistically significant.

4

1 **Results**

2 The presence of LE (1% and 2% Intralipid) did not produce a significant change in the contraction

3 caused by EPI or NOR in endothelium-intact aorta (Fig. 1A and Fig. 2A). In addition, the LE (1 and

4 2%) did not significantly affect vasoconstriction induced by EPI (10^{-8} to 10^{-5} M) or NOR (3×10^{-8} to

5 10^{-5} M) (Fig. 1B and Fig. 2B) in endothelium-denuded aorta. However, in the endothelium-denuded

6 aorta, the presence of 1% LE slightly increased the contraction induced by EPI (at concentrations of

7 10^{-9} and 3×10^{-9} M) (Fig. 1B). The P values were less than 0.001 and 0.021 versus the control at 10^{-9}

8 ^{9} and 3×10^{-9} M EPI, respectively, with the corresponding 95% CI of 3.04 to 10.76 and 0.67 to 8.38.

9 A similar trend was seen for NOR: 1% LE slightly increased 10^{-8} M NOR-induced contraction in

10 endothelium-denuded aorta (Fig. 2B; $P < 0.001$ versus control: 95% CI: 3.30 to 10.57). In contrast,

11 2% LE slightly decreased 10^{-9} M EPI-induced contraction in endothelium-denuded aorta (Fig. 1B; P

12 $= 0.030$ versus control; 95% CI: -0.41 to -8.13).

13 Both EPI (10^{-5} M; 10 μM) and NOR (10^{-5} M; 10 μM) elevated the intracellular calcium level in

14 vascular smooth muscle cells (Figs. 3A and 3B; $P < 0.001$ versus control). Furthermore, the

15 combination of LE (1% or 2%) with EPI (10^{-5} M), or LE (1% and 2%) with NOR (10^{-5} M), resulted

16 in an elevation of intracellular calcium (Figs. 3A and 3B; $P < 0.001$ versus control). However, the

17 mixture of LE and EPI or NOR did not significantly change the increase in calcium level caused by

18 EPI or NOR alone (Figs. 3A and 3B).

19 The K_{DS} of EPI and NOR between a buffered aqueous phase and 1% LE were determined by

20 liquid-liquid extraction. The concentrations in the aqueous phase after the extractions were

21 quantitatively determined by CE with UV-absorption detection. The migration times and peak areas

22 of EPI and NOR were recorded. The linearity curves for both compounds were determined in spiked

23 aqueous buffers taken from liquid-liquid extractions without any drugs. The curves were highly linear

24 (Figs. 4A and 4B) over the tested concentration range (5–200 μg/ml), giving a coefficient of

1   correlation (R^2) of more than 0.997 for both compounds. The corresponding linear equations were y

2   = 2.21x – 7.41 and y = 2.68x – 11.71 for EPI and NOR, respectively (Figs. 4A and 4B), where y is

3   the migration time-corrected analyte peak area and x is the analyte concentration (µg/ml). Using these

4   calibration curves, the unknown concentrations were determined. From the obtained concentrations

5   the K_D values were determined using equation (1). The data in Fig. 4C shows that the K_D values of

6   EPI decreased slightly when changing the 20 mM PHB to higher ionic strength PHB solutions (50

7   and 100 mM). Even lower K_D values were observed for NOR, with a slight increase in the value at

8   the highest ionic strength (100 mM). The corresponding logarithmic values (log K_D) of K_D were

9   negative for all investigated buffer solutions (20, 50, and 100 mM ionic strength), being in the range

10   of -0.71 to -1.00 for EPI and -0.96 to -1.25 for NOR. The log K_D values were in all cases clearly

11   negative suggesting highly hydrophilic compounds.

1 **Discussion**

2 This study suggests that LE (Intralipid) does not significantly alter vasoconstriction induced by EPI

3 or NOR. The major findings of this study are the following: 1) LE did not significantly affect

4 vasoconstriction caused by EPI or NOR in endothelium-intact aorta; 2) LE did not significantly affect

5 the calcium level increased by EPI or NOR in vascular smooth muscle cells; 3) EPI and NOR had

6 only negligible interactions with the LE due to their hydrophilic properties.

