รายงานวิจัยฉบับสมบูรณ์

โครงการ

ผลของการกระตุ้นเส้นประสาทเวกัสต่อภาวะการบาดเจ็บของกล้ามเนื้อหัวใจในภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันที่ได้รับเลือดกลับมาเลี้ยงใหม่

The Effects of Vagus Nerve Stimulation on Acute Cardiac Ischemia/Reperfusion Injury

โดย

ผู้ช่วยศาสตราจารย์ ดร. นายแพทย์ เกริกวิชช์ ศิลปวิทยาทร และคณะ
ศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ และ
ภาควิชาสรีรวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

กรกฎาคม พ.ศ. 2561

รายงานวิจัยฉบับสมบูรณ์

โครงการ: ผลของการกระตุ้นเส้นประสาทเวกัสต่อภาวะการบาดเจ็บของกล้ามเนื้อหัวใจในภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันที่ได้รับเลือดกลับมาเลี้ยงใหม่

คณะผู้วิจัย	**สังกัด**
ผศ. ดร. นพ. เกริกวิชช์ ศิลปวิทยาทร	ศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ, ภาควิชาสรีรวิทยา,คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
ศ.(เชี่ยวชาญพิเศษ) ดร. ทพญ. สิริพร ฉัตรทิพากร	ภาควิชาชีววิทยาช่องปากและพิเคราะห์โรคช่องปาก คณะทันตแพทยศาสตร์, ศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ, คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
ศ.(เชี่ยวชาญพิเศษ) ดร. นพ. นิพนธ์ ฉัตรทิพากร	ศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ, ภาควิชาสรีรวิทยา,คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

คำนำ

ภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันเป็นปัญหาที่สำคัญอันดับต้นๆ ในหลายๆ ประเทศทั่วโลก อีกทั้งยังเป็นสาเหตุหลักของการเสียชีวิตของประชากรในกลุ่มประเทศอุตสาหกรรมรวมทั้งประเทศไทย ปัจจุบันวิธีการรักษาผู้ป่วยที่มีภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลัน คือ การปล่อยให้เลือดกลับไปเลี้ยงกล้ามเนื้อหัวใจบริเวณที่ขาดเลือดโดยการใช้ยา หรือ การใส่สายสวนทางหลอดเลือดแดงเพื่อทำการขยายหลอดเลือดที่ไปเลี้ยงหัวใจบริเวณที่มีการอุดตันเพื่อให้เลือดสามารถไหลผ่านได้ อย่างไรก็ตามจากงานวิจัยก่อนหน้านี้พบว่าการปล่อยให้เลือดกลับไปเลี้ยงกล้ามเนื้อหัวใจบริเวณที่ขาดเลือด ยังส่งผลทำให้กล้ามเนื้อหัวใจบางส่วนได้รับบาดเจ็บหรือตายได้ โดยภาวะดังกล่าวเรียกว่า “ภาวะการบาดเจ็บของกล้ามเนื้อหัวใจในภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันที่ได้รับเลือดกลับมาเลี้ยงใหม่” ซึ่งผู้ป่วยที่รอดชีวิตมีโอกาสที่จะเกิดภาวะหัวใจล้มเหลวตามมาได้ในเวลาต่อมา ดังนั้นจึงมีความจำเป็นที่จะต้องทำการวิจัยเพื่อที่จะเข้าใจถึงกลไกในการเกิด “ภาวะการบาดเจ็บของกล้ามเนื้อหัวใจในภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันที่ได้รับเลือดกลับมาเลี้ยงใหม่” อีกทั้งยังมีความจำเป็นในการทำวิจัยเพื่อหาวิธีการรักษาแบบใหม่เพื่อนำมาใช้ในการป้องกัน การเกิดภาวะดังกล่าวจากงานวิจัยในสัตว์ทดลองก่อนหน้านี้พบว่าการกระตุ้นเส้นประสาทเวกัสบริเวณลำคอ สามารถป้องกันการบาดเจ็บของกล้ามเนื้อหัวใจจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลัน และมีเลือดกลับมาหล่อเลี้ยงใหม่ได้ แต่ทว่ากลไกการป้องกันการบาดเจ็บของกล้ามเนื้อหัวใจจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลัน และมีเลือดกลับมาหล่อเลี้ยงใหม่โดยการกระตุ้นเส้นประสาทเวกัสยังไม่เป็นที่ทราบแน่ชัด

โดยงานวิจัยในฉบับนี้เป็นการศึกษาถึง ผลของการกระตุ้นเส้นประสาทเวกัสระดับลำคอ ต่อภาวะการบาดเจ็บของกล้ามเนื้อหัวใจในภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันที่ได้รับเลือดกลับมาเลี้ยงใหม่ ซึ่งทางทีมผู้วิจัยพบว่าการกระตุ้นเส้นประสาทเวกัสข้างซ้ายระดับลำคอแบบ intermittent ทั้งในช่วงที่หัวใจพึ่งถูกเหนี่ยวนำให้เกิดภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลัน หรือแม้กระทั่งทำการกระตุ้นเส้นประสาทเวกัสในช่วงที่เกิดภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันผ่านมาแล้วเป็นระยะเวลา 30 นาที สามารถป้องกันการบาดเจ็บของหัวใจจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลันและมีเลือดกลับมาหล่อเลี้ยงใหม่ โดยทางทีมผู้วิจัยพบว่าการกระตุ้นเส้นประสาทเวกัสทำให้การทำงานและการเปลี่ยนแปลงรูปร่างของไมโทคอนเดรียดีขึ้น อีกทั้งยังทำให้เกิดการเปลี่ยนแปลงกระบวนการเผาผลาญกรดไขมันในไมโทคอนเดรียไปสู่กระบวนการเบต้าออกซิเดชั่น โดยข้อมูลทั้งหมดที่ได้จากการศึกษานี้สามารถสรุปได้ว่าผลในการป้องกันกล้ามเนื้อหัวใจจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันและมีเลือดกลับมาหล่อเลี้ยงใหม่ของการกระตุ้นเส้นประสาทเวกัส ผ่านทางการนำกระแสประสาทขาออกไปยังหัวใจ (efferent) เป็นหลัก และยิ่งไปกว่านั้นหากต้องการประสิทธิภาพสูงสุดในการป้องกันการ

เกิดการบาดเจ็บจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลันและมีเลือดกลับมาหล่อเลี้ยงใหม่ จากการกระตุ้นเส้นประสาทเวกัส จะต้องมีการส่งสัญญาณกระแสประสาทขาออกไปยังหัวใจ (efferent) ผ่านเส้นประสาทเวกัสทั้งสองข้าง ดั้งนั้นการกระตุ้นเส้นประสาทเวกัสมีความเป็นไปได้ที่จะนำมาใช้ในการป้องกันการเกิดการบาดเจ็บจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลันและมีเลือดกลับมาหล่อเลี้ยงใหม่ในผู้ป่วยที่มีภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลัน ซึ่งจะเป็นการช่วยลดโอกาสในการเกิดภาวะหัวใจล้มเหลว และเป็นการช่วยให้ผู้ป่วยมีคุณภาพชีวิตที่ดีขึ้น

ผู้วิจัยขอขอบคุณ นายวัฒนา นันต๊ะภูมิ, น.ส.พ. ดร. วันพิทักษ์ ป้องกัน, ดร. สุวคนธ์ วงศ์ใจคำ, ดร. สาวิตรี ชารุนันทกร, ดร. พงษ์พันธ์ ธนะจักร, นางสาวจุฑามาศ คำสีแก้ว, นางสาวกรรณาภรณ์ อินทชัย, นางรจนา ทาวัน ในฐานะผู้ช่วยวิจัยที่ช่วยดำเนินการทดลองและให้ความช่วยเหลือในทุกทางตลอดโครงการ นอกจากนี้ ขอขอบคุณ ศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ (CERT CENTER) ภายใต้การดูแลของ ศาสตราจารย์ (เชี่ยวชาญพิเศษ) ดร. นายแพทย์นิพนธ์ ฉัตรทิพากร และ ศาสตราจารย์ (เชี่ยวชาญพิเศษ) ดร. ทพญ. สิริพร ฉัตรทิพากร คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ที่ให้ความอนุเคราะห์ในการใช้เครื่องมือสำหรับงานวิจัยครั้งนี้ การศึกษานี้ได้รับการสนับสนุนจากกองทุนวิจัยจาก Thailand Research Fund (RSA5880015) and Faculty of Medicine Endowment, Chiang Mai University

ผศ. ดร. นพ. เกริกวิชช์ ศิลปวิทยาทร

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สารบัญรูปภาพ

สารบัญรูปภาพ

สารบัญตาราง

บทคัดย่อภาษาไทย

จากงานวิจัยในสัตว์ทดลองก่อนหน้านี้พบว่าการกระตุ้นเส้นประสาทเวกัส บริเวณลำคอ สามารถป้องกันการบาดเจ็บของกล้ามเนื้อหัวใจ จากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลัน และมีเลือดกลับมาหล่อเลี้ยงใหม่ได้ แต่ทว่ากลไกการป้องกันการบาดเจ็บของกล้ามเนื้อหัวใจจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลัน และมีเลือดกลับมาหล่อเลี้ยงใหม่โดยการกระตุ้นเส้นประสาทเวกัสยังไม่เป็นที่ทราบแน่ชัด โดยงานวิจัยในฉบับนี้เป็นการศึกษาถึง ผลของการกระตุ้นเส้นประสาทเวกัสระดับลำคอ ต่อภาวะการบาดเจ็บของกล้ามเนื้อหัวใจในภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันที่ได้รับเลือดกลับมาเลี้ยงใหม่ โดยทำการศึกษาในสุกรจำนวน 90 ตัว โดยสุกรจะถูกเหนี่ยวนำให้เกิดภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลัน โดยการผูกหลอดเลือดแดงโคโรนารีข้างซ้าย เป็นระยะเวลา 60 นาที หลังจากนั้นจะทำการปล่อยเหลือดกลับไปเลี้ยงใหม่เป็นเวลา 120 นาที ซึ่งทางทีมผู้วิจัยพบว่าการกระตุ้นเส้นประสาทเวกัสข้างซ้ายระดับลำคอแบบ intermittent ทั้งในช่วงที่หัวใจพึ่งถูกเหนี่ยวนำให้เกิดภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลัน หรือแม้กระทั่งทำการกระตุ้นเส้นประสาทเวกัสในช่วงที่เกิดภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันผ่านมาแล้วเป็นระยะเวลา 30 นาที สามารถป้องกันการบาดเจ็บของหัวใจจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลันและมีเลือดกลับมาหล่อเลี้ยงใหม่

โดยทางทีมผู้วิจัยพบว่าการกระตุ้นเส้นประสาทเวกัสทำให้การทำงานและการเปลี่ยนแปลงรูปร่างของไมโทคอนเดรียดีขึ้น อีกทั้งยังทำให้เกิดการเปลี่ยนแปลงกระบวนการเผาผลาญกรดไขมันในไมโทคอนเดรียไปสู่กระบวนการเบต้าออกซิเดชั่น ซึ่งผลดังกล่าวถูกยับยั้งภายหลังจากการฉีดยาอะโทรปีน โดยข้อมูลทั้งหมดที่ได้จากการศึกษานี้สามารถสรุปได้ว่า ผลในการป้องกันกล้ามเนื้อหัวใจ จากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันและมีเลือดกลับมาหล่อเลี้ยงใหม่ของการกระตุ้นเส้นประสาทเวกัส ผ่านทางการนำกระแสประสาทขาออกไปยังหัวใจ (efferent) เป็นหลัก และยิ่งไปกว่านั้นหากต้องการประสิทธิภาพสูงสุดในการป้องกันการเกิดการบาดเจ็บจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลัน และมีเลือดกลับมาหล่อเลี้ยงใหม่จากการกระตุ้นเส้นประสาทเวกัส จะต้องมีการส่งสัญญาณกระแสประสาทขาออกไปยังหัวใจ (efferent) ผ่านเส้นประสาทเวกัสทั้งสองข้าง ดั้งนั้นการกระตุ้นเส้นประสาทเวกัสมีความเป็นไปได้ ที่จะนำมาใช้ในการป้องกันการเกิดการบาดเจ็บ จากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลันและมีเลือดกลับมาหล่อเลี้ยงใหม่ในผู้ป่วยที่มีภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลัน

ABSTRACT

Vagus nerve stimulation (VNS) has been shown to exert cardioprotection against myocardial ischemia/reperfusion (I/R) injury. However, the mechanisms by which VNS produces its protective effects are not yet entirely known. Therefore, in this study we hypothesized that intermittent VNS (I-VNS) exerts cardioprotection against myocardial I/R injury predominantly through its efferent vagal fibers. Ninety swine (30-35 kg) were used in our study. VNS was applied, at the onset of ischemia, during ischemia, at the onset on reperfusion and continued until the end of reperfusion. Ischemia was induced by left anterior descending (LAD) coronary artery occlusion for 60 minutes, followed by 120 minutes of reperfusion. Cardiac function, infarct size, myocardial connexin43 levels, apoptotic markers, oxidative stress markers, inflammatory markers and cardiac mitochondrial morphology and function were determined. Our data demonstrated that I-VNS applied at the onset of ischemia or during ischemia, but not at the onset of reperfusion, exerted cardioprotection against myocardial I/R injury via improvement of mitochondrial function and dynamics and shifted cardiac fatty acid metabolism toward beta oxidation. These beneficial effects of VNS were abolished by atropine. In summary, our finding suggested that I-VNS exerted cardioprotection against cardiac I/R injury predominantly through its efferent vagal fibers. Furthermore, VNS required both ipsilateral and contralateral efferent vagal activities to fully provide its cardioprotection against I/R injury. Thus, our findings suggested that VNS might be a promising therapeutic modality for protecting myocardium at risk of I/R injury.

EXECUTIVE SUMMARY

ชื่อโครงการ:

ผลของการกระตุ้นเส้นประสาทเวกัสต่อภาวะการบาดเจ็บของกล้ามเนื้อหัวใจในภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันที่ได้รับเลือดกลับมาเลี้ยงใหม่

The Effects of Vagus Nerve Stimulation on Acute Cardiac Ischemia/Reperfusion Injury

ชื่อหัวหน้าโครงการ หน่วยงานที่สังกัด ที่อยู่ หมายเลขโทรศัพท์ โทรสาร และ e-mail

ผู้ช่วยศาสตราจารย์ ดร. นายแพทย์เกริกวิชช์ ศิลปวิทยาทร

ศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ,

ภาควิชาสรีรวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

อ.เมือง จ.เชียงใหม่ 50200

โทร: 053-935329 Fax: 053-935365

E-mail: kshiplap@gmail.com

สาขาวิชาที่ทำการวิจัย: Cardiac Electrophysiology

งบประมาณรวมทั้งโครงการ: 1,500,000.00 บาท

ระยะเวลาดำเนินงาน: 3 ปี

ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

Despite major advances in treatment of cardiovascular disease, acute myocardial infarction (MI) remains a common cause of heart damage throughout the world.[1] Although restoration of blood flow to ischemic tissue is essential to prevent irreversible tissue damage and cell death, sudden reperfusion may trigger a devastating cascade of rapidly-evolving, biological events ending with the activation of intracellular pathways that accelerate cell death. This phenomenon, so-called myocardial

ischemia/reperfusion (I/R) injury, can paradoxically reduce the beneficial effects of myocardial reperfusion.[2] Moreover, myocardial I/R injury is considered a major concern in patients with acute myocardial infarction or those undergoing coronary artery bypass grafting (CABG) and transplantation.[3, 4] Therefore, a therapeutic modality used as an adjunct to percutaneous coronary intervention (PCI) to attenuate reperfusion injury could potentially improve the long-term outcome of MI patients. Over the past several years, enhancing parasympathetic activation of the autonomic nervous system by delivering electrical stimulation to the cervical vagus nerve (VNS) has emerged as a promising therapy for various conditions, including brain and heart diseases. Over the past decade, VNS has been shown to exert cardioprotection in both chronic heart failure and ischemic heart diseases.[5-10] In ischemic hearts, the effects of VNS have been shown to improve cardiac function, limit dispersion of repolarization, prevent reperfusion injury, attenuate cardiac remodeling, improve defibrillation efficacy, and decrease the infarct size.[11, 12] These emerging data led us to hypothesize that VNS might be used as adjunctive myocardial salvage approach to current percutaneous coronary and pharmacological interventions designed to protect myocardium at risk of I/R injury.

The outcome of this research has important implications. Although advances in percutaneous coronary technology, reduced procedure times and appropriate use of pharmacological approaches (antiplatelet and antithrombotic) for maintaining coronary flow have improved outcomes, attenuation of myocardial reperfusion injury remains unresolved. Therefore, novel therapeutic strategies are needed to prevent myocardial reperfusion injury and further reduce the infarct size, improve left ventricular function and clinical outcomes in patients with acute MI. Augmentation of vagus nerve activity

with electrical stimulation exerts a broad spectrum of cardioprotective effects such as anti-oxidative stress, anti-inflammation, anti-apoptosis, and also preserves mitochondrial function. Based on data from numerous preclinical studies in a wide range of species and experimental models, as well as a observations from clinical studies, it seems logical that VNS might be a promising therapeutic modality for protecting myocardium at risk of I/R injury. Therefore, in this study we tested our hypothesis that VNS exerts direct cardioprotective effects through a muscarinic ACh receptor by amelioration of cardiac mitochondrial dysfunction in a time- and duration-dependent manner.