7 EPI that is an alpha-1 adrenoceptor agonist and a beta-1 adrenoceptor agonist is used to treat

8 hypotension and cardiac arrest [21]. The strong peripheral vasoconstrictor NOR that acts on alpha-1

9 and beta-1 adrenoceptor depending on the dose (less than 2 μg/min: beta-1 effect versus higher than

10 3 μg/min: alpha-1 effect) is used to treat hypotension unresponsive to fluid administration [22]. Thus,

11 for managing refractory cardiovascular collapse caused by toxic doses of local or non-local

12 anesthetics, LE and vasopressors like EPI or NOR can be concurrently administered. LE did not

13 significantly alter EPI-induced contraction in endothelium-intact aorta. The results from liquid-liquid

14 extraction experiments indicate that the portion of total EPI that is distributed into the LE at 20, 50,

15 and 100 mM ionic strength is 16.16, 9.26, and 9.07%, respectively. These values are small, suggesting

16 that EPI is strongly water soluble. Higher ionic strengths did not significantly affect the distribution

17 that is of importance considering that the calculated ionic strength of the Krebs solution was 158 mM

18 and that of plasma 161 mM. This LE mediated no significant change of EPI-induced vasoconstriction

19 that can be explained by the relatively low K_D of EPI. The K_D of NOR at ionic strengths of 20 and 50

20 mM was even lower than that of EPI. The portion of total NOR distributed in the lipid phase was only

21 5.72 and 5.34% at 20 mM and 50 mM ionic strength, respectively. At 100 mM ionic strength the

22 percentual distribution was slightly higher (9.83%). No significant alternation of NOR-induced

23 contraction by LE was observed that can be explained by the very low K_D at all studied ionic strengths

24 (20 to 100 mM). Considering the discussion above, most parts of EPI and NOR will be distributed in

the aqueous phase, indicating that the vasopressor effect induced by EPI or NOR, when used in conjunction with LE resuscitation for systemic toxicity due to local anesthetics or non-local anesthetics, may remain unchanged. LE suppresses the vasodilation mediated by nitric oxide that is stimulated by acetylcholine and the adenosine triphosphate-sensitive potassium channel agonist levcromakalim [23,24]. Moreover, LE impedes the phosphorylation of endothelial nitric oxide synthase induced by acetylcholine that plays a role in nitric oxide generation [23]. Furthermore, LE administration alone raises blood pressure and peripheral vascular resistance in humans, while it reduces flow-mediated vasodilation and vascular compliance in humans [25,26]. All these previous reports suggest that LEs inhibit endothelial nitric oxide release that is spontaneous or stimulated by some drugs [23-26]. However, further *in vivo* studies regarding the effect of LE on the increased blood pressure caused by various doses of EPI or NOR are needed. Endothelial nitric oxide, generated from L-arginine by nitric oxide synthase, induces vasodilation through a pathway that involves guanylate cyclase, cyclic guanosine monophosphate (cGMP), and cGMP-dependent protein kinase [17]. Thus, endothelial nitric oxide attenuates vasoconstriction induced by an agonist. As residual endothelium after endothelial denudation affects contraction caused by EPI or NOR, the endothelium-denuded aortas were pretreated with L-NAME (10^{-4} M; 100 μM) to examine the effect of LE on the contraction caused by EPI or NOR in endothelium-denuded aorta. While this study employed L-NAME to reduce nitric oxide release triggered by the remaining endothelium following endothelial denudation, the extent of residual endothelium may produce a slight alteration in contraction induced by low concentrations of EPI or NOR solely in the endothelium-denuded aorta in response to LE. Furthermore, since long-chain fatty acids enhance voltage-dependent calcium currents, the slight increase in vasoconstriction induced by low doses of EPI or NOR when using LE might be attributed to the LE itself causing an increase in calcium levels [27]. Additional research is warranted to investigate the underlying mechanism behind the slight modification of vasoconstriction induced by

1   low concentrations of EPI and NOR in endothelium-denuded aorta mediated by LE.

2   Contraction caused by agonists such as EPI or NOR produces increased intracellular calcium

3   levels and subsequently vasoconstriction via activation of myosin light chain kinase that is mediated

4   by calcium influx via receptor-operated calcium or voltage-operated calcium channels and calcium

5   release from sarcoplasmic reticulum [28]. The Intralipid emulsion (1 and 2%) did not significantly

6   alter the calcium increase caused by EPI or NOR. Thus, considering the results from the tension study

7   and the low K_D of EPI and NOR, these results suggest that the LE does not significantly absorb EPI

8   or NOR, leading to sustained vasoconstriction produced by EPI or NOR used in LE resuscitation.