วัตถุประสงค์

The autonomic imbalance is deleterious for the cardiac function, and this effect is associated with a poor outcome of cardiovascular diseases including acute MI. One of the strategies that have been used in recent times to modulate this imbalance is electrical VNS. Over the past decade, chronic, intermittent VNS has been shown to exert cardioprotective effects in chronic ischemic cardiomyopathy as well as in heart failure in both clinical and experimental animal studies. However, the mechanisms by which VNS produces its protective effects are not yet entirely known. Therefore, our general hypothesis is that VNS exerts direct cardioprotective effects through a muscarinic ACh receptor by amelioration of cardiac mitochondrial dysfunction in a time- and duration-dependent manner. Thus the specific aims of this proposal are to:

1. Determine whether low-amplitude, left cervical vagus nerve stimulation (VNS) applied either intermittently or continuously imparts cardioprotection against acute ischemia-reperfusion injury

2. Determine whether VNS applied later during ischemia or at the onset of reperfusion exerts differential cardioprotection against cardiac I/R injury

3. Determine whether VNS exerts cardioprotection predominantly through its efferent vagal fibers.

ระเบียบวิธีวิจัย

Animal Preparation: All experiments will be approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. The domestic pigs (25-30 kg) will be anesthetized by an intramuscular injection of a combination of 4.4 mg/kg zoletil® (Vibbac Laboratories, Carros, France) and 2.2 mg/kg xylazine (Laboratorios Calier, S.A., Barcelona, Spain). After endotracheal intubation, anesthesia will be maintained by 1.5–3.0% isoflurane (Abbott Laboratories Ltd., Queenborough, UK) delivered in 100% oxygen. Surface electrocardiogram (lead II), femoral arterial blood pressure (BP), heart rate (HR), and rectal temperature will be continuously monitored, and all data will be recorded for subsequent analysis. Arterial blood gases and electrolytes will be also monitored every 30 minutes and maintained within acceptable physiological ranges.[13] Under fluoroscopic guidance, platinum coated titanium coil electrodes (34- and 68-mm) will be advanced into and positioned at the right ventricular apex and the junction between the right atrium and superior vena cava, respectively, to deliver electrical shocks when malignant ventricular arrhythmias spontaneously occurred during ischemia-reperfusion (I/R).[13] The chest will be opened through a left thoracotomy. The left anterior descending artery (LAD) will be isolated and occluded by ligature (3-0 silk) three

centimeters from the left main coronary artery. Ischemia will be confirmed by an ST elevation on the ECG and the change in color of myocardial tissues on the ischemic area. Complete LAD occlusion will be maintained for 60 minutes followed by 120 minutes of reperfusion. Heart rate (HR), PR interval, QRS complex duration (an indicator of ventricular activation time), QT interval (an indicator of ventricular repolarization time), and time from T-wave peak to end (Tpe; an indicator of transmural dispersion of repolarization) will be measured. ECG traces will be analyzed with Chart 6 (AD Instruments).

Vagus Nerve Stimulation (VNS) Protocol: The left vagus nerve will be surgically isolated (~ 3 cm, C5-6 level) from the carotid sheath. A VNS lead (Model 304, Cyberonics, Houston, TX, USA) with bipolar electrodes (platinum-iridium, 4 mm^2 surface area, 6-mm interelectrode spacing) will be attached to the vagus nerve using helical fixation elements to assure electrode stability. The cathodic electrode will be oriented closest to the heart. The proximal terminal pin of VNS lead will be attached to a pulse generator (Demipulse, Model 103, Cyberonics) for delivery of VNS. Prior to onset of ischemia, the mean PR interval will be determined from an average of ten consecutive sinus beats. We will verify that VNS will be engaging the autonomic nervous system by briefly stimulating the vagus nerve and observing a significant increase in the PR intervals. The VNS parameters (3.5 mA, 500 μs pulse width and 20 Hz) are based on FDA-approved VNS parameters and our previous study.[14, 15] During the I/R study in each pig, cardiac hemodynamic performance will be continuously monitored and recorded using the pressure-volume (P-V) loop recording system (Model

ADV500/ADVantage System, Scisense Inc., London, Canada) as described previously.[16] For ECG analysis, the mean baseline PR interval will be determined from an average of ten sinus beats just prior to LAD occlusion. The mean PR intervals during the ischemia and reperfusion periods will be analyzed from an average of ten consecutive beats before the end of occlusion and the end of reperfusion, respectively.

Evaluation of Rhythm Disturbances: Premature ventricular contractions (PVCs), VT, and VF will be defined according to the Lambeth Convention criteria[17] with more rigorous modifications for the entire 180 minutes I/R period. Specifically, PVCs will be defined as ventricular contractions without atrial depolarization. VT will be defined as more than six consecutive PVCs. VF will be characterized by a loss of synchronicity of electrocardiogram plus decreased amplitude and a precipitous fall in blood pressure (BP) for more than one second.

Infarct Size Determination: After 120 minutes of reperfusion, the LAD will be re-occluded by the LAD ligation, and the heart will be removed and irrigated with normal saline to wash out blood from chambers and vessels. The infarct size will be assessed with 0.5% Evans Blue and 1.0% Triphenyltetrazolium Chloride (TTC) staining as previously described.[15] The area at risk (AAR) will be defined as the area not stained by the Evan blue dye, and the infarcted area will be defined as the area not stained by TTC. An area measurement will be performed using the Image Tool software version 3.0. The mass-ratio of the AAR to total ventricular mass, and the infarct size normalized to the AAR, will be calculated as described previously.[15]

Western Blot Analysis: At the end of each experiment, the heart will be rapidly excised, and then the remote and ischemic areas of ventricular tissues will be collected, quickly frozen in liquid nitrogen, and stored at -80ºC until analysis. Heart proteins will be lysed with extraction buffer (Tris–HCl 20 mmol/L, Na_3VO_4 1 mmol/L, NaF 5 mmol/L) and separated by electrophoresis on 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then will be transferred onto nitrocellulose membranes. After immunoblots will be blocked for one hour with 5% nonfat dry milk in Tris-buffer saline (pH 7.4) containing 0.1% Tween 20, they will be probed overnight at 4ºC with the primary antibodies that recognize phospho-connexin43 (p-Cx43)(Ser368); a marker of intercellular electrical communication, cytochrome c (Cell Signaling Technology, Danvers, MA); a marker of apoptosis, Akt (1:1,000) (Cell Signaling Technology), Ser473phospho-Akt (1:1,000) (Cell Signaling Technology), GSK-3β (1:1,000) (Santa Cruz Biotchnology Inc.), Ser9phospho-GSK-3β (1:300) (Cell Signaling Technology), and actin (Sigma-Aldrich, Tokyo, Japan); a loading control, followed by one hour of incubation at room temperature with the horseradish peroxidase–conjugated secondary antibody. The blots will be visualized by ECL reagent. The film images of the Western blots will be scanned and analyzed using ImageJ (NIH image) analysis software. For quantitation of the proteins of interest, the ratio of ischemic (I) area per remote (R) area will be determined, and normalized with actin.

Cardiac Inflammatory and Anti-inflammatory Cytokine Assay: Myocardial protein will be extracted by the homogenization of myocardial tissues in a homogenization buffer

(PBS containing 0.5% Triton X-100 and a protease inhibitor cocktail, pH 7.2 at 4ºC), and subsequently be centrifuged at 14,359g for ten minutes.[18] Then, the supernatant will be collected to measure the level of tumor necrosis factor-α (TNF-α) and interleukin-4 (IL-4) by using an enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Inc., Camarillo, CA, USA).

Transmission Electron Microscopy for Cardiac Mitochondrial Morphology: Cardiac mitochondrial morphology will be determined by transmission electron microscopy. Isolated cardiac mitochondria from both ischemic and remote areas will be fixed overnight by mixing 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 4ºC. Then, the pellet will be postfixed in 1% cacodylate-buffered osmium tetroxide for two hours at room temperature. The pellets will be dehydrated in a graded series of ethanol and embedded in Epon-Araldite and cut by a diamond knife into ultra-thin sections (60-80 nm thick), placed on copper grids and stained with the combination of uranyl acetate and lead citrate. Finally, the mitochondrial morphology will be observed with a transmission electron microscope.[19]

TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay will be performed using One Step TUNEL Apoptosis Assay Kit (Key GNE BioTECH, Nanjing, China) according to the manufacture's protocol. Briefly, cells will be grown I 96-well plates. After hypoxia/reoxygenation (H/R) treatment, cells will be fixed with 4% paraformaldehyde and permeabilized using 0.1% Triton X-100 in 0.1% sodium citrate. Then, cells will be incubated in TUNEL reaction working solution for 1

hr at 37°C, and will be followed by incubation with the fluorescent label DAPI (Beyotime, Haimen, China). The fluorescence will be analyzed using an inverted fluorescence microscope (TE-2000U, Nikon, Japan). TUNEL-positive nuclei will be expressed as the percentage of apoptotic cells (apoptotic nuclei/total nuclei x 100%).

ผลการวิจัย

Low-Amplitude, Left Vagus Nerve Stimulation Significantly Attenuates Ventricular Dysfunction and Infarct Size Through Prevention of Mitochondrial Dysfunction During Acute Ischemia-Reperfusion Injury

BACKGROUND: Right cervical VNS provides cardioprotective effects against acute ischemia-reperfusion injury in small animals. However, inconsistent findings have been reported.

OBJECTIVE: The purpose of this study was to determine whether low-amplitude, left cervical vagus nerve stimulation (VNS) applied either intermittently or continuously imparts cardioprotection against acute ischemia-reperfusion injury.

METHODS: Thirty-two isoflurane-anesthetized swine (25-30 kg) were randomized into 4 groups: Control (sham operated, no VNS), Continuous-VNS (C-VNS, 3.5mA, 20Hz), Intermittent-VNS (I-VNS, continuously recurring cycles of 21-s ON, 30-s OFF), and I-VNS+Atropine (1mg/kg). Left cervical VNS was applied immediately after left anterior ascending artery (LAD) occlusion (60 min), and continued until the end of reperfusion (120 min). The ischemic and non-ischemic myocardium was harvested for cardiac mitochondrial function assessment.

RESULTS: VNS significantly reduced infarct size, improved ventricular function, decreased VF episodes, and attenuated cardiac mitochondrial reactive oxygen species (ROS) production, depolarization and swelling, compared to Control. However, I-VNS produced the most profound cardioprotective effects, particularly infarct size reduction and decreased ventricular fibrillation episodes, compared to Control and C-VNS. These beneficial effects of VNS were abolished by atropine.

CONCLUSIONS: During ischemia-reperfusion injury, both C-VNS and I-VNS provide significant cardioprotective effects compared to Control. These beneficial effects were abolished by muscarinic blockade, suggesting the importance of muscarinic receptor modulation during VNS. The protective effects of VNS could be due to its protection of mitochondrial function during ischemia-reperfusion.

Vagus Nerve Stimulation Initiated Late During Ischemia, but not Reperfusion, Exerts Cardioprotection via Amelioration of Cardiac Mitochondrial Dysfunction

BACKGROUND: We previously reported that vagus nerve stimulation (VNS) applied immediately at the onset of cardiac ischemia provides cardioprotection against cardiac ischemia-reperfusion (I/R) injury.

OBJECTIVES: To determine whether VNS applied later during ischemia or at the onset of reperfusion exerts differential cardioprotection against cardiac I/R injury.

METHODS: Twenty-eight swine (25-30 kg) were randomized into four groups: Control (sham-operated, no VNS), VNS-Ischemia (VNS applied during ischemia), VNS-Reperfusion (VNS applied during reperfusion), and VNS-Ischemia+Atropine (VNS applied during ischemia with 1 mg/kg Atropine administration). Ischemia was induced by left anterior descending coronary artery (LAD) occlusion for 60 minutes, followed by 120 minutes of reperfusion. VNS was applied either 30 minutes after LAD occlusion or at the onset of reperfusion and continued until the end of reperfusion. Cardiac function, infarct size, myocardial levels of connexin43, cytochrome c, tumor necrosis factor-α, interleukin-4 (IL-4) and cardiac mitochondrial function were determined.

RESULTS: VNS applied 30 minutes later after LAD occlusion, but not at reperfusion, markedly reduced ventricular fibrillation incidence and the infarct size (~59%), improved cardiac function, and attenuated cardiac mitochondrial reactive oxygen species production, depolarization, swelling, cytochrome c release, and increased the amount of phosphorylated connexin43 and IL-4, compared to the controls. These beneficial effects of VNS were abolished by Atropine.

CONCLUSIONS: VNS could provide significant cardioprotective effects even when initiated later during ischemia, but was not effective after reperfusion. These findings indicate the importance of timing for VNS initiation, and warrant the potential clinical application of VNS in protecting myocardium at risk of I/R injury.

Vagus Nerve Stimulation Exerts Cardioprotection Against Myocardial Ischemia/Reperfusion Injury Predominantly Through its Efferent Vagal Fibers

BACKGROUND: Vagus nerve stimulation (VNS) has been shown to exert cardioprotection against myocardial ischemia/reperfusion (I/R) injury. However, whether the cardioprotection of VNS are mainly due to direct activation through its ipsilateral efferent fibers (motor) rather than indirect effects mediated by the afferent fibers (sensory) have not been clearly understood.

OBJECTIVES: To determine whether VNS exerts cardioprotection predominantly through its efferent vagal fibers.

METHODS: Thirty swine (30-35 kg) were randomized into 5 groups: I/R no VNS (I/R), and left mid-cervical VNS with both vagal trunks intact (LC-VNS), with left vagus nerve transection (LtVNX), with right vagus nerve transection (RtVNX) and with atropine

pretreatment (Atropine), respectively. VNS was applied at the onset of ischemia (60 minutes) and continued until the end of reperfusion (120 minutes). Cardiac function, infarct size, arrhythmia score, myocardial connexin43 expression, apoptotic markers, oxidative stress markers, inflammatory markers (TNF-α and IL-10) and cardiac mitochondrial function, dynamics and fatty acid oxidation (MFN2, OPA1, DRP1, PGC1α and CPT1) were determined.

RESULTS: LC-VNS exerted cardioprotection against myocardial I/R injury via improvement of mitochondrial function and dynamics and shifted cardiac fatty acid metabolism toward beta oxidation. However, LC-VNS and LtVNX, both efferent vagal fibers are intact, produced more profound cardioprotection, particularly infarct size reduction, decreased arrhythmia score, oxidative stress and apoptosis and attenuated mitochondrial dysfunction compared to RtVNX. These beneficial effects of VNS were abolished by atropine.

CONCLUSIONS: Our findings suggest that selective efferent VNS may potentially be effective in attenuating myocardial I/R injury. Moreover, VNS required the contralateral efferent vagal activities to fully provide its cardioprotection.

เนื้อหางานวิจัย

บทที่ 1: Low-Amplitude, Left Vagus Nerve Stimulation Significantly Attenuates Ventricular Dysfunction and Infarct Size Through Prevention of Mitochondrial Dysfunction During Acute Ischemia-Reperfusion Injury

บทนำ

Acute myocardial infarction is one of the leading causes of death worldwide.[20] Early and successful myocardial reperfusion with either thrombolytic therapy or primary percutaneous coronary intervention (PCI) is the most effective modality for reducing the infarct size and improving clinical outcomes.[1] However, the process of abruptly restoring blood flow to the ischemic tissue during PCI may result in a devastating cascade of biological processes resulting in the production of several toxic compounds.[2, 21] This phenomenon, so-called myocardial ischemia-reperfusion injury, can paradoxically reduce the beneficial effects of myocardial reperfusion.[2] Therefore, myocardial ischemia-reperfusion injury is considered a major concern in patients with acute myocardial infarction or those undergoing coronary artery bypass grafting (CABG) and transplantation.[3, 4]

Over the past decade, vagus nerve stimulation (VNS) has been shown to exert cardioprotection in both chronic heart failure and ischemic heart diseases.[5-10] In ischemic hearts, the effects of VNS have been shown to improve cardiac function, limit dispersion of repolarization, prevent reperfusion injury, attenuate cardiac remodeling, improve defibrillation efficacy, and decrease the infarct size.[11, 12] Despite these well-documented beneficial effects of VNS, inconsistent findings have been reported.

Currently, more than 100,000 patients worldwide have already been implanted with the left cervical VNS system for suppression of epilepsy and depression.[22] Left cervical VNS is preferred over the right because of the greater number of cardiac efferent fibers from the right vagus nerve[23], whose stimulation may elicit more frequent undesireable effects. Furthermore, the effect of low-amplitude VNS on cardiac mitochondria has been rarely investigated. Since cardiac mitochondria have been indicated as a major determinant of both cardiac cell survival and arrhythmias, it is important that the role of left cervical VNS on cardiac mitochondria during ischemia-reperfusion be explored. Therefore, in the present study we sought to determine whether left cervical VNS applied either intermittently or continuously imparts cardioprotection against acute ischemia-reperfusion injury in a swine model.

วิธีการทดลอง

Animal preparation

All experiments were approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Thirty-two domestic pigs (25-30 Kg) were anesthetized by intramuscular injection of a combination of 4.4 mg/kg zoletil® (Vibbac Laboratories, Carros, France) and 2.2 mg/kg xylazine (Laboratorios Calier, S.A., Barcelona, Spain). After endotracheal intubation, anesthesia was maintained by 1.5–3.0% isoflurane (Abbott Laboratories Ltd., Queenborough, UK) delivered in 100% oxygen. Surface electrocardiogram (lead II), femoral arterial blood pressure (BP), heart rates (HR), rectal temperature were continuously monitored and all data were recorded for subsequent analysis. Arterial

blood gases and electrolytes were also monitored every 30 min and maintained within acceptable physiological ranges.[13] Platinum coated titanium coil electrodes (34- and 68-mm) were used to deliver electrical shocks.[13] The chest was opened through a left thoracotomy. The left anterior descending artery (LAD) was isolated and occluded by ligature (3-0 silk) 3 centimeters from the left main coronary artery. Complete LAD occlusion was maintained for 60 min and then reperfusion was allowed for the next 120 min.

Vagus nerve stimulation protocol

The left vagus nerve was surgically isolated (~ 3 cm, C5-6 level) from the carotid sheath. A VNS lead (Model 304, Cyberonics) with bipolar electrodes (platinum-iridium, 4 mm^2 surface area, 6-mm interelectrode spacing) was attached to the vagus nerve using helical fixation elements to assure electrode stability. The cathodic electrode was oriented closest to the heart. The proximal terminal pin of VNS lead was attached to a pulse generator (Demipulse, Model 103, Cyberonics) which was used to deliver electrical stimulation during VNS. At the beginning of the study, the mean PR interval was determined from an average of 10 sinus beats. Then, the 3.5 mA with 500 μs pulse width and 20 Hz (based on the FDA approved for clinical use in epilepsy treatment) current was set for the left cervical VNS.[14] This left VNS was sufficient to produce a 5-10 ms increase in PR intervals, and that this effect of VNS was completely disappeared as indicated by the return of the PR interval to the pre-stimulation values within 20 seconds after the cessation of VNS. Pigs were randomly divided into 4 groups (n = 8/group, Figure 1). All pigs in each group underwent 60-min ischemia

followed by 120-min reperfusion. Group 1 was the control (sham operated) group without VNS. Group 2 received continuous left cervical VNS (C-VNS, 3.5 mA amplitude, 500 μs pulse duration, 20 Hz pulse frequency) immediately after LAD occlusion and continued until the end of reperfusion. Group 3 received intermittent left cervical VNS (I-VNS, continuous recurring cycles of 21-sec ON and 30-sec OFF) immediately after LAD occlusion and continued until the end of reperfusion. Group 4 was done similar to group 3 except that atropine (1 mg/kg) was intravenously administered 15 min prior to left cervical VNS protocol to block parasympathetic actions on the heart.[24] During the ischemia-reperfusion study in each pig, the cardiac function was continuously monitored and recorded using the pressure-volume (P-V) loop recording system (Model ADV500/ADVantage System, Scisense Inc., London, Canada) as described previously.[16] For ECG analysis, the mean baseline PR interval was determined from an average of 10 sinus beats just prior to LAD occlusion. The mean PR intervals during the ischemia and reperfusion periods were analyzed from an average of 10 consecutive beats before the end of occlusion and the end of reperfusion, respectively.

Evaluation of rhythm disturbances

Premature ventricular contractions (PVCs), ventricular tachycardia (VT), and ventricular fibrillation (VF) were defined according to the Lambeth convention criteria[25] with more rigorous modifications. Specifically, PVCs was defined as ventricular contraction without atrial depolarization. VT was defined as more than six consecutive

PVCs. VF was characterized by a loss of synchronicity of electrocardiogram plus decreased amplitude and a precipitous fall in blood pressure (BP) for >1 s.

Infarct size determination

At the end of each experiment, the heart was removed and irrigated with normal saline to wash out blood from chambers and vessels. The infarct size was assessed with 0.5% Evans Blue and 1.0% Triphenyltetrazolium Chloride (TTC) staining as previously described.[13] An area measurement was performed using Image Tool software version 3.0. The mass-ratio of the area at risk (AAR) to total ventricular mass, and the infarct size normalized to the AAR, were calculated.

Isolated cardiac mitochondria study protocol.

Cardiac mitochondria were isolated from the ischemic and non-ischemic regions, using the technique as previously described[26], and the protein concentration was determined according to the bicinchoninic acid assay. Isolated cardiac mitochondrial morphology was confirmed by using a transmission electron microscope. The measurement of reactive oxygen species (ROS) production and mitochondrial membrane potential changes ($\Delta\Psi_m$) were determined using a fluorescent microplate reader in all groups as previously described.[26] In short, dichlorohydro-fluorescein diacetate (DCFDA) dye was used to determine the level of ROS production in cardiac mitochondria. The DCFDA could pass through the mitochondrial membrane, and was oxidized by ROS in the mitochondria into DCF. The dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide (JC-1) was used to determine the changes

in the mitochondrial membrane potential. JC-1 was characterized as a cation and remained in the mitochondrial matrix as a monomer (green fluorescence) form. However, it could interact with anions in the mitochondrial matrix to form an aggregate (red fluorescence) form. Cardiac mitochondrial depolarization was indicated by a decrease in the red/green fluorescence intensity ratio. Moreover, isolated mitochondrial swelling was assessed by measuring changes in the absorbance of the suspension wavelength at 540 nm using a microplate reader. Cardiac mitochondria (0.4 mg/ml) were incubated in 2 ml of respiration buffer: 150 mM KCl, 5 mM HEPES, 5 mM $K_2HPO_4.3H_2O$, 2 mM L-glutamate and 5 mM pyruvate sodium salt. Mitochondrial swelling is associated with decreasing absorbance.

Statistical Analysis

All data are expressed as mean ± standard deviation. The normality and equality of variance were tested using the Shapiro-Wilk test and Levene's test, respectively. The mean values between two groups were compared using the unpaired Student's *t*-test. One-way ANOVA with Dunnett's multiple-comparison test using the statistical program SPSS17 (SPSS, Inc., Chicago, IL, USA) was used for multiple sets of data. The level of significance for all statistical tests was $P<0.05$.

ผลการทดลอง

Effect of VNS on ECG parameters and LV function during ischemia-reperfusion period

The electrophysiological effects of VNS were examined in thirty-two pigs where HR, PR interval and LV function were measured continuously during the ischemia-reperfusion period. In the control group, HR during the ischemic period significantly increased when compared with the baseline (Figure 2A). Interestingly, HR at baseline and during ischemia and reperfusion periods were not different in VNS treated groups (n=8/group) either in the presence or absence of muscarinic blockade by atropine, indicating that the effects of VNS prevented increased HR during ischemia-reperfusion injury independent of muscarinic receptor activation. Nevertheless, while statistically there is a difference in HR (Figure 2A), it is possible that physiologically there appears to be no difference between groups and conditions. PR interval during the baseline, ischemic and reperfusion periods was not different in Control, C-VNS and I-VNS groups (Figure 2B). However, PR intervals during ischemic and reperfusion periods were significantly reduced in the I-VNS+Atropine group, suggesting that sympathetic activity might be relatively increased by muscarinic blockade. The effect of VNS on LV functional performance is shown in Table 1. In the Control group, the stroke volume (SV) and the ejection fraction (EF) were significantly decreased during the ischemia and reperfusion periods compared with the baseline. The end-diastolic pressure (EDP) was significantly increased during the ischemia and reperfusion periods compared with the baseline. C-VNS attenuated the decline in SV and EF only during the reperfusion period. Interestingly, I-VNS preserved LV functional performance during the ischemia

and reperfusion periods. However, the beneficial effect of VNS was completely abolished by the administration of atropine.

VNS and the occurrence of cardiac arrhythmia during ischemia-reperfusion injury

Figure 3A shows examples of ECG tracings at baseline and after LAD occlusion. Premature ventricular contractions (PVCs) were markedly decreased for both C-VNS and I-VNS groups during ischemic and reperfusion periods, compared with the Control group (Figure 3B). This effect was significantly reduced by atropine, indicating that VNS reduces the frequency of spontaneous PVCs during the ischemic and reperfusion periods through a pathway that at least partially involves muscarinic receptor activation. Moreover, the number of ventricular tachycardia/ventricular fibrillation (VT/VF) episodes was significantly reduced in the I-VNS group during the reperfusion period, whereas I-VNS+Atropine significantly increased the number of VT/VF during ischemic and reperfusion periods compared with the I-VNS group (Figure 3C). However, time to VT/VF onset was not significantly different among groups during both ischemic and reperfusion periods (Figure 3D). In the present study, PVCs did not return to control values with atropine, whereas VT/VF did return to control values, suggesting that some other factors might suppress PVCs during reperfusion with VNS, and that PVCs might not be related to VT/VF occurrence during reperfusion with VNS.

Myocardial infarct size

Myocardial infarct size was expressed as the percentage of the area at risk (AAR). The AAR, expressed as a percentage of total ventricular mass, was not

different among groups (Control 32.8±4.9%, C-VNS 37.1±2.0%, I-VNS 35.6±5.8% and I-VNS+Atropine 38.1±4.7%; *P*=NS). C-VNS significantly reduced mean infarct size by 60%, whereas and I-VNS significantly reduced mean infarct size by 89%. The mean infarct size reduction for I-VNS was 48% more than that observed for C-VNS. Administration of atropine totally abolished the cardioprotective effects of VNS with respect to infarct size (Figure 4).

Effect of VNS on cardiac mitochondria after ischemia-reperfusion period

Cardiac mitochondrial dysfunction including increased ROS production, mitochondrial membrane depolarization and mitochondrial swelling has been shown to participate in myocyte dysfunction and degradation of cardiac contractile performance, arrhythmias, and myocytes apoptosis and infarct size during and subsequent to ischemia-reperfusion injury. Our results demonstrated that both C-VNS and I-VNS decreased cardiac mitochondrial ROS production (Figure 5A) and prevented cardiac mitochondrial membrane depolarization (Figure 5B) and cardiac mitochondrial swelling (Figure 5C) in the ischemic myocardium, compared with the Control group. Again, these beneficial effects on cardiac mitochondria were abolished by atropine, suggesting the importance of muscarinic receptor activation during VNS. However, mitochondrial ROS production (Figure 5A) and mitochondrial membrane depolarization (Figure 5B) were partially reversed by atropine in the VNS treated groups, suggesting the presence of an additional non-muscarinic modulation. Electron photomicrographs demonstrated that in the ischemic area of the control group, ischemia-reperfusion induced severe mitochondrial damage as indicated by the loss of cristae (Figure 6). Interestingly, the

cardiac mitochondria in the ischemic myocardium in both C-VNS and I-VNS groups were significantly preserved following ischemia-reperfusion injury, and this effect was not present following administration of atropine (Figure 6).

อภิปรายผลการทดลอง

To the best of our knowledge, this is the first demonstration that I-VNS provides more robust efficacy than C-VNS with respect to the prevention of cardiac mitochondrial dysfunction during ischemia-reperfusion period. Moreover, our finding confirms previous reports[7, 12, 27, 28], which also indicated that VNS exerted cardioprotection and improved LV function during ischemia-reperfusion injury. Our findings on the better efficacy of I-VNS than C-VNS suggests that intermittent VNS may prevent adaptation of neural structures involved in cardioprotection, sometimes referred to as “fade”.[29] In the present study, our finding that VNS significantly decreased the occurrence of PVCs and the number of spontaneous VT/VF episodes is consistent with previous studies that VNS exerted the anti-arrhythmic effects during ischemia-reperfusion period. Waxman and colleagues[30] provided the first evidence that some ventricular tachycardia could be modulated by vagal activation, and that ventricular automaticity was reduced by enhanced vagal tone. Moreover, an elegant study by Schwartz and colleagues in conscious dogs clearly demonstrated that enhanced vagus nerve activity by means of right VNS prevented spontaneous ventricular tachyarrhythmias in a model with healed MI, exercise testing and intermittent ischemia.[7] In the present study, left cervical VNS exerted cardioprotective effects without HR alteration, supporting that the benefits of VNS could be independent of HR changes. Recently, left-sided low-level VNS has

been shown to suppress stellate ganglion nerve activity and reduce the incidences of paroxysmal atrial tachyarrhythmias in ambulatory dogs.[31] In addition, left-sided low-level VNS has been shown to upregulate small conductance calcium activated potassium channels in the stellate ganglion.[32] These changes might be the mechanistic insight underlying the antiarrhythmic effect of low-level VNS.

In the present study, our finding that VNS preserved LV functional performance is consistent with previous studies. In rats subjected to global ischemia-reperfusion with intact vagal innervation, right cervical VNS-treated LV showed significantly better performance throughout the 120-min reperfusion period, and that VNS exerted a marked anti-infarct effect irrespective of the heart rate compared with sham stimulation.[33] Thus, the preserved LV function provided by VNS might exert through a reduction in the myocardial infarct size. In the present study, both C-VNS and I-VNS significantly reduced myocardial infarct size by 60% and 89%, respectively. This finding is consistent with previous both *in vitro* and *in vivo* studies showing that increased vagus nerve activity mainly via right cervical VNS reduced the ratio of infarct size to the area at risk, suggesting that VNS has an anti-apoptotic effect in myocardium during and after the ischemia-reperfusion period. Kakinuma and colleagues[27] have reported that infarct size was significantly reduced by 25% in right cervical VNS-treated rat hearts. Moreover, in a global ischemia-reperfusion rat model (30-minute of ischemia and 120-minute of reperfusion) with an intact vagal innervation, the right-sided VNS has been shown to exert a marked anti-infarct effect irrespective of the HR.[33] Furthermore, early VNS has been shown to reduce infarct size and ventricular remodeling by inhibiting the

increase in myocardium interstitial myoglobin[34], norepinephrine[35], and matrix metalloproteinase levels[36] associated with myocardial ischemia-reperfusion injury. Recently, it has been shown that the “efferent VNS” could inhibit the myocardial ischemia-reperfusion injury mainly through its nicotinic inhibition.[12] Although we did not determine the effects of right VNS, a large number of the experimental studies have been reported the cardioprotective effects of the right VNS, including infarct size and arrhythmia reduction.[37, 38] Therefore, right-sided VNS could have provided similar benefits as observed in the present study. However, left-sided VNS would be less likely to interfere the heart rate.

In the present study, we have shown that one potential possible mechanism of the pronounced cardioprotective effect of VNS during ischemia-reperfusion could be due to its effects on the cardiac mitochondria. Increased ROS production and oscillation of the mitochondrial membrane potential ($\Delta\Psi_m$) have been shown to play a crucial role in the genesis of cardiac arrhythmias and myocardial infarction.[39] Our data indicate that mitochondrial membrane potential is stabilized by VNS, thereby protecting mitochondria from aberrant changes in membrane potential. This could lead to the reduction of arrhythmias occurred during ischemia-reperfusion in the VNS group. Moreover, mitochondrial ROS reduction and decreased mitochondrial swelling could be responsible for decreased infarct size in the VNS group. These beneficial effects of VNS were abolished by atropine, suggesting a dominant muscarinic receptor involvement.

Conclusions and Study Limitation

We have thus provided compelling evidence that during acute ischemia-reperfusion injury, both C-VNS and I-VNS provide significant cardioprotection and improve LV function in a large animal model of ischemia-reperfusion injury. However, left I-VNS provides more robust efficacy than left C-VNS with respect to infarct size reduction and reperfusion arrhythmia prevention. A potential mechanism of such cardioprotection of VNS is associated with its prevention of cardiac mitochondrial dysfunction during ischemia-reperfusion. Thus, these data strongly support the notion that VNS is emerging as a promising therapeutic modality in combination with reperfusion therapy for protecting myocardium at risk of ischemia-reperfusion injury due to coronary artery disease. There are several limitations in the present study. Our study was conducted in anesthetized healthy animals, whereas most victims of cardiac arrest have significant coronary lesions. Furthermore, VNS treatment was conducted immediately after LAD occlusion. The time schedule of treatment in this experimental study may differ from the clinical condition.

Table 1. Effects of VNS and Muscarinic Receptor Inhibition on Pressure-Volume (P-V) Loop Derived Functional Parameters

	Control			C-VNS			I-VNS			I-VNS + Atropine		
	Baseline	**Ischemia**	**Reperfusion**	**Baseline**	**Ischemia**	**Reperfusion**	**Baseline**	**Ischemia**	**Reperfusion**	**Baseline**	**Ischemia**	**Reperfusion**
SV (ml)	24±1	8±2*	8±2*	23±2	10±2*	16±6	22±2	15±5	19±7	24±3	10±3*	10±1*
EF (%)	55±6	26±6*	26±4*	46±4	28±2*	35±8	52±5	38±6	42±7	54±4	27±7*	29±8*
ESP (mmHg)	85±9	77±6	87±6	84±4	75±4	82±3	76±6	69±3	72±5	83±4	74±3	79±3
EDP (mmHg)	14±4	22±2*	24±5*	17±8	23±6*	25±5*	13±3	15±3	16±2	12±2	21±2*	22±2*

Summary of left ventricular (LV) functional parameters at baseline, at the end of ischemia, and at the end of reperfusion (n = 8/group). Values are mean±SD. *$P < 0.05$ vs baseline. VNS= vagus nerve stimulation; C-VNS; continuous-VNS; I-VNS= intermittent-VNS; SV = stroke volume; EF = ejection fraction; ESP = end-systolic pressure; EDP = end-diastolic pressure

Figure 1: **Schematic representation of the study protocols.** The ischemic period (60 min) was induced by rapid, complete ligation of the left anterior descending coronary artery (LAD). The reperfusion period was 120 min in duration. In Group 1, the pigs were sham operated and then subjected to ischemia (60 minutes) and reperfusion (120 minutes) without VNS. In Groups 2-4, VNS was initiated immediately after the LAD ligation and continued until the end of reperfusion. In Group 4, pigs were given a bolus injection intravenously of atropine (1 mg/kg) 15 minutes prior to initiation of VNS.