9   The study has the following limitations: First, blood pressure, predominantly influenced by

10   peripheral vascular resistance and cardiac output, is mainly regulated by small resistance arterioles

11   [29]. However, this study utilized the aorta that is a conduit vessel. Second, *in vivo* experiments would

12   be more appropriate as the blood pressure is affected by the cardiac output and the sympathetic

13   nervous system, and the LE increases the blood pressure *in vivo*. However, in this work *in vitro*

14   experiments were done. Third, liquid-liquid extraction experiments used slightly higher

15   concentrations of EPI (5.46×10^{-4} M) and NOR (5.91×10^{-4} M). One suggested dosing regimen of

16   LE treatment as adjuvant drug for drug toxicity is 1% plasma triglyceride that provides positive

17   inotropic and scavenging effect [30-32]. Thus, we used 1% Intralipid emulsion in isometric tension

18   measurements and liquid-liquid extractions. Even with these limitations, the results suggest that

19   Intralipid emulsion has negligible effect on the vasoconstriction produced by EPI and NOR due to a

20   clinically negligible amount of EPI or NOR sequestration into the LE. This leads to a beneficial

21   sustained hemodynamic support produced by EPI and NOR used during LE resuscitation for

22   intractable cardiovascular collapse caused by drug toxicity due to local anesthetic or non-local

23   anesthetics. Taking these results into consideration, cardiovascular support induced by EPI or NOR

24   remains unaltered in patients undergoing LE administration for parenteral nutrition in the intensive

1   care unit.

2   In conclusion, based on our tension study and liquid-liquid extraction experiments, the studied

3   LE (Intralipid) does not significantly alter the vasoconstriction induced by EPI or NOR due to their

4   hydrophilic properties that may lead to a well sustained vasopressor effect during LE resuscitation

5   for drug toxicity.

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Table 1. Physiochemical properties of EPI and NOR

Drug	log P[*]	pK$_a$[†]	NIF (%)[‡] at pH 7.4	NIF (%)[‡] at pH 7.3	NIF (%)[‡] at pH 7.2	NIF (%)[‡] at pH 7.1
EPI	-1.37	8.59	6.06	4.88	3.91	3.13
NOR	-1.24	8.58	6.20	4.99	4.00	3.21

[*]log P: log octanol/water partition coefficient from PubChem (https://pubchem.ncbi.nlm.nih.gov/).

[†]pK$_a$: negative logarithm of the acid dissociation constant (K$_a$) from PubChem (https://pubchem.ncbi.nlm.nih.gov/).