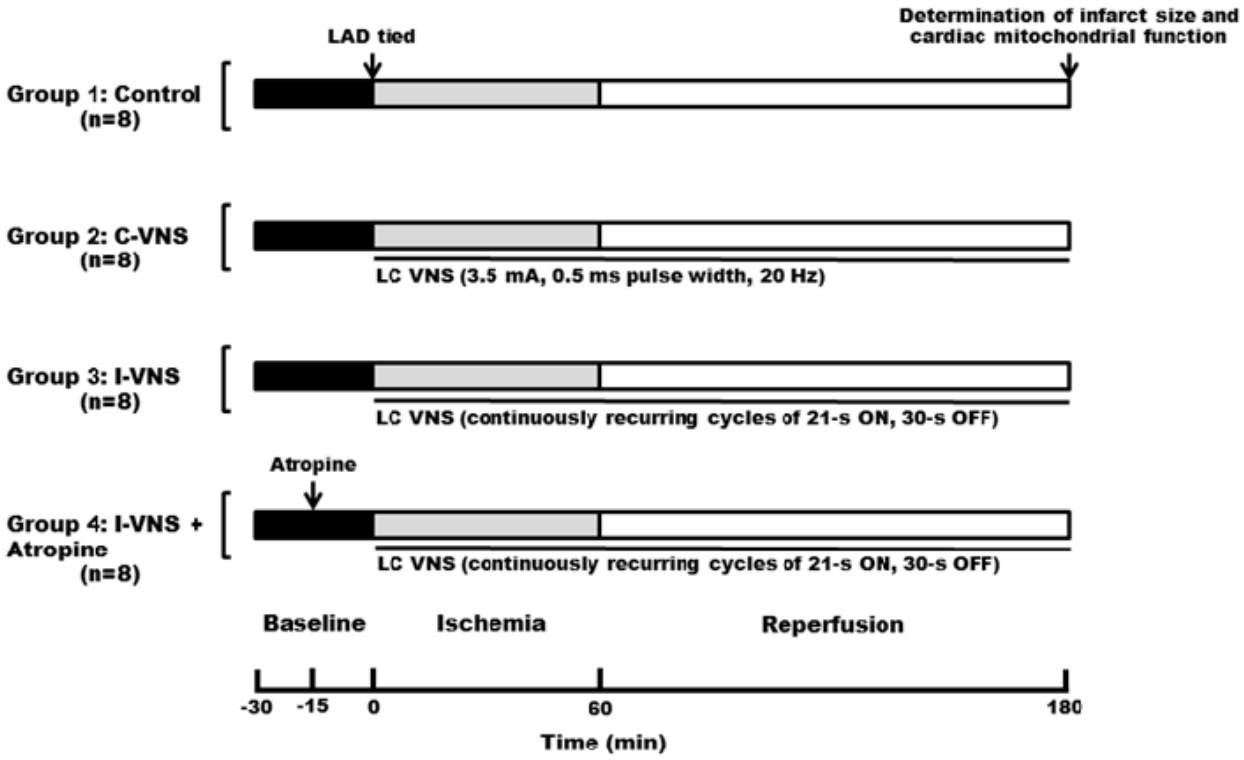


Figure 2: **Effect of vagus nerve stimulation (VNS) on heart rate and PR interval during ischemia-reperfusion period**. In the Control group, heart rate during the ischemic period was significantly increased when compared with the baseline period. Interestingly, heart rate during baseline and during ischemia and reperfusion periods were not different in VNS treated groups (n=8/group), indicating that VNS prevented the increase in HR during the ischemic period independent of muscarinic receptor activation (panel A). Panel B, mean PR interval during the baseline, ischemic and reperfusion periods was not different in Control, C-VNS and I-VNS groups. However, PR intervals during ischemic and reperfusion periods were significantly reduced in the I-VNS+Atropine group (panel B). Data are presented as mean±SD. *P < 0.05 vs baseline. C-VNS = Continuous-VNS; I-VNS = Intermittent-VNS.

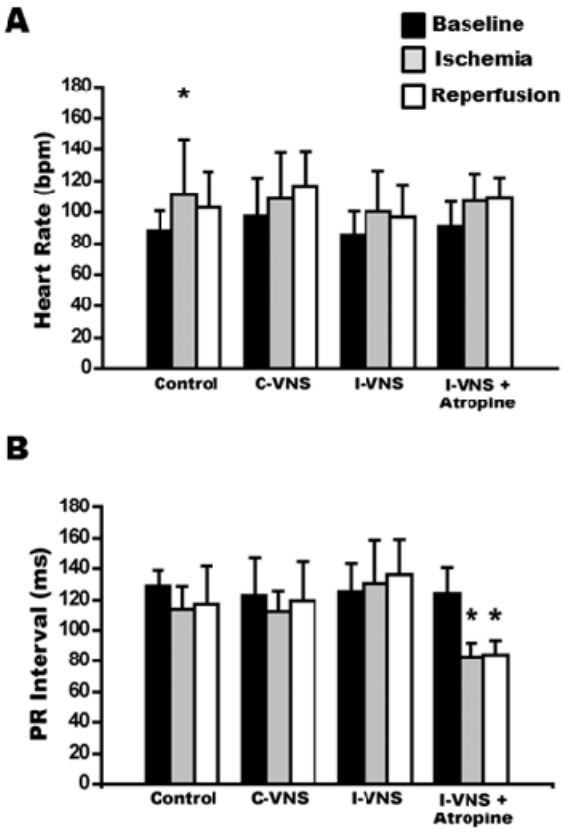


Figure 3: **VNS and the occurrence of cardiac arrhythmia during ischemia-reperfusion injury.** The ECG (lead II) recorded before and after LAD occlusion (panel A). PVCs were markedly decreased in both C-VNS and I-VNS groups during the ischemic and reperfusion periods, compared with Control group (panel B). This effect was attenuated by atropine, indicating that VNS prevented formation of PVCs during the ischemic and reperfusion periods through muscarinic receptor activation. The number of VT/VF episodes was significantly reduced in I-VNS group during the reperfusion period,whereas, I-VNS+Atropine significantly increased the number of VT/VF episodes during ischemic and reperfusion periods, compared with I-VNS group (panel C). However, time to VT/VF episode onset was not significantly different between groups during ischemic and reperfusion periods (panel D). Data are presented as mean±SD. **P* < 0.05 vs control, †*P* < 0.05 vs I-VNS. C-VNS = Continuous-VNS; I-VNS = Intermittent-VNS; PVC = premature ventricular contraction; VT = Ventricular tachycardia; VF = Ventricular fibrillation.

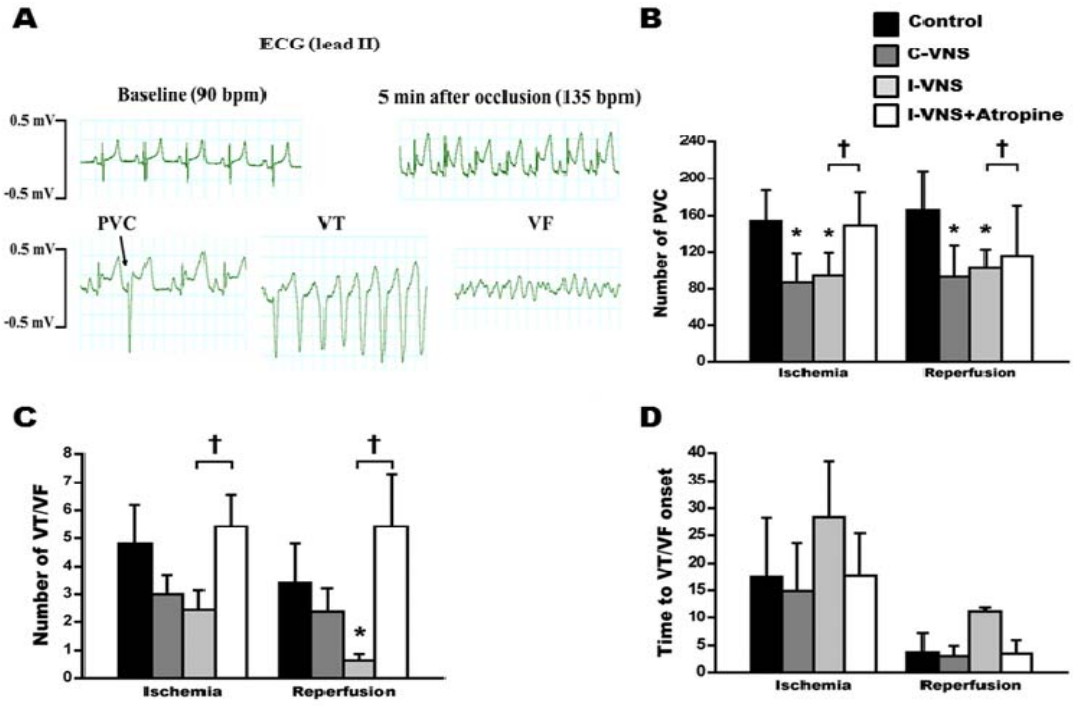


Figure 4: **Determination of myocardial infarct size.** Myocardial infarct size (IS) was expressed as the percentage of area at risk (AAR). Regional myocardial blood flow during ischemia and the AAR, expressed as a percentage of total ventricular mass, were not different between groups (control 32.8±4.9%, C-VNS 37.1±2.0%, I-VNS 35.6±5.8% and I-VNS+atropine 38.1±4.7%; *P*=NS). Interestingly, VNS significantly reduced myocardial IS and this effect was reversed by atropine. Control (45.7±14.2%, n=7); C-VNS (18.5±10.5%, n=8); I-VNS (5.1±3.1%, n=6); I-VNS+Atropine (52.9±8.7%, n=6). Representative pictures after Evan Blue (Sigma-Aldrich) and triphenyltetrazolium chloride staining are shown in the inset. Blue indicates non-threatened myocardium, red indicates the non-infarcted area within the area at risk, and white indicates myocardial infarction. Data are presented as mean±SD. **P* < 0.05 vs control, †*P* < 0.05 vs C-VNS, and ‡*P* < 0.05 vs I-VNS. C-VNS = Continuous-VNS; I-VNS = Intermittent-VNS; NS = Not significant.

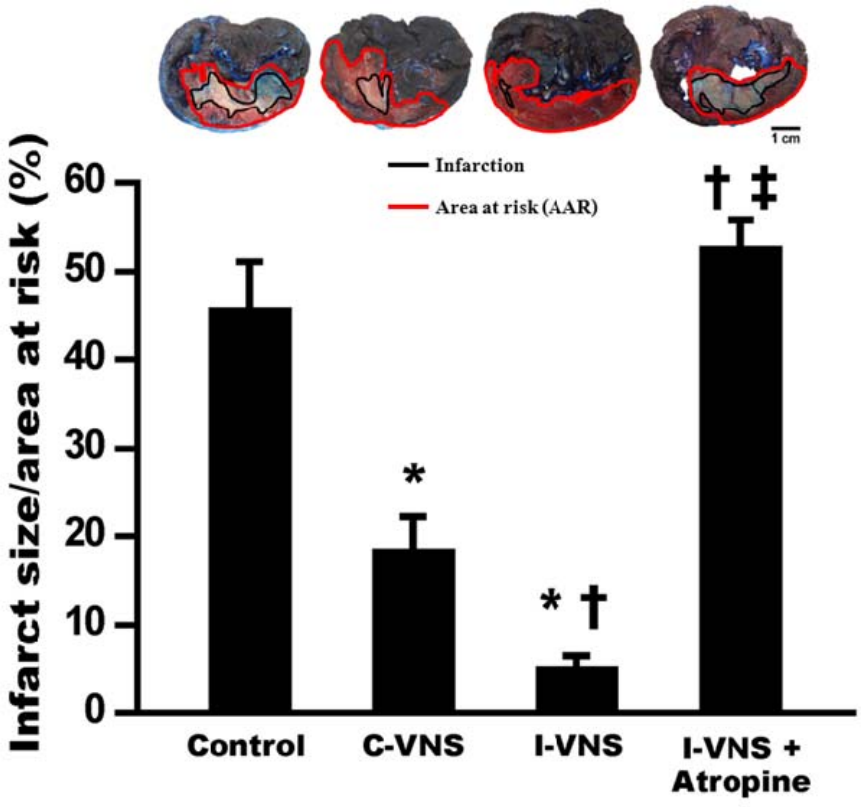


Figure 5: **Effect of VNS on cardiac mitochondria after ischemia and reperfusion periods.** Both C-VNS and I-VNS decreased mitochondrial ROS production (panel A) and prevented mitochondrial membrane depolarization (panel B) and mitochondrial swelling (panel C) in the ischemic myocardium, compared with the Control group. Again, these effects were diminished by atropine, suggesting the importance of muscarinic modulation during VNS. However, mitochondrial ROS production (panel A) and mitochondrial membrane depolarization (panel B) were only partially attenuated by atropine in the VNS treated groups, suggesting the presence of additional non-muscarinic modulation. Data are presented as mean±SD. **P* < 0.05 vs control, †*P* < 0.05 vs I-VNS. C-VNS = Continuous-VNS; I-VNS = Intermittent-VNS.

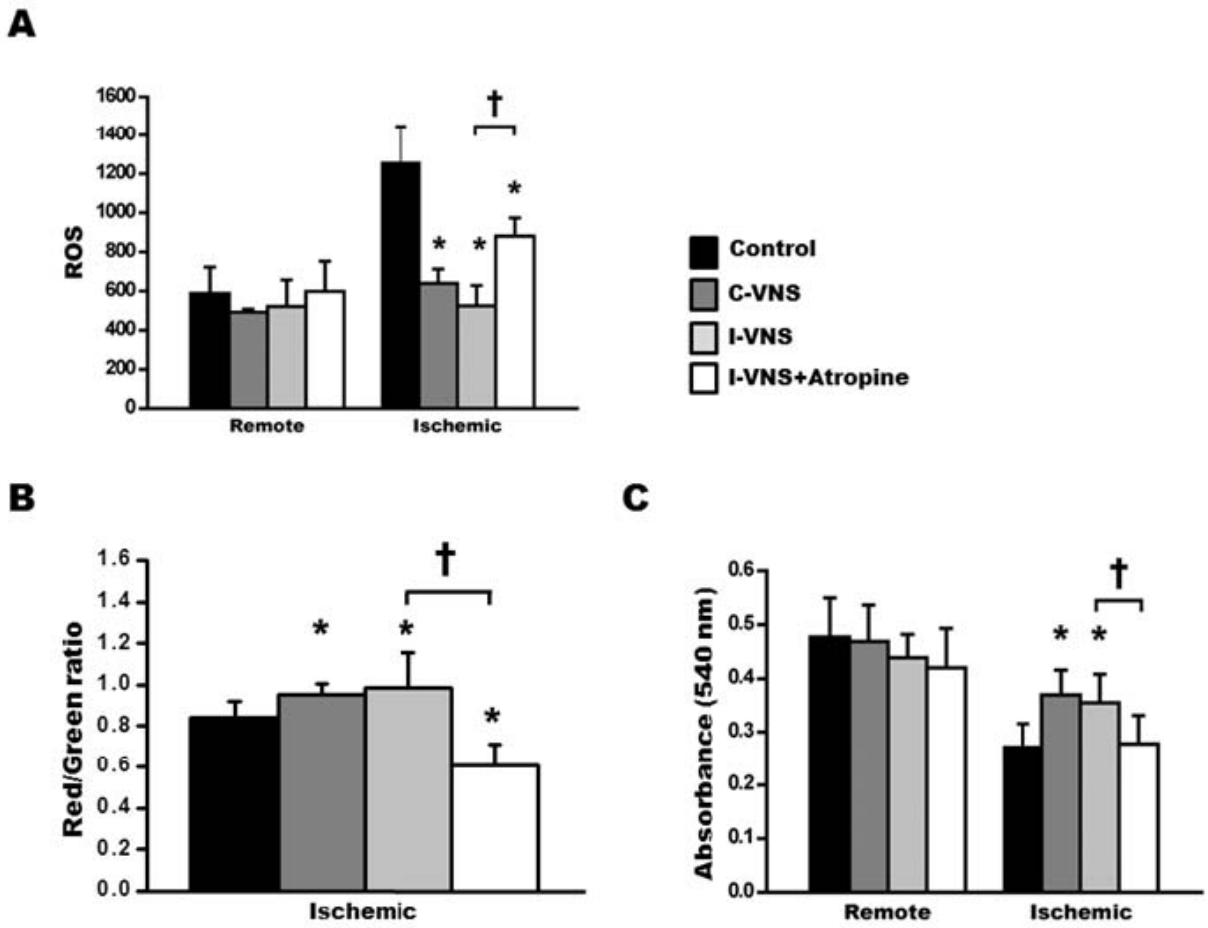
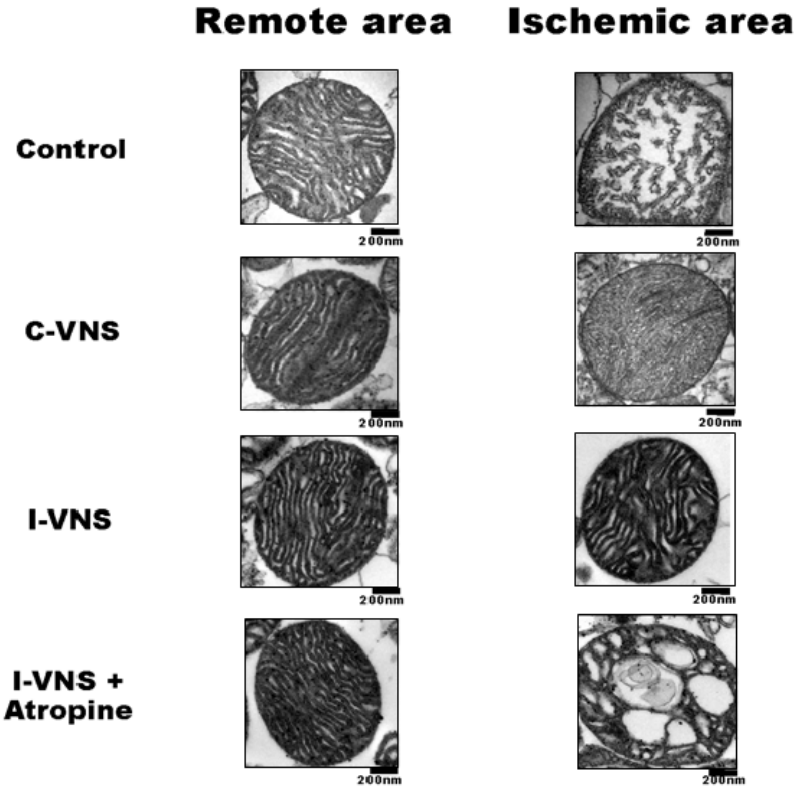


Figure 6: Representative electron photomicrographs of cardiac mitochondrial ultrastructure. In ischemic area of the Control group, ischemia-reperfusion induced severe mitochondrial damage was observed. Note mitochondrial swelling accompanied by disruption in membrane integrity. Whereas, both C-VNS and I-VNS significantly protected cardiac mitochondrial swelling following ischemia-reperfusion injury and this effect was abolished by atropine. C-VNS = Continuous-VNS; I-VNS = Intermittent-VNS.



บทที่ 2: Vagus Nerve Stimulation Initiated Late During Ischemia, but not Reperfusion, Exerts Cardioprotection via Amelioration of Cardiac Mitochondrial Dysfunction

บทนำ

Acute myocardial infarction (MI) is a major cause of morbidity and mortality worldwide.[20] In MI patients, timely and effective myocardial reperfusion with either thrombolytic therapy or primary percutaneous coronary intervention (PCI) is the most effective modality for salvaging viable myocardium.[1] However, the process of abruptly restoring blood flow to the ischemic tissues can itself induce further death of the myocardium, a phenomenon known as myocardial ischemia-reperfusion (I/R) injury.[2, 21] Although advances in PCI technology, reduced procedure times and appropriate use of pharmacological agents (antiplatelet and antithrombotic) for maintaining coronary flow have improved outcomes, attenuation of myocardial reperfusion injury remains unresolved.[40]

The autonomic nervous system has been shown to play an important role in the regulation of cardiac function.[41] During acute myocardial I/R injury, cardiac adrenergic receptor activation contributes to cellular dysfunction that, if untreated, promotes degradation of cardiac performance and upregulation of apoptotic signaling in affected cardiac myocytes.[42, 43] Over the past decade, chronic, intermittent vagus nerve stimulation (VNS) has been shown to be feasible in humans and exerts cardiprotective effects in both chronic ischemic cardiomyopathy and heart failure.[9, 10, 44] Specifically, in ischemic hearts, VNS applied before ischemia has been shown to limit the infarct size

and attenuate I/R injury by its nicotinic pathway independent from heart rate changes.[12] Furthermore, we recently demonstrated that VNS applied immediately at the onset of ischemia markedly reduced the infarct size, improved ventricular function, and caused a dramatic reduction in ventricular tachycardia/ventricular fibrillation (VT/VF) episodes.[15] However, a direct translation of these experimental observations to clinical reality is hampered by the fact that, in acute MI patients, treatment is typically initiated only after the onset of ischemia. In the present study, we hypothesized that VNS applied late after myocardial ischemia but prior to reperfusion exerts cardioprotective effects against I/R injury. Furthermore, we also determined whether VNS applied at the onset of reperfusion exerted differential cardioprotection against I/R injury.

วิธีการทดลอง

Detailed methods are available in the Online Appendix. Briefly, twenty-eight swine (25-30 kg) were randomized into four groups (Figure 7A): Control (sham operated, no VNS), VNS-Ischemia (VNS applied during ischemia), VNS-Reperfusion (VNS applied during reperfusion), and VNS-Ischemia+Atropine (VNS applied during ischemia with 1 mg/kg Atropine administration). Ischemia was induced by left anterior descending coronary artery (LAD) occlusion for 60 minutes, followed by 120 minutes of reperfusion. VNS (3.5 mA, 20 Hz, continuously recurring cycles of 21-s ON, 30-s OFF) was applied either 30 minutes after LAD occlusion or at the onset of reperfusion and continued until the end of reperfusion. A bipolar pacing lead and anchor lead were placed around the mid-cervical left vagus nerve (Figure 7B). During the ischemic and reperfusion periods, arrhythmia incidence and left ventricular function were continuously

monitored and recorded by using ECG and the pressure-volume (P-V) loop recording system as previously described.[15] After 120 minutes of reperfusion, heart was removed and sectioned for infarct size, cardiac mitochondrial function and western blot analysis. Data were presented as mean ± standard deviation. A value of $P<0.05$ was considered statistically significant. All experiments were approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

ผลการทดลอง

Effect of VNS on heart rate and ECG parameters during the ischemia-reperfusion period

The electrophysiological effects of VNS were examined in twenty-eight pigs where HR and PR interval were measured continuously during the I/R period. In the Control and VNS-Reperfusion groups, HR during the ischemic period significantly increased when compared with the baseline (Figure 8A). When VNS was applied during ischemia, HR was not different than baseline HR. However, in the VNS-Ischemia+Atropine group, HR during ischemic and reperfusion periods increased, suggesting that sympathetic activity might be relatively increased by a muscarinic blockade. Nevertheless, while statistically there was a difference in HR (Figure 8A), it is possible that physiologically there appears to be no difference between groups and conditions. There was no significant difference in the PR interval during the baseline, ischemic and reperfusion periods among groups (Figure 8B). However, PR intervals during ischemic and reperfusion periods were significantly reduced in the VNS-

Ischemia+Atropine group, suggesting a relative sympathetic predominance after the muscarinic blockade. Mean QRS duration during the baseline, ischemic and reperfusion periods was not different among groups (Figure 8C). QT intervals during ischemia and reperfusion were significantly decreased in all groups (Figure 8D). However, during ischemia and reperfusion, time from T-wave peak to end (Tpe) was significantly decreased only in VNS-Ischemia group and this effect was abolished by Atropine (Figure 8E and 8F).

VNS applied during ischemia protected the heart against reperfusion arrhythmias by increasing Cx43 phosphorylation

Figure 9A represents a PVC initiated self-terminating ventricular tachycardia in four different groups. However, the PVC and VT cycle lengths were not different among groups (Figure 9B). The total number of PVCs was markedly decreased in both VNS-Ischemia and VNS-Reperfusion groups, compared with the Control group (Figure 10A). This effect was abolished by Atropine, indicating that VNS reduced the frequency of spontaneous PVCs during the ischemic and reperfusion periods through muscarinic receptor activation. Moreover, the total number of VT/VF episodes was significantly reduced in the VNS-Ischemia group, whereas Atropine administration abolished the antiarrhythmic effect of VNS (Figure 10B). However, the time to spontaneous VT/VF onset was not significantly different among groups during both ischemic and reperfusion periods (Figure 10C). Figure 4D shows the effect of VNS on connexin43 phosphorylation. Quantitative densitometric analysis revealed that the levels of connexin43 phosphorylation at serine368 residue in the VNS-Ischemia and VNS-

Reperfusion groups were significantly increased compared with the Control group (Figure 10D). Interestingly, Atropine inhibited the increased connexin43 phosphorylation in the VNS-Ischemia+Atropine group, suggesting that the level of phosphorylated connexin43 during acute I/R period was enhanced by muscarinic receptor activation.

VNS applied during ischemia preserved LV function and reduced infarct size

The effect of VNS on LV contractile function is shown in Table 2. In the Control group, the stroke volume (SV) and the ejection fraction (EF) were significantly decreased, and the end-diastolic pressure (EDP) was significantly increased during the I/R periods, compared with the baseline. VNS applied during ischemia, but not at the onset of reperfusion, preserved LV functional performance during the ischemia and reperfusion periods. However, the beneficial effect of VNS was completely abolished by the administration of Atropine. Figure 11 illustrates the effect of VNS on the infarct size. Myocardial infarct size was expressed as the percentage of the area at risk (AAR). The AAR, expressed as a percentage of total ventricular mass, was not different between groups (Control 32.8±5%, VNS-Ischemia 33.5±5%, VNS-Reperfusion 34.2±1% and VNS-Ischemia+Atropine 38.9±4%; *P*=NS). Interestingly, VNS applied during ischemia, but not at the onset of reperfusion, significantly reduced myocardial infarct size (mean of 59%) and this effect was completely abrogated by Atropine.

VNS applied during ischemia protected cardiac mitochondrial integrity by mitigating cytochrome c release

Cardiac mitochondrial dysfunction including increased ROS production, decreased the red/green fluorescent intensity ratio (which indicated mitochondrial membrane depolarization), and decreased absorbance (indicative of mitochondrial swelling) has been shown to participate in myocyte dysfunction and degradation of cardiac contractile performance, arrhythmias, and myocytes apoptosis and infarct size during and subsequent to I/R injury. Our results demonstrated that VNS applied during ischemia significantly decreased mitochondrial ROS production (Figure 12A) and prevented mitochondrial membrane depolarization (Figure 12B) and mitochondrial swelling (Figure 12C) in the ischemic myocardium, compared with the control ischemic area. VNS applied during ischemia, but not at the onset of reperfusion, also significantly decreased the cytochrome c release (Figure 12D), compared with the control group. Again, these effects were abolished by Atropine, suggesting the importance of muscarinic modulation during VNS.

Electron photomicrographs demonstrated that in the ischemic area of the control group, I/R induced severe mitochondrial damage was observed as demonstrated by noted mitochondrial swelling accompanied by a disruption in membrane integrity (Figure 13). VNS applied during ischemia, but not reperfusion, significantly attenuated cardiac mitochondrial swelling following I/R injury, and that this effect was abolished by Atropine. Moreover, in order to determine the effect of VNS on the inflammatory response, the levels of anti-inflammatory cytokine IL-4 and the pro-inflammatory

cytokine TNF-α were determined. VNS applied during ischemia significantly increased the anti-inflammatory cytokine IL-4, compared with the Control group (176.8±34.5 pg/ml vs. 84.7±11.4 pg/ml; *P*<0.05). The IL-4 levels of both the VNS-Reperfusion and VNS-Ischemia+Atropine groups were not different compared to the Control group (123.8±20.8 pg/ml and 72.2±22.1 pg/ml, respectively; *P*=NS). The level of the pro-inflammatory cytokine TNF-α in the heart was not different between groups (Control 537.4±52.5 pg/ml, VNS-Ischemia 498.2±45.1 pg/ml, VNS-Reperfusion 529.2±32.6 pg/ml and VNS-Ischemia+Atropine 493.1±73.5 pg/ml; *P*=NS).

อภิปรายผลการทดลอง

In the present study, we investigated whether VNS applied late during ischemia or at the onset of reperfusion exerted differential cardioprotection against I/R injury. The major findings of this study are as follows. VNS applied during ischemia provided cardioprotective effects against I/R injury by (1) preventing reperfusion arrhythmias by increasing the phosphorylation of connexin43 at the serine368 residue and reducing time from T-wave peak to end (an indicator of transmural dispersion of repolarization), and (2) preserving LV function by reducing infarct size and via its action on cardiac mitochondrial integrity through its anti-apoptotic and anti-inflammatory effects. However, VNS applied at the onset of reperfusion did not produce cardioprotection against I/R injury, suggesting that the timing of VNS initiation with respect to the onset of myocardial ischemia is an important determinant of its therapeutic efficacy. Our results provide novel evidence that VNS applied later during an ischemia episode provides significant cardioprotection and prevents the attenuation of LV function in a large animal

model of I/R injury. Moreover, our finding strengthens and extends the mechanistic explanation of previous reports, which indicated that VNS applied before ischemia or at the onset of coronary occlusion exerted cardioprotection and improved LV function during the I/R period.[7, 12, 15, 27]

The anti-arrhythmogenic effects of VNS during ischemia-reperfusion

Previous studies demonstrated that VNS exerts anti-arrhythmogenic effects during the ischemic period. Zuanetti and colleagues provided the first evidence that some ventricular tachycardia could be modulated and reduced by vagal activation.[45] Moreover, an elegant study by Schwartz and colleagues in conscious dogs clearly demonstrated that enhanced vagus nerve activity by means of right cervical VNS prevented spontaneous VT in a model with healed MI, exercise testing and intermittent ischemia.[7] In the present study, we found that VNS applied late during ischemia, but not at the onset of reperfusion, still significantly decreased the occurrence of PVCs, the number of spontaneous VT/VF episodes and decreased time from T-wave peak to end. It is well recognized that Connexin43, a major cardiac gap junction in the ventricles, contributes to intercellular communication and electrical coupling.[46, 47] Moreover, marked dephosphorylation of ventricular connexin43 has been observed during the process of electrical uncoupling induced by ischemia.[48] A recent study by Ando and colleagues demonstrated that VNS applied before ischemia effectively inhibits the loss of the phosphorylated isoform of connexin43 during acute MI.[37] Thus, we hypothesized that VNS applied during ischemia exerts anti-arrhythmogenic effects by modulating the level of connexin43 phosphorylation. It has been shown previously that the

phosphorylation of connexin43 at the serine368 residue could lead to the prevention of phosphorylation at the other connexin43 residues, thus maintaining electrical coupling with ventricular myocytes.[48, 49] In the present study, we demonstrated that VNS applied during ischemia markedly increased connexin43 phosphorylation at the serine368 residue during I/R. Therefore, functional preservation of connexin43 at serine368 by VNS might play a crucial role in the anti-arrhythmogenic effects during I/R. In our study, VNS applied at the onset of reperfusion significantly increased connexin43 phosphorylation at the serine368 residue. However, it could attenuate only the number of PVCs, but not the number of VT/VF episodes as observed in the VNS-Ischemia group. This observation might be explained by the difference in the structural substrate, such as the infarct size, which was larger in the VNS-Reperfusion group and, due to the wave length theory, influenced the VT/VF incidence.[50-52] Moreover, our findings suggested that, during ischemia and reperfusion, VNS mainly improved ventricular repolarization, but not depolarization since there were no significant differences in QRS intervals (a parameter of depolarization) in all timing of VNS. However, our results showed that VNS increased the phosphorylation of connexin43. Future cardiac mapping study should be done to assess this discrepancy.

VNS effects on LV function and infarct size

Our finding that VNS applied during ischemia, but not at the onset of reperfusion, preserved LV functional performance is consistent with previous studies. In rats subjected to global I/R with intact vagal innervation, right cervical VNS-treated LV showed significantly better performance throughout the 120-minutes reperfusion period, and that VNS exerted a marked anti-infarct effect irrespective of the heart rate compared with sham stimulation.[33] The preserved LV function provided by VNS might be explained by a reduction in the myocardial infarct size. In the present swine study, VNS applied during ischemia, but not at the onset of reperfusion, significantly reduced myocardial infarct size by 59%. However, we recently reported that VNS applied immediately at the onset of ischemia dramatically reduced myocardial infarct size by 89%.[15] These findings suggested that the timing of VNS initiation with respect to the onset of myocardial ischemia is an important determinant of VNS therapeutic efficacy, and that the earlier the VNS is applied the smaller the infarct size.

Mechanisms responsible for infarct size reduction by VNS

Myocardial I/R injury leads to the activation of cell death programs, including cytochrome c-mediated apoptosis and necrosis. Thus, the beneficial effect of VNS in reducing the infarct size suggests that it may have an anti-apoptotic effect. In the present study, we found that VNS applied during ischemia, but not at the onset of reperfusion, markedly reduced the level of cytochrome c release, suggesting that it may be involved in the suppression of mitochondrial permeability transition pore opening that prevented myocardial apoptosis.[33] A previous study in rats demonstrated that VNS

prevented down regulation of the anti-apoptotic protein Bcl-2, and suppressed cytochrome c release, and reperfusion-induced opening of mitochondrial permeability transition pore[33], which is also consistent with our finding. Therefore, the decreased cytochrome c release could be responsible for the infarct size reduction observed in this study. Moreover, we have shown that one potential mechanism of the pronounced cardioprotective effect of VNS during I/R could be due to its prevention of cardiac mitochondrial dysfunction. Increased ROS production and oscillation of the mitochondrial membrane potential ($\Delta\Psi_m$) have been shown to play an important role in the genesis of cardiac arrhythmias and myocardial infarction.[39] Our data indicate that VNS stabilized cardiac mitochondrial membrane potential by protecting the depolarization of mitochondrial membrane potential. This could lead to the reduction of arrhythmias that occurred during I/R in the VNS group. Moreover, mitochondrial ROS reduction and decreased mitochondrial swelling could be responsible for the decreased infarct size in the VNS group. Our findings that these beneficial effects of VNS were abolished by Atropine suggest a dominant muscarinic receptor involvement. In addition to its anti-apoptosis, previous studies also demonstrated that VNS exerts an anti-inflammatory effect.[12, 53, 54] Inflammatory processes have been shown to play a critical role in myocardial I/R injury. In the present study, VNS applied during ischemia, but not at the onset of reperfusion, significantly increased myocardial anti-inflammatory cytokine IL-4. A recent clinical study has shown that serum IL-4 concentrations on admission and immediately after PCI were significantly lower in patients who subsequently developed severe cardiac dysfunction.[55] Another experimental study also reported that IL-4 protects B cells from apoptosis and prevents mitochondrial damage as shown by

the maintenance of $\Delta\Psi_m$.[56] Therefore, the anti-inflammatory effect of VNS could also be responsible for the reduction of the infarct size reported in this study. Moreover, our finding also suggested that myocardial insult leading to myocardial cell death occurred early during ischemia as shown by a smaller infarct size when VNS was applied immediately at the onset of ischemia[15] or later during ischemia compared to that at the onset of the reperfusion period.