[‡]NIF (%): non-ionized fraction of total drug (EPI and NOR) is calculated using Henderson-Hasselbalch equation.
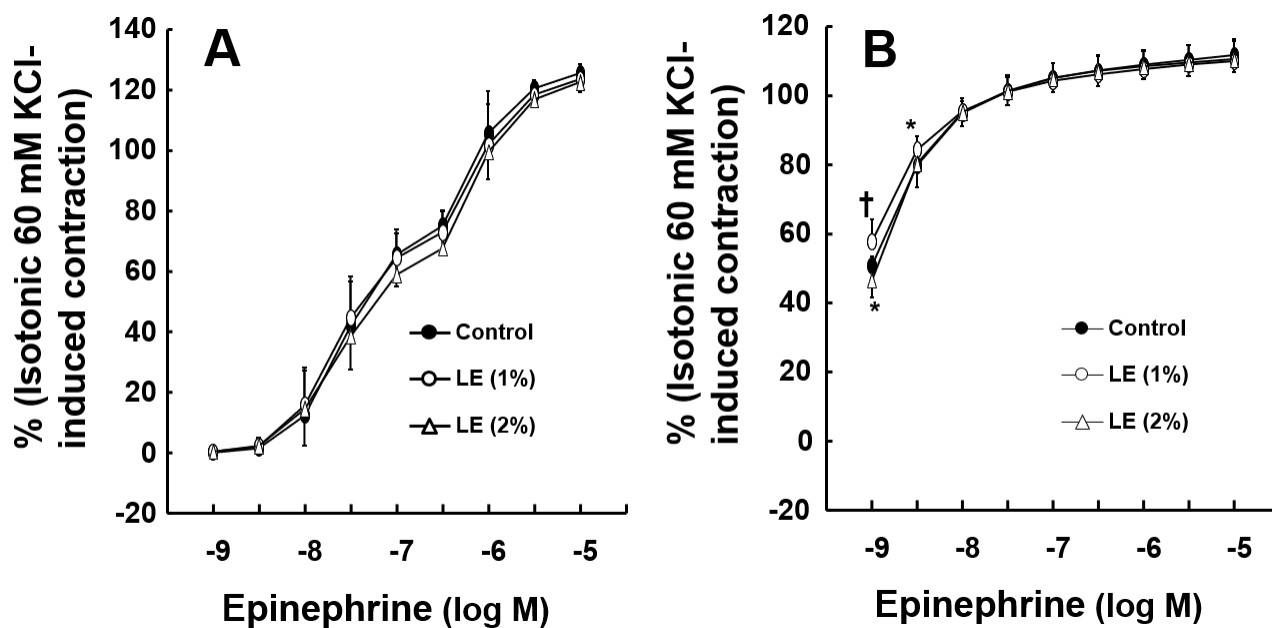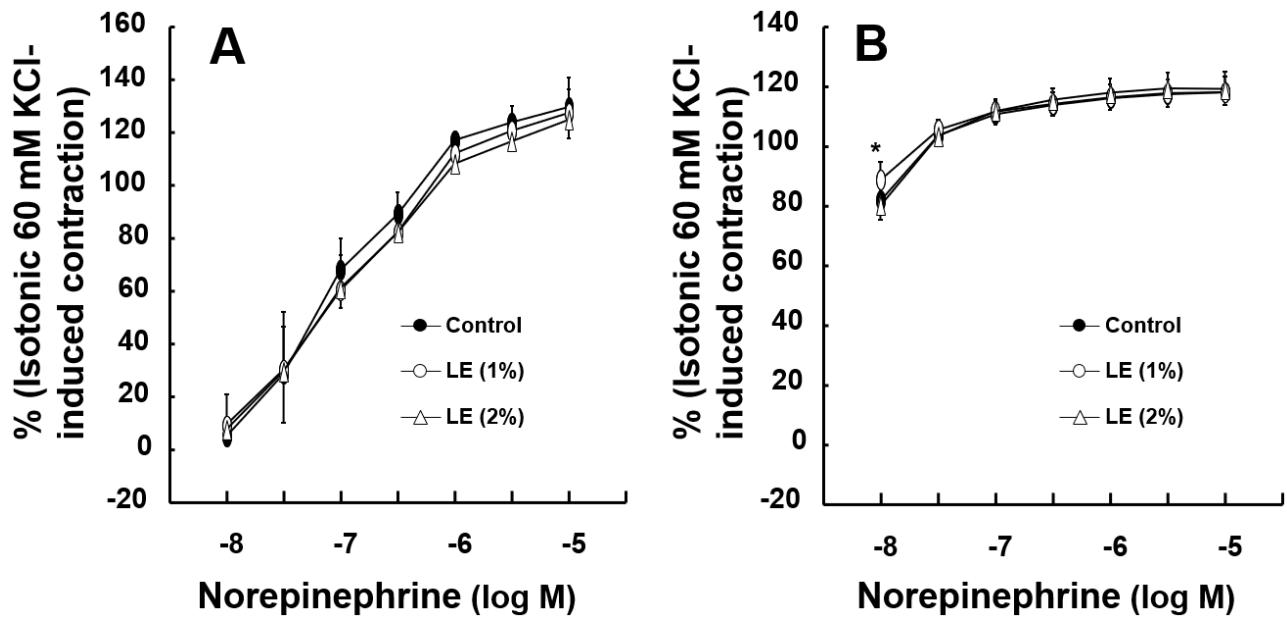
EPI: epinephrine; NOR: norepinephrine.