Conclusions and study limitations

We have thus provided compelling evidence that VNS after myocardial ischemia onset provided significant cardioprotection and improved LV function in a large animal model of I/R injury. However, cardioprotection associated with VNS is diminished or absent when VNS was applied at the onset of reperfusion. These findings suggest that timing of VNS initiation with respect to the onset of ischemia is an important determinant of its therapeutic efficacy. The underlying potential mechanisms of such cardioprotection of VNS are associated with its prevention of reperfusion arrhythmias by increasing the phosphorylation of connexin43 at the serine368 residue, decreased time from T-wave peak to end, preserving LV function by reducing infarct size and protecting cardiac mitochondrial integrity through its anti-apoptotic and anti-inflammatory effects. Thus, these data strongly support the notion that VNS is emerging as a promising therapeutic modality in combination with reperfusion therapy for protecting myocardium at risk of I/R injury due to coronary artery disease.

Some limitations of the present study are also important to note. Our study was conducted in anesthetized healthy animals, whereas most victims of cardiac arrest have significant coronary lesions. A majority of the large animal studies on the effects of VNS have been performed in canine models. In contrast to dogs, pigs have no collateralized coronary arteries.[57] Thus, our model might be more similar to the patients who have a first coronary event accompanied by fatal ischemia. Furthermore, we did not measure the myocardial interstitial level of Acetylcholine at the ischemic area during VNS. However, a previous study demonstrated that the electrical stimulation of the vagal efferent during acute MI produced the significant additive release of Acetylcholine to the myocardial interstitial space.[58] Therefore, such an additional release of Acetylcholine in response to VNS during ischemia would play a critical role in the cardioprotection against I/R injury. Moreover, in the present study, there was no significant change in heart rate during VNS which is contrary to a previous study.[31] This discrepancy may be due to different VNS parameters used in that study and ours. It is known that VNS-induced bradycardia is dependent on stimulation parameters (voltage, current, duration, and frequency). By adjusting the VNS parameters, graded sinus bradycardia could be achieved, and that strong VNS can produce long pauses and sinus arrest. Nevertheless, a recent study[59] suggested that “low-dose” stimulation with no cardiac synchronization may be equally effective than higher amplitude stimulation coupled to the heart rate, and a heart rate reduction may not necessarily be an ideal surrogate marker of the favorable effects at ventricular level[60]. In view of the present study, VNS initiating during ischemia may thus offer a novel adjunctive

myocardial salvage approach to current percutaneous coronary and pharmacological interventions designed to reduce myocardial reperfusion injury.

Table 2. Effects of VNS and Muscarinic Receptor Blocker on Pressure-Volume (P-V) Loop Derived Functional Parameters

	Control			VNS-Ischemia			VNS-Reperfusion			VNS-Ischemia+Atropine		
	Baseline	**Ischemia**	**Reperfusion**	**Baseline**	**Ischemia**	**Reperfusion**	**Baseline**	**Ischemia**	**Reperfusion**	**Baseline**	**Ischemia**	**Reperfusion**
SV (ml)	25±2	9±4*	9±4*	23±4	18±6	20±6	22±2	7±2*	10±2*	23±3	7±2*	6±3*
EF (%)	56±5	27±9*	27±8*	54±9	42±4	43±6	53±8	28±9*	27±4*	55±8	28±6*	24±8*
ESP (mmHg)	80±11	83±6	81±13	73±7	74±6	81±14	74±14	73±11	79±16	81±11	81±9	82±8
EDP (mmHg)	9±6	17±7*	20±8*	7±2	8±4	10±1	7±2	16±5*	13±1*	9±5	20±7*	21±7*

Summary of left ventricular (LV) functional parameters at baseline, at the end of ischemia, and at the end of reperfusion (n = 6-8/group). Values are mean ± SD. *$P < 0.05$ vs baseline. VNS= vagus nerve stimulation; SV = stroke volume; EF = ejection fraction; ESP = end-systolic pressure; EDP = end-diastolic pressure

Figure 7: **Schematic representation of the study protocols and electrodes placement on left cervical vagus nerve.** Panel A represents the study protocols. The ischemic period (60 minutes) was induced by rapid, complete ligation of the left anterior descending coronary artery (LAD). The reperfusion period was 120 minutes. A bipolar pacing lead and anchor lead were placed around the mid-cervical left vagus nerve (panel B). LC-VNS = Left cervical vagus nerve stimulation.

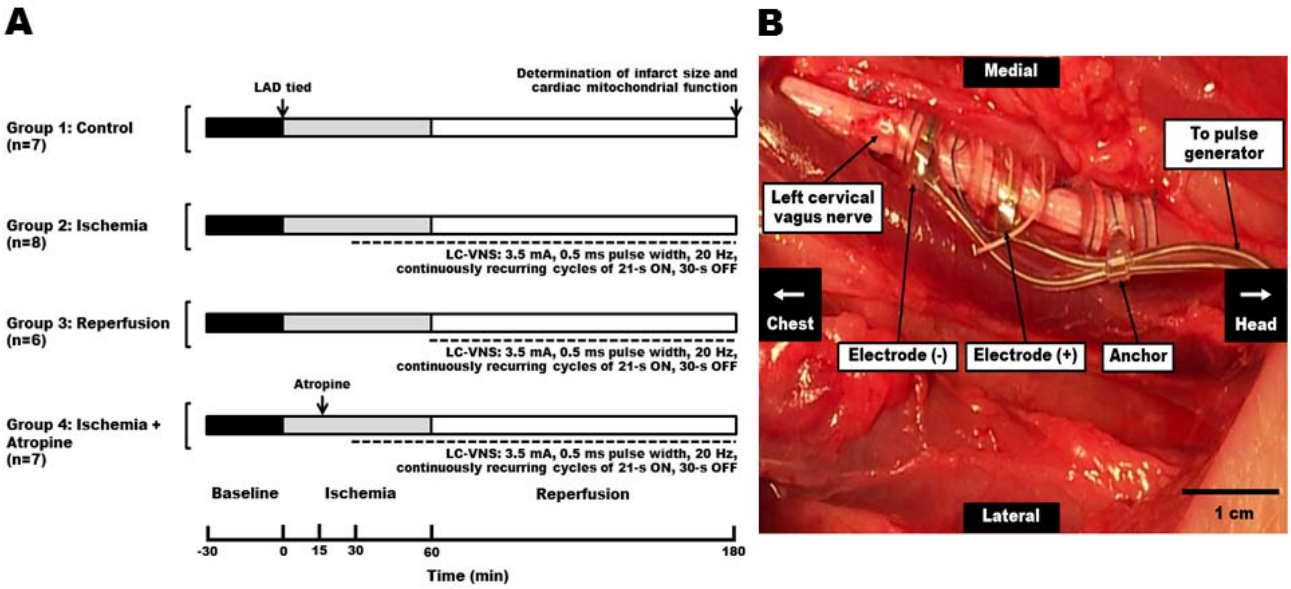


Figure 8: **Effect of vagus nerve stimulation (VNS) on heart rate and ECG parameters during the ischemia-reperfusion period.** Panel A shows the effect of VNS on heart rate. Panel B represents the effect of VNS on mean PR interval. Panel C shows the effect of VNS on mean QRS duration. The effect of VNS on QT intervals was shown in panel D. Panel E represents the effect of VNS on T-wave peak to end (Tpe). Representative of Tpe interval seen in group 2 at baseline, during ischemic and reperfusion periods (panel F). Data are presented as mean ± SD. **P* < 0.05 vs baseline. VNS = Vagus nerve stimulation.

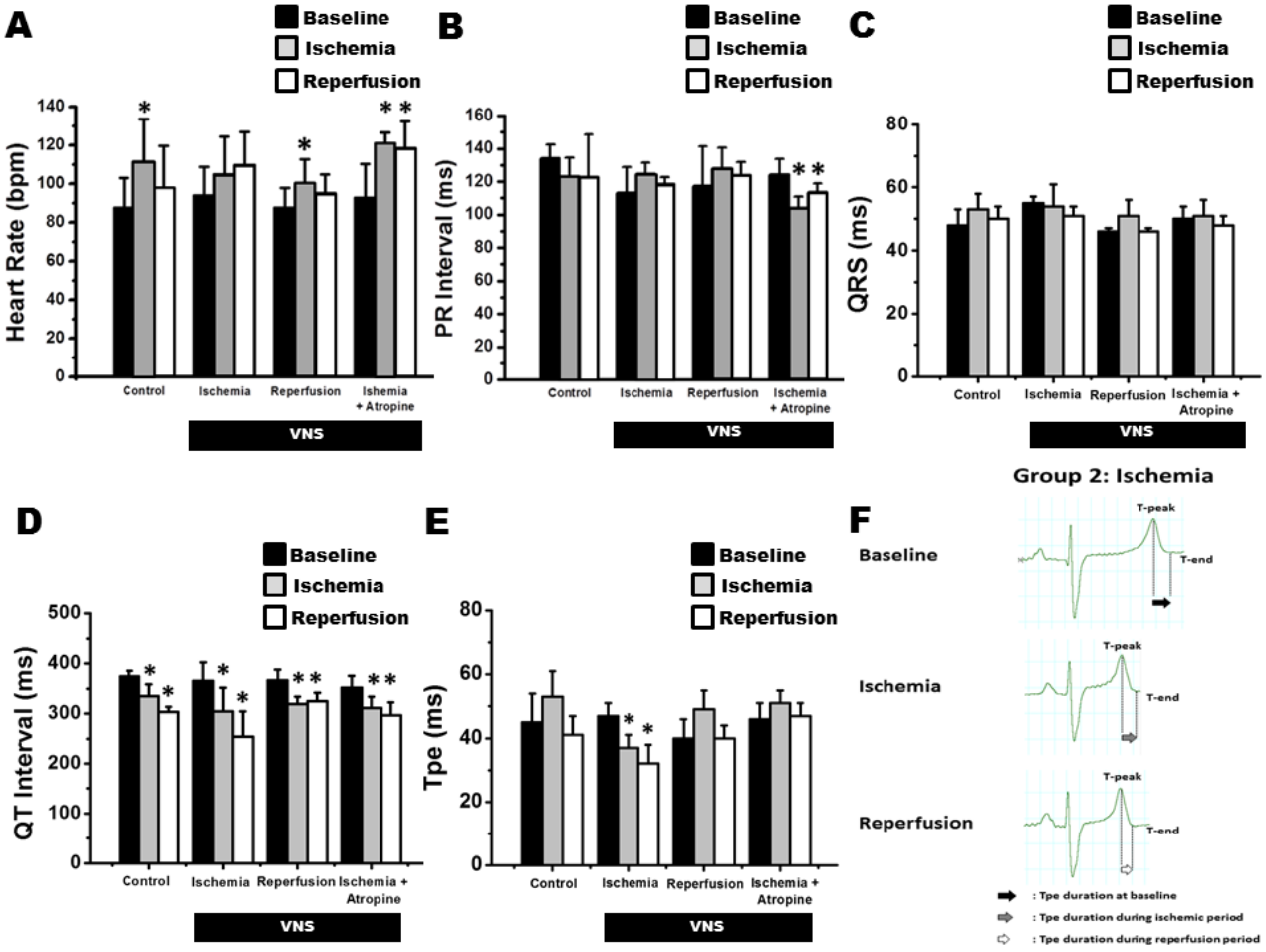


Figure 9: **Representative ECG tracings illustrating PVC induced ventricular tachycardia and PVC and VT cycle lengths**. Panel A represents a PVC initiated self-terminating ventricular tachycardia in four different groups. The PVC and VT cycle lengths were not different among groups (panel B). Data are presented as mean ± SD. PVC = Premature ventricular contraction; VT = Ventricular tachycardia

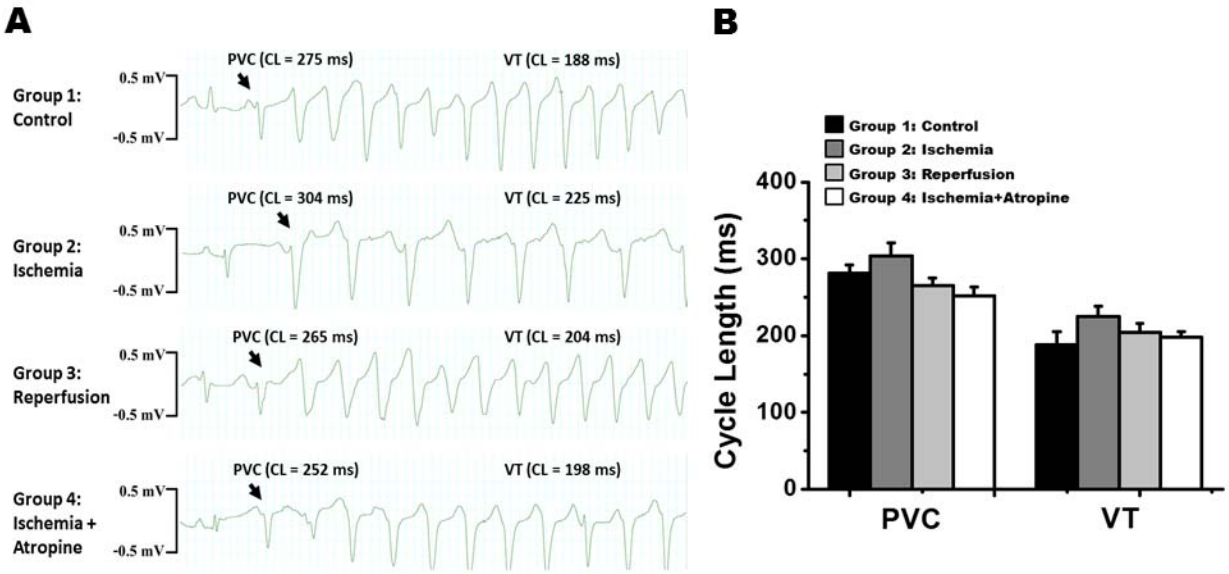


Figure 10: **VNS applied during ischemia protected the heart against reperfusion arrhythmias by increasing Cx43 phosphorylation.** The total number of PVCs were markedly decreased in both VNS-Ischemia and VNS-Reperfusion groups, compared with the Control group (panel A). The total number of VT/VF episodes was significantly reduced in the VNS-Ischemia group compared with the VNS-Ischemia group (panel B). However, the time to VT/VF onset was not significantly different between groups during ischemic-reperfusion periods (panel C). Both VNS-Ischemia and VNS-Reperfusion groups significantly increased the phosphorylation of connexin43 at serine368 in the ischemic myocardium compared with the control group (panel D, n=4/group). Data are presented as mean ± SD. **P* < 0.05 vs Control, †*P* < 0.05 vs VNS-Ischemia+Atropine, ‡*P* < 0.05 vs VNS-Ischemia+Atropine group. PVC = premature ventricular contraction; VNS = Vagus nerve stimulation; VT = Ventricular tachycardia; VF = Ventricular fibrillation.

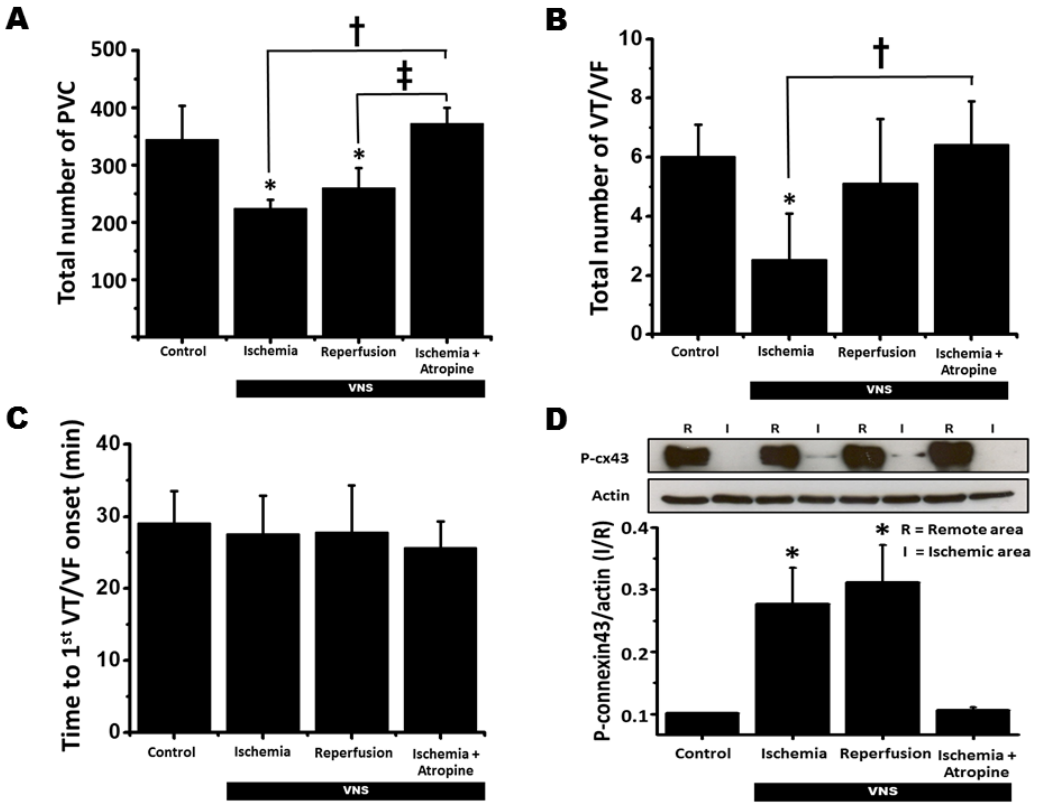


Figure 11: **VNS applied during ischemia markedly reduced the infarct size.** Myocardial infarct size was expressed as the percentage of area at risk (AAR). The AAR, expressed as a percentage of total ventricular mass, were not different between groups (Control 32.8±5%, VNS-Ischemia 33.5±5%, VNS-Reperfusion 34.2±1% and VNS-Ischemia+Atropine 38.9±4%; *P*=NS). Interestingly, VNS applied during ischemia significantly reduced myocardial infarct size and this effect was reversed by Atropine. Control (45.7±14%, n=7); VNS-Ischemia (18.6±8%, n=8); VNS-Reperfusion (43.6±6%, n=6); VNS-Ischemia+Atropine (46.4±11%, n=7). Representative pictures after Evan Blue and triphenyltetrazolium chloride staining are shown in the inset. Blue indicates non-threatened myocardium, red indicates the non-infarcted area within the area at risk, and white indicates myocardial infarction. Data are presented as mean ± SD. **P* < 0.05 vs Control, †*P* < 0.05 vs VNS-Ischemia group. NS = Not significant.

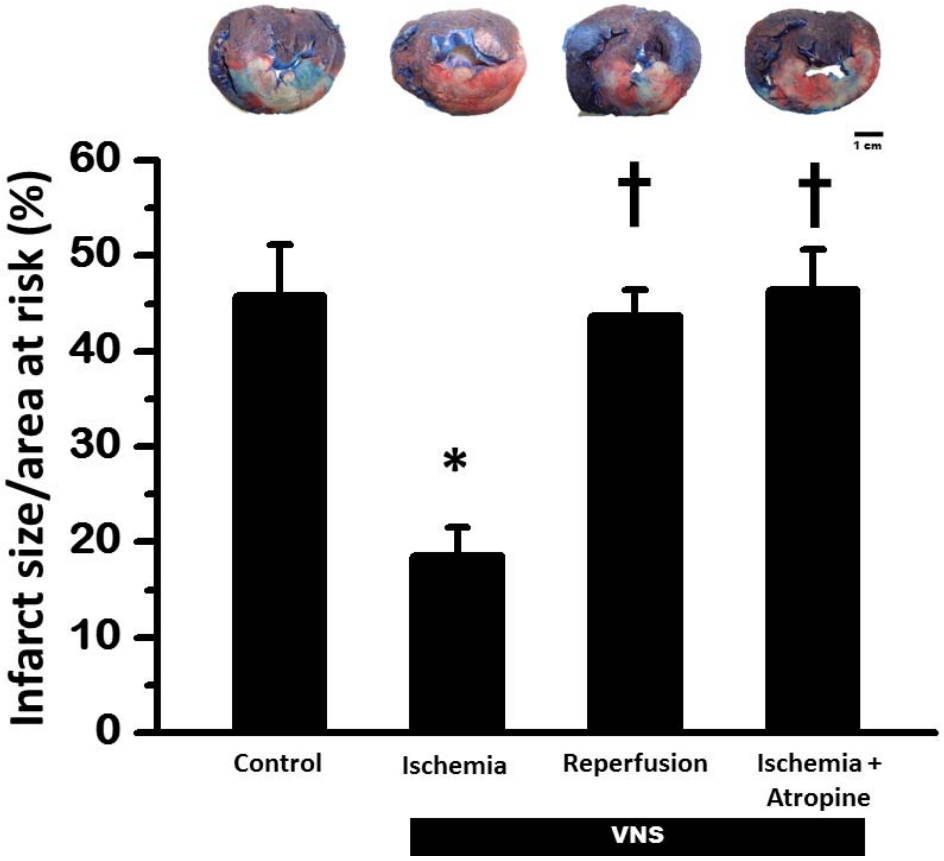


Figure 12: **VNS applied during ischemia protected cardiac mitochondrial integrity by mitigating cytochrome c release.** VNS applied during ischemia significantly decreased mitochondrial ROS production (panel A, n=4/group) and prevented mitochondrial membrane depolarization (panel B, n=4/group) and mitochondrial swelling (panel C, n=4/group) in the ischemic myocardium, compared with the control ischemic area. VNS applied during ischemia significantly decreased cytochrome c release (panel D, n=4/group), compared with the Control group. Again, these effects were abolished by Atropine, suggesting the importance of muscarinic modulation during VNS. Data are presented as mean ± SD. **P* < 0.05 vs remote area (panel A-C) or vs control (panel D), †*P* < 0.05 vs control ischemic area (panel A-C) or vs VNS-Ischemia+Atropine group (panel D), ‡*P* < 0.05 vs VNS-Reperfusion group (panel D). VNS = Vagus nerve stimulation.

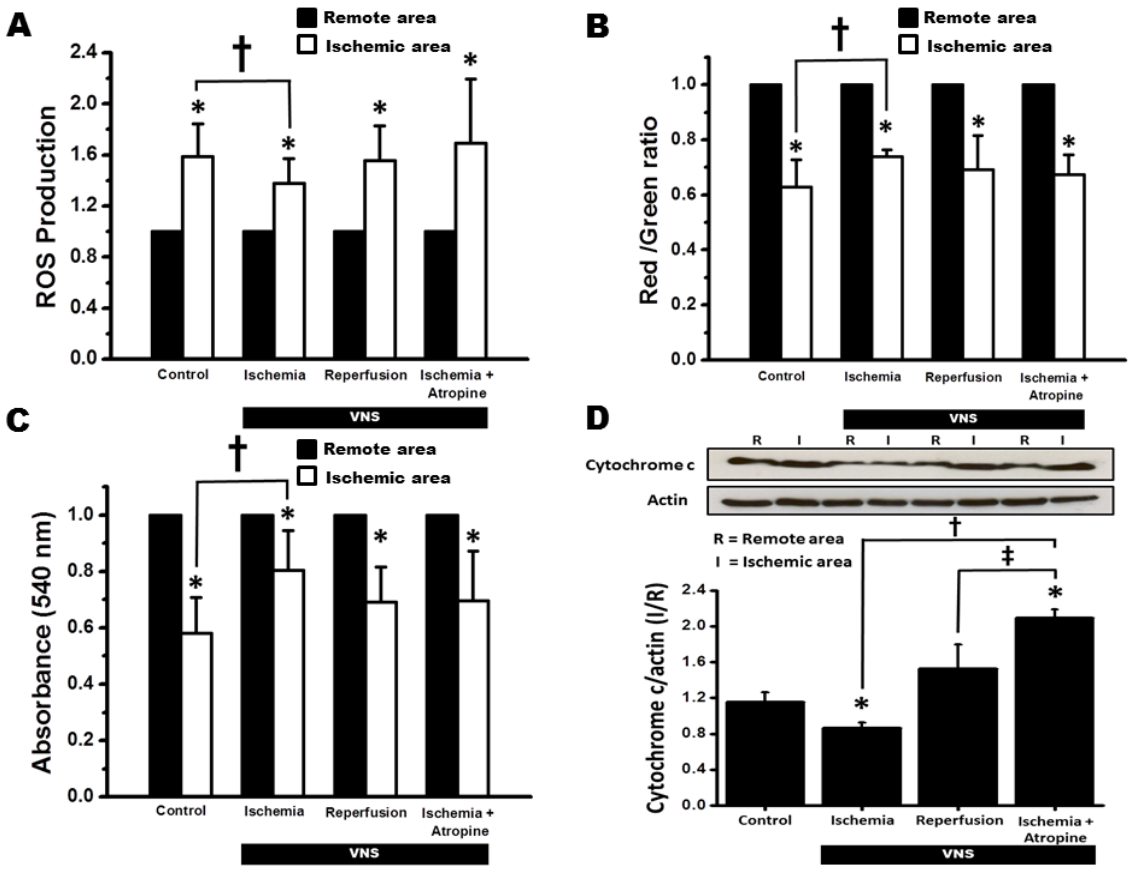
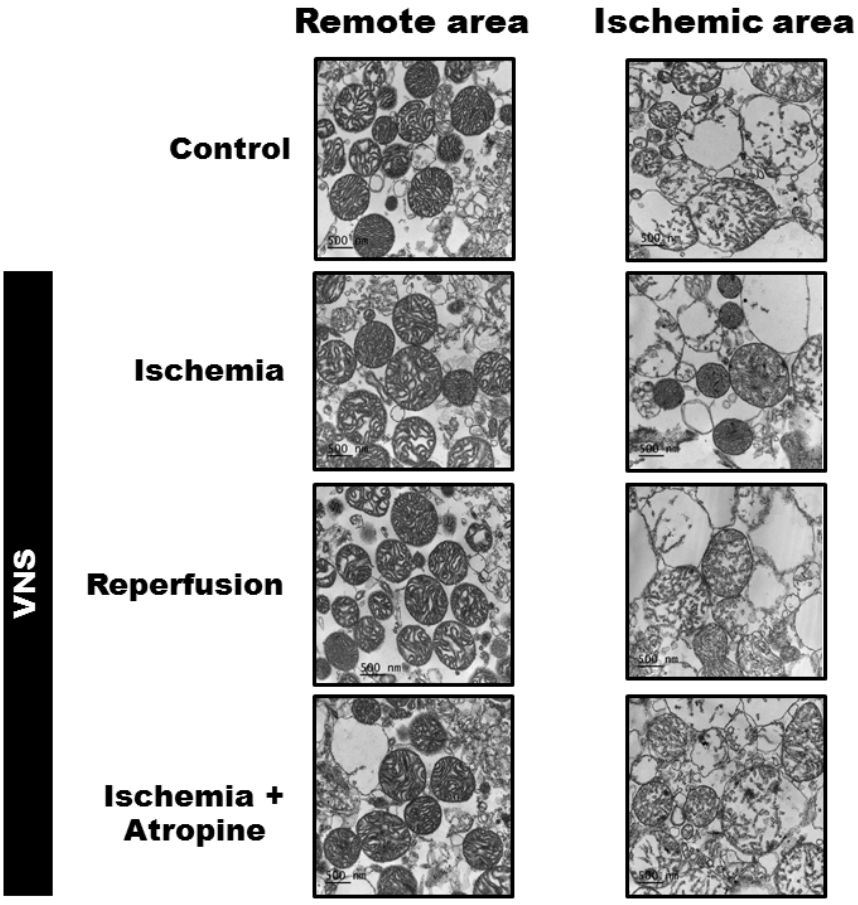


Figure 13: Representative electron photomicrographs of cardiac mitochondrial ultrastructure. In the ischemic area of the control group, I/R-induced severe mitochondrial damage was observed as indicated by marked cardiac mitochondrial swelling accompanied by the disruption in membrane integrity. However, VNS applied during ischemia, but not reperfusion, significantly protected cardiac mitochondrial swelling following I/R injury and this effect was abolished by Atropine. VNS = Vagus nerve stimulation.



บทที่ 3: Vagus Nerve Stimulation Exerts Cardioprotection Against Myocardial Ischemia/Reperfusion Injury Predominantly Through its Efferent Vagal Fibers

บทนำ

Acute myocardial infarction (AMI) is a major cause of morbidity and mortality worldwide.[61] Although early and rapid reperfusion is the most effective strategy to reduce myocardial injury and limit the infarct size, reperfusion itself can induce cardiomyocyte death. This phenomenon is known as myocardial ischemia/reperfusion (I/R) injury.[61] During I/R, reactive oxygen species (ROS) is dramatically increased[62], which causes oxidative damage and cell apoptosis.[63] Moreover, I/R injury can induce mitochondrial dysfunction and structur al change including impaired mitochondrial dynamics and metabolism.[64] These deleterious effects lead to myocardial cell death, increased infarct size, cardiac arrhythmia and impaired left ventricular and hemodynamic parameters.[15, 65] Therefore, cardioprotection beyond that by timely reperfusion is needed to reduce infarct size and improve the prognosis of the affected AMI patients.[66] Previous study demonstrated that myocardial infarction increases sympathetic tone, especially the relative ischemia distal to a severe coronary stenosis which in turn results in poststenotic vasoconstriction and an aggravation of ischemia, and decreases parasympathetic tone.[67] Therefore, rebalanced autonomic activity by augmenting vagal activity may be a potential therapeutic intervention for the affected MI patients. A growing body of literature has shown that enhancing parasympathetic activity by electrical stimulation of the cervical vagus nerve (both afferent and efferent fibers) has emerged as a promising therapy for various conditions, including brain and heart diseases.[68, 69] Specifically in the heart, both invasive and non-invasive vagus

nerve stimulation (VNS) has been shown to exert cardioprotection in patients with chronic heart failure (the ANTHEM-HF trial) and ischemic heart diseases.[9, 10, 44, 70, 71]

The vagus nerve is a mixed nerve which contains 80% afferent fibers (sensory) and 20% efferent fibers (motor).[72] Recently, in I/R swine model, we have demonstrated that left cervical VNS (anode cephalad to cathode to stimulate cardiac efferent vagal fibers) applied at the onset or during the ischemic periods can improve cardiac function, decrease myocardial infarct size, reduce dispersion of repolarization via amelioration of cardiac mitochondrial dysfunction.[15, 65] Moreover, VNS (caudal end to stimulate cardiac efferent vagal fibers) can improve mitochondrial function through the activation of M_3 Receptor/CaMKKβ/AMPK pathway in isoproterenol-induced cardiac damage in rats.[73] In addition, previous study demonstrated that VNS also activated the ipsilateral afferent vagal fibers, which reflexively reduced cardiac efferent parasympathetic effects.[74] Although VNS has been shown to exert cardioprotection against myocardial I/R injury in both preclinical and clinical studies, it is still remained unclear whether the cardioprotection of VNS is mainly due to direct vagal activation through its ipsilateral efferent vagal fibers (motor) or indirect effects mediated by the afferent vagal fibers (sensory). Additionally, the majority of publications describing cardioprotection by VNS were performed in small animal (rodents or rabbits), which are sympathetically dominant.[75, 76] However, the importance of vagal tone for the heart in small animal is less than that in larger mammals (canine or pigs) or humans.[77] Thus, it is important to study role of vagus nerve activation in cardioprotection in a larger mammal model. Therefore, the objectives of this study were to determine whether the cardioprotective effects against myocardial I/R injury of VNS were mainly due to direct ipsilateral efferent

vagal fibers activation or indirect effects mediated by the afferent vagal fibers in a large animal model of AMI. Furthermore, roles of the contralateral efferent vagal fibers during VNS were also investigated. We hypothesized that VNS exerts cardioprotection against myocardial I/R injury predominantly through its ipsilateral efferent vagal fibers.

วิธีการทดลอง

Animal preparation

All experiments were approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Thirty domestic pigs (30-35 kg) were anesthetized by an intramuscular injection of a combination of 4.4 mg/kg zoletil® (Vibbac Laboratories, Carros, France) and 2.2 mg/kg xylazine (Laboratorios Calier, S.A., Barcelona, Spain). After endotracheal intubation, anesthesia was maintained by 1.5–3.0% isoflurane (Abbott Laboratories Ltd., Queenborough, UK) delivered in 100% oxygen. Surface electrocardiogram (lead II), femoral arterial blood pressure (BP), heart rate (HR), and rectal temperature were continuously monitored, and all data were recorded for subsequent analysis. Arterial blood gases and electrolytes were also monitored every 30 minutes and maintained within acceptable physiological ranges.[78] Furthermore, under fluoroscopic guidance, platinum coated titanium coil electrodes (34- and 68-mm) were advanced into and positioned at the right ventricular apex and the junction between the right atrium and superior vena cava, respectively, to deliver electrical shocks when malignant ventricular arrhythmias spontaneously occurred during I/R periods.[78]

Ischemia/reperfusion (I/R) protocol

The heart was exposed through a left thoracotomy. The left anterior descending artery (LAD) were isolated and occluded by ligature (3-0 silk) three centimeters from the left main coronary artery. Ischemia was confirmed by an ST elevation on the ECG (lead II) and the change in color of myocardial tissues on the ischemic area. I/R were performed by 60 minutes of a complete LAD occlusion followed by 120 minutes of reperfusion.

Vagus nerve stimulation (VNS) protocol

The left vagus nerve was surgically isolated (~ 3 cm length, at C5-6 level) from the carotid sheath. A VNS lead (Model 304, Cyberonics, Houston, TX, USA) with bipolar electrodes (platinum-iridium, 4 mm^2 surface area, 6-mm interelectrode spacing) were attached to the vagus nerve using helical fixation elements to assure electrode stability. The cathodic electrode was oriented closest to the heart. The proximal terminal pin of VNS lead was attached to a pulse generator (Demipulse, Model 103, Cyberonics) for delivery of VNS. Prior to onset of ischemia, the mean PR interval were determined from an average of ten consecutive sinus beats. We verified that VNS were engaging the autonomic nervous system by briefly stimulating the vagus nerve and observing a significant increase in the PR intervals. In the present study, we sought to determine whether the cardioprotection of VNS are mainly due to direct activation through its ipsilateral efferent fibers (motor) or through the indirect effects mediated by the afferent fibers (sensory) by using infarct size as the primary endpoint. Thus, we intended to use the I/R protocol and the VNS parameters (3.5 mA, 500 μs pulse width, 20 Hz and continuous recurring cycles of 21-seconds ON and 30-seconds OFF) which

provided the most potent anti-infarct effect (about 89% reduction) based on our previous studies.[15, 65]

Experimental protocols

Pigs were randomly divided into five groups (n = 6/group) as shown in Figure 14. All pigs in each group were subjected to I/R protocol. Group 1 (I/R): pigs were sham operated without VNS. Group 2 (LC-VNS): the vagal nerve was left intact for combined afferent and efferent stimulation, pigs were received intermittent left cervical VNS at the onset of LAD occlusion and continued until the end of reperfusion. Group 3 (LtVNX): pigs were assigned to left vagus nerve transection at middle cervical level and received intermittent left cervical VNS 2 cm under the point of cut for selective efferent vagal nerve stimulation. Group 4 (RtVNX): to study roles of the contralateral efferent vagal fibers during VNS, pigs were assigned to right vagus nerve transection at middle cervical level and received intermittent left cervical VNS. Group 5 (Atropine) pigs were received Atropine (1 mg/kg) by administered intravenous 15 minutes prior to initiation of left cervical VNS to inhibit parasympathetic actions on the heart.