Fig. 1. Effect of lipid emulsion (LE) on the contraction caused by epinephrine in both endothelium-intact rat aorta (A) and endothelium-denuded rat aorta (B). Data (n = 5) are shown as mean ± SD and expressed as the percentage of isotonic 60 mM KCl-induced contraction. n indicates the number of rats from which isolated rat aortae were obtained. *P = 0.030 and 0.021 at 10^{-9} and 3 x 10^{-9} M epinephrine versus control, respectively. †P < 0.001 versus control.

Fig. 2. Effect of lipid emulsion (LE) on the contraction caused by norepinephrine in both endothelium-intact rat aorta (A) and endothelium-denuded rat aorta (B). Data (n = 5) are shown as mean ± SD and expressed as the percentage of isotonic 60 mM KCl-induced contraction. n indicates the number of rats from which isolated rat aortae were obtained. *P < 0.001 versus control.
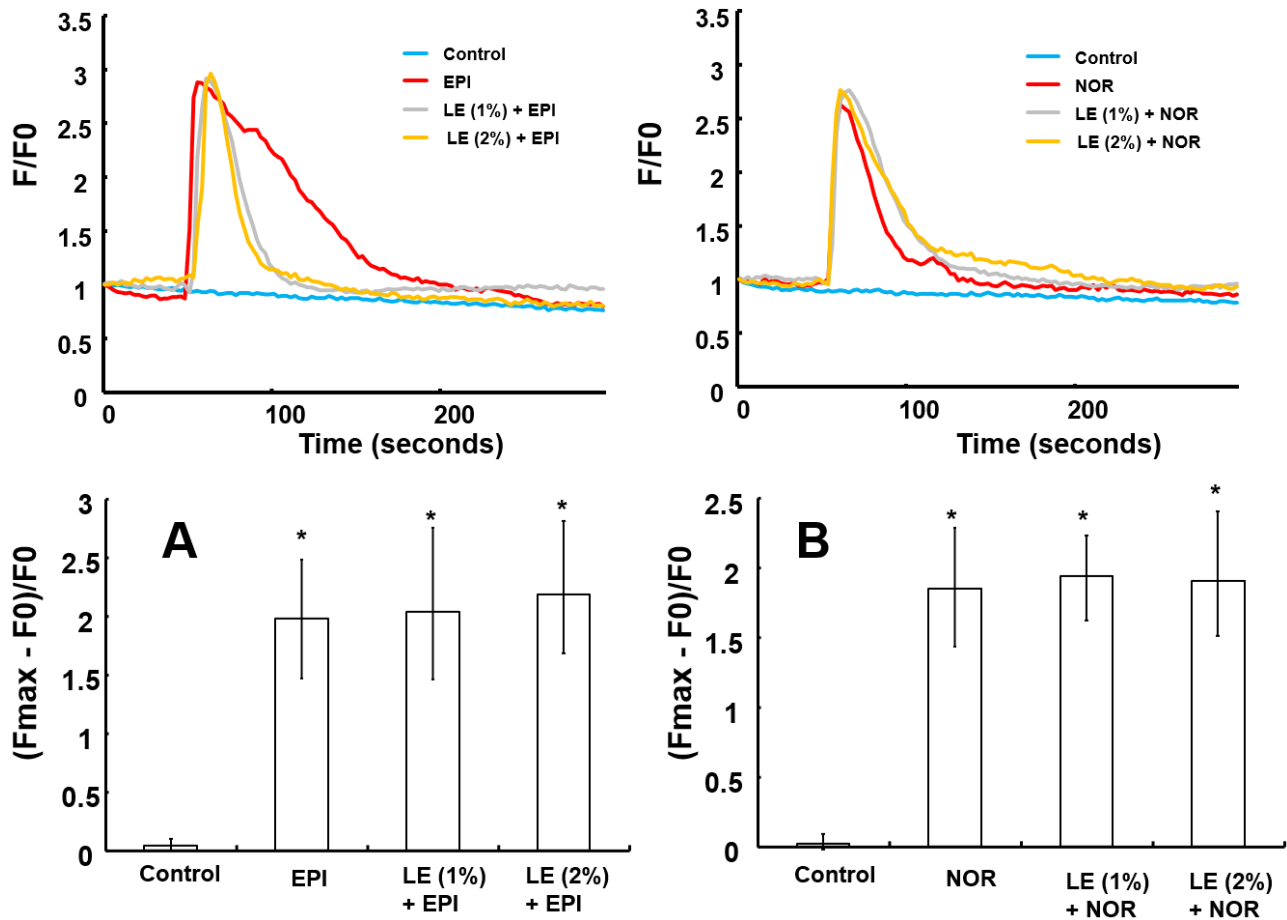
Fig. 3. Effect of lipid emulsion (LE) on the increased intracellular calcium level induced by 10^{-5} M epinephrine (EPI, A) or 10^{-5} M norepinephrine (NOR, B) in the vascular smooth muscle cells. Data (n = 4) are shown as median and interquartile range (25 to 75%). n indicates the number of independent experiments. $^{*}P < 0.001$ versus control.
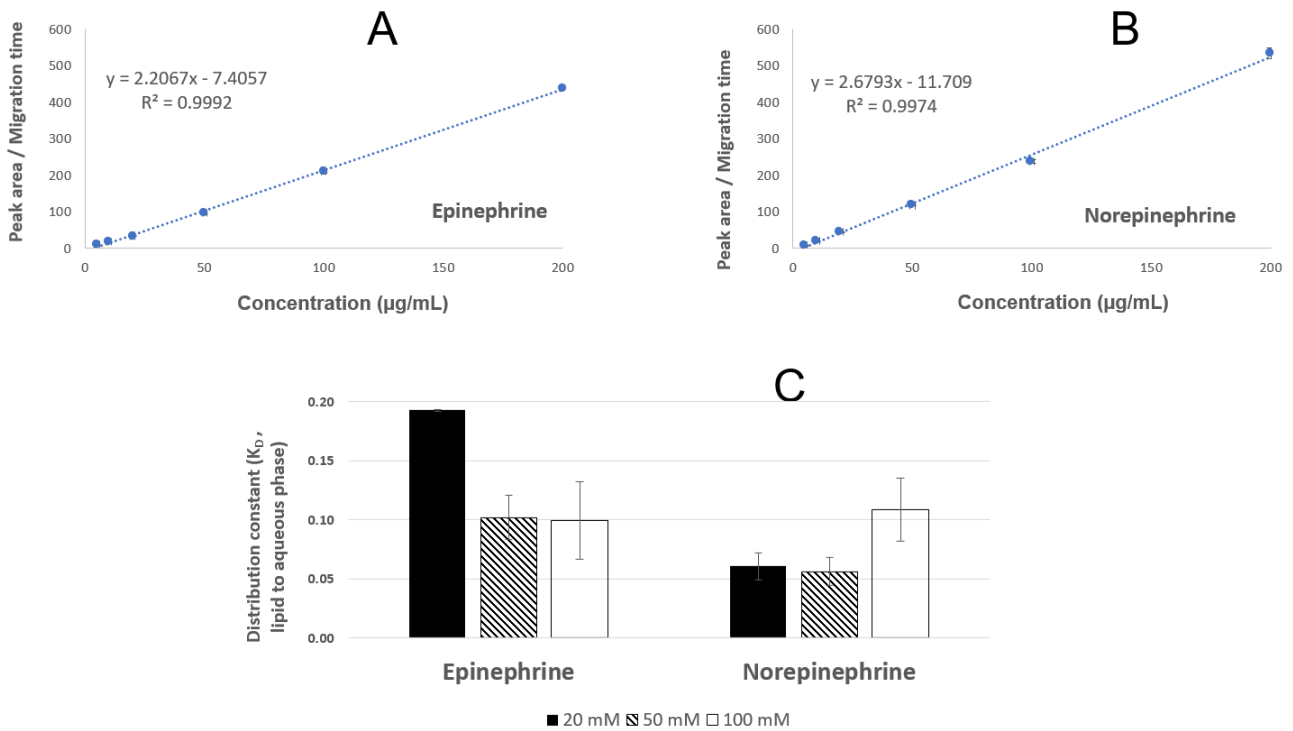
Fig. 4. Calibration curves for epinephrine (A) and norepinephrine (B) by capillary electrophoresis. (C) Distribution constants (K_D: lipid to aqueous phase) of epinephrine and norepinephrine at 25°C by liquid-liquid extraction at 20, 50, and 100 mM ionic strength of phosphate buffer. The data is presented as mean ± SD from three experiments.