Evaluation of ECG parameters

Heart rate (HR), PR interval, QRS complex duration (an indicator of ventricular activation time), QT interval (an indicator of ventricular repolarization time), time from T-wave peak to end (Tpe; an indicator of transmural dispersion of repolarization), and T-wave peak to end per QT interval ratio (Tpe/QT ratio; an indicator of dispersion of repolarization relative to the total duration of repolarization) were measured. ECG traces were analyzed with Chart 6 (AD Instruments). The mean baseline all of parameters were determined from an average of twenty sinus beats just prior to LAD

occlusion. The mean parameters during the ischemia and reperfusion periods were analyzed from an average of twenty consecutive beats before the end of occlusion and the end of reperfusion, respectively.

Evaluation of rhythm disturbances

Premature ventricular contractions (PVC), Ventricular tachycardia (VT), and Ventricular fibrillation (VF) were defined according to the Lambeth Convention criteria[17] with more rigorous modifications for the entire 180 minutes I/R period. Specifically, PVC was defined as ventricular contractions without atrial depolarization. VT was defined as more than six consecutive PVC. VF was characterized by a loss of synchronicity of electrocardiogram plus decreased amplitude and a precipitous fall in blood pressure (BP) for more than one second. ECG traces were analyzed with Chart 6 (AD Instruments). Furthermore, the arrhythmia scores and mortality rate were determined. The arrhythmia scores all correlated with the incidences of PVC, VT, and VF.[79]

Evaluation of left ventricular (LV) functions parameters

During the I/R periods, the left ventricular function including stroke volume (SV), ejection fraction (EF), end-systolic pressure (ESP), and end-diastolic pressure (EDP), and stroke work (SW) were continuously monitored and recorded using the pressure-volume (P-V) loop recording system (Model ADV500/ADVantage System, Scisense Inc., London, Canada) as described previously.[18]

Infarct size determination

After 120 minutes of reperfusion, the LAD were re-occluded by the LAD ligation, and the heart were removed and irrigated with normal saline to wash out blood from

chambers and vessels. The infarct size were assessed with 0.5% Evans Blue and 1.0% Triphenyltetrazolium Chloride (TTC) staining as previously described.[15] The area at risk (AAR) were defined as the area not stained by the Evan blue dye, and the infarcted area were defined as the area not stained by TTC. An area measurement was performed using the Image Tool software version 3.0. The area of infarct size was normalized to the AAR and calculated as %infarct size/AAR as described previously.[15]

Isolated cardiac mitochondria

Cardiac mitochondria were isolated from the ischemic and non-ischemic regions, using the technique previously described[26], and the protein concentration was determined according to the bicinchoninic acid assay. Cardiac mitochondrial functions were determined by measuring the cardiac mitochondrial reactive oxygen species (ROS) production, cardiac mitochondrial membrane potential change ($\Delta\Psi_m$) and cardiac mitochondrial swelling. Cardiac mitochondrial ROS production was determined using a fluorescent microplate reader in all groups. The dye dichlorohydro-fluorescein diacetate (DCFDA) was used to determine the level of ROS production in cardiac mitochondria. The DCFDA can pass through the mitochondrial membrane, and was oxidized by ROS in the mitochondria into the fluorescent form of DCF. Thus, increased fluorescent intensity indicates increased ROS production in the mitochondria. A cardiac mitochondrial membrane potential change was determined using a fluorescent microplate reader in all groups. The dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide (JC-1) was used to determine the change in the mitochondrial membrane potential. JC-1 is characterized as a cation and remains in the mitochondrial matrix as a monomer (green fluorescence) form. However, it can interact

with anions in the mitochondrial matrix to form an aggregate (red fluorescence) form. Cardiac mitochondrial depolarization was indicated by a decrease in the red/green fluorescence intensity ratio. Cardiac mitochondrial swelling was assessed by measuring changes in the absorbance of the suspension wavelength at 540 nm using a microplate reader. Cardiac mitochondria (0.4 mg/ml) were incubated in 2 ml of respiration buffer: KCl 150 mM, HEPES 5 mM, $K_2HPO_4.3H_2O$ 5 mM, L-glutamate 2 mM and pyruvate sodium salt 5 mM. Mitochondrial swelling was indicated by a decrease in the absorbance of the mitochondrial suspension. Isolated cardiac mitochondrial morphology was confirmed by using a transmission electron microscope.

Transmission electron microscopy for cardiac mitochondrial morphology

Cardiac mitochondrial morphology was determined by transmission electron microscopy. Isolated cardiac mitochondria from both ischemic and remote areas were fixed overnight by mixing 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 4ºC. Then, the pellets were post fixed in 1% cacodylate-buffered osmium tetroxide for two hours at room temperature. The pellets were dehydrated in a graded series of ethanol and embedded in Epon-Araldite and cut by a diamond knife into ultra-thin sections (60-80 nm thick), placed on copper grids and stained with the combination of uranyl acetate and lead citrate. Finally, the mitochondrial morphology was observed with a transmission electron microscope.[19]

Western blot analysis

At the end of each experiment, the hearts were rapidly excised, and then the remote and ischemic areas of ventricular tissues were collected, quickly frozen in liquid nitrogen, and stored at -80ºC until analysis. Heart proteins were lysed with extraction

buffer (Tris–HCl 20 mmol/L, Na_3VO_4 1 mmol/L, NaF 5 mmol/L) and separated by electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then were transferred onto nitrocellulose membranes. After immunoblots were blocked for one hour with 5% nonfat dry milk in Tris-buffer saline (pH 7.4) containing 0.1% Tween 20, they were probed overnight at 4ºC with the primary antibodies that recognize phospho-connexin43 (P-Cx43)(Ser 368) and total connexin43 (Total-Cx43) (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA); a marker of intercellular electrical communication, Bax, Bcl-2, Pro caspase-3 and Cleaved caspase-3 (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA); a marker of apoptosis, Mitofusin-2 (MFN2), optic atrophy protein 1 (OPA1), dynamin related protein 1 (DRP1), phospho-dynamin related protein 1 at serine 616 (P-DRP1 Ser 616) and serine 637 (P-DRP1 Ser 637) (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA); a marker of mitochondrial fission and fusion, phospho-AMPK-activated protein kinase (P-AMPK) and total AMPK (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA); a marker of cardiac cellular energy homeostasis, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1α), Carnitine palmitoyltransferase 1 (CPT1) (1:200 dilution, Santa Cruz biotechnology, TX, USA); a marker of mitochondrial biogenesis and fatty acid oxidation mitochondrial complex I-V (1:2000 dilution, Cell Signaling Technology, Danvers, MA, USA); a marker of cardiac mitochondrial respiration and actin (1:4000 dilution, Santa Cruz biotechnology, TX, USA); a loading control, followed by one hour of incubation at room temperature with the horseradish peroxidase–conjugated secondary antibody (1:2000 dilution, Cell Signaling Technology, Danvers, MA, USA). The blots were visualized by ECL reagent

(Bio-Rad Laboratories, CA, USA). The western blot pictures were carried out using the ChemiDoc Touching system (Bio-Rad Laboratories, CA, USA). The densitometric analysis was performed using NIH Image J analysis software. For quantitation of the proteins of interest, the ratio of ischemic (I) area per remote (R) area was determined, and normalized with actin.

HPLC-based assay of malondialdehyde (MDA) concentration

Malondialdehyde (MDA) concentration in cardiac tissue was measured by HPLC system.[80] A 0.5 ml aliquot of samples were mixed with 1.1 ml of 10% trichloroacetic acid (TCA) containing BHT (50 ppm), heated at 90 °C for 30 minutes and cooled down to room temperature. The mixture was centrifuged at 6,000 rpm, 10 minutes. The supernatant (0.5 ml) was mixed with 0.44 M H_3PO_4 (1.5 ml) and 0.6% thiobabituric acid (TBA) solution (1.0 ml) and then incubated at 90°C for 30 minutes to generate a pink-colored products called thiobarbituric acid reactive substances (TBARS). The solution was filtered through a syringe filter (polysulfone type membrane, pore size 0.45 μm, Whatman International, Maidstone, United Kingdom) and analyzed with HPLC system. The TBARS was fractionated on the adsorption column (Water Spherosorb ODS2 type, 250×4.3 mm, 5 μm), eluted with mobile-phase solvent of 50 mM KH_2PO_4 : methanol at flow rate 1.0 ml/min and online detected at 532 nm. Data was recorded and analyzed with BDS software (BarSpec Ltd., Rehovot, Israel). A standard curve was constructed from the peak from height of standard 1,1,3,3-tetramethoxypropane (standard reagent for malondialdehyde) at different concentrations (0-10 μM). TBARS concentration was determined directly from standard curve and reported as MDA equivalent concentration. MDA concentration was expressed in μM.[80]

Cardiac inflammatory and anti-inflammatory cytokine assay

Myocardial protein was extracted by the homogenization of myocardial tissues in a homogenization buffer (PBS containing 0.5% Triton X-100 and a protease inhibitor cocktail, pH 7.2 at 4ºC), and subsequently be centrifuged at 14,359 g for 10 minutes. Then, the supernatant and plasma were collected to measure the level of tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10), a marker of pro-inflammatory and anti-inflammatory cytokines, by using an enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Inc., Camarillo, CA, USA).

TUNEL

To quantitatively determine cardiomyocyte apoptosis, TUNEL staining (terminal Deoxynucleotidyl transferase-mediated dUTP nick end labeling) was performed. TUNEL staining of cardiomyocyte was performed with a TdT-Blue Label apoptosis detection kit. The enzyme terminal deoxynucleotidyl transferase was used to incorporate biotinylated-conjugated dUTP to the ends of DNA fragments. At the end of the experiment, the hearts were perfused first with 0.9% NaCl for 5 minutes and then with 4% paraformaldehyde in PBS (pH 7.4) for 20 minutes. The ventricles were removed and further fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 hours at room temperature. The ventricles were cut into 10 μm sections for the TUNEL assay in a cryostat. Immunohistochemical procedures for detecting apoptotic cardiomyocytes were performed using an In Situ Apoptosis Detection Kit (Trevigen, Maryland, USA) according to the manufacturer's instructions. For the negative control, TdT was omitted from the reaction mixture. After washing, the label incorporated at the damaged sites of the DNA was visualized by fluorescence microscopy. Five images per heart (3-5 hearts

per genotype group) were acquired and positive cells were counted individually. Results were expressed as the percentage of apoptotic cells among the total cell population.[81]

Statistical Analysis

Data were expressed as mean ± standard error. The normality and equality of variance were tested using the Shapiro-Wilk test and Levene's test, respectively. Chi-square test was performed to compare mortality rate. The mean values between the two groups were compared using the paired student's t-test. One-way ANOVA with Dunnett's multiple-comparison or LSD tests using the statistical program SPSS22 (SPSS, Inc., Chicago, IL, USA) were used for multiple sets of data. A value of $p < 0.05$ was considered statistically significant.

ผลการทดลอง

Effects of VNS on the ECG parameters during I/R

Figure 15a shows examples of ECG tracing at baseline, a marked elevation of the ST-segment after 5 minutes of LAD occlusion and ECG tracing returned to baseline after reperfusion. The electrophysiological effects of VNS were examined in 30 pigs in which heart rate (HR), PR interval, QRS duration, QT interval, T-wave peak to end (Tpe), and Tpe/QT ratio were continuously measured during the I/R period. In I/R group, HR during the ischemic period increased significantly compared with the baseline and returned to the baseline after reperfusion (Figure 15b). HR at the baseline, during ischemia, and reperfusion periods was not different in all VNS-treated groups. Interestingly, HR significantly increased in the LtVNX group during ischemia. In

contrast, HR significantly increased during reperfusion period in the RtVNX group. However, in the atropine group, HR during ischemic and reperfusion periods significantly increased when compared with baseline. PR interval was no significant difference among all groups at the baseline, ischemic, and reperfusion periods (Figure 15c). In contrast, PR interval in the atropine group significantly decreased PR during ischemic and reperfusion periods when compared with baseline. QRS duration was not significant difference among all groups at the baseline, ischemic, and reperfusion periods (Figure 15d). QT interval during ischemia and reperfusion were significantly decreased in all groups (Figure 16b). Tpe were significantly decreased in all VNS-treated groups, except during ischemic period in RtVNX group and this effect was abolished by atropine (Figure 16c). Tpe/QT ratio in the I/R group during the ischemic period increased significantly compared with the baseline and atropine significantly increased Tpe/QT ratio during both ischemic and reperfusion periods (Figure 16d). Interestingly, there was no significant difference in Tpe/QT ratio during the baseline, ischemic, and reperfusion periods in all VNS treated groups (Figure 16d).

Effects of VNS on the occurrence of cardiac arrhythmia and mortality rate during/after myocardial I/R

Representative tracings of premature ventricular contractions (PVC), ventricular tachycardia (VT), and ventricular fibrillation (VF) have been shown in Figure 17a. The total number of PVC markedly decreased in both LC-VNS and LtVNX groups, but not RtVNX group, compared with the I/R group (Figure 17b) The total number of VT/VF episodes were significantly reduced in all VNS treated groups compared with the I/R group (Figure 17c). However, time to 1st VT/VF onset was not significantly different

among groups (Figure 17d). The arrhythmia scores were significantly decreased in both LC-VNS and LtVNX groups compared with the I/R group (Figure 18a). The mortality rate after I/R induction surgery was significantly lower only in the LC-VNS group compared with the I/R group (Figure 18b), with all mortality occurring during ischemia or immediately on reperfusion. The effect of VNS on connexin43 phosphorylation at serine 368 has been shown in Figure 18c. The connexin43 phosphorylation was significantly increased only in the LC-VNS group compared with the I/R group.

Effects of VNS on LV function and myocardial infarct size

The effect of VNS on LV function has been shown in Table 3. In the I/R group, the stroke volume, ejection fraction and stroke work were significantly decreased and the end-diastolic pressure was significantly increased during the ischemic and reperfusion periods compared with the baseline. Interestingly, all VNS treated groups, the LV functions were preserved during the ischemic and reperfusion periods. The beneficial effects of VNS on LV function were completely abolished by atropine. The percentage of the area at risk (AAR), a percentage of the total ventricular mass, was used to indicate myocardial infarct size. The AAR was not different among groups (I/R 32.8% ± 1.8%; LC-VNS 35.6% ± 2.4%; LtVNX 32.2% ± 1.7%; RtVNX 32.9 % ±0.8 %; Atropine 38.1% ± 1.9%; $p > 0.05$) (Figure 19a). All VNS treated groups significantly reduced myocardial infarct size compared with the I/R group and this effect was reversed by atropine. Interestingly, the myocardial infarct size was significantly increased in the RtVNX group when compared with the LC-VNS and the LtVNX groups. In contrast, the infarct sizes was not different between the LC-VNS and the LtVNX

group (I/R 45.7% ± 5.4%; VNS 5.1% ± 1.3%; LtVNX 7.1% ± 1.8%; RtVNX 17.6% ± 1.4%; Atropine 52.9% ± 3.6%; $p < 0.05$) (Figure 19b).

Effects of VNS on cardiomyocyte apoptosis

TUNEL staining was performed to detect cardiomyocyte apoptosis. TUNEL positive cells, reported as the percentage of total nuclei, were significantly increased in ischemic area when compared with remote area in the I/R injury group. In contrast, TUNEL positive cells were significantly decreased in all VNS treated groups and this effect was reversed by atropine (Figure 19c and 19d). Interestingly, % TUNEL positive cell was significantly increased in the RtVNX group when compared with the LC-VNS and the LtVNX groups. In contrast, % TUNEL positive cell was not different between the LC-VNS and the LtVNX group (Figure 19c and 19d). Additionally, the mechanism underlying the anti-infarct effect of VNS was determined by measuring the key apoptotic markers (Figure 20). The expression of Bax and the Cleaved caspase-3/Pro caspase-3 ratio were significantly decreased in the VNS treated groups. However, the levels of Bcl-2 were significantly increased in the LC-VNS and the LtVNX groups, but not in the RtVNX, compared with the I/R group. The administration of atropine totally abolished the anti-apoptotic effects of VNS.

Effects of VNS on oxidative stress activity (MDA) and inflammation

The changes of MDA levels in myocardium between ischemic and remote areas have been shown in Figure 21a. VNS significantly decreased the level of MDA in the myocardium compared with the I/R group. However, there was a statistic difference between the RtVNX group compared with the LC-VNS and LtVNX groups. This effect was abolished by atropine. Figure 21b shows the changes of TNF-α levels in

myocardium between ischemic and remote areas. Only the LC-VNS group significantly decreased the levels of TNF-α in the myocardium compared with the I/R group. There was no statistically significant difference among the groups with respect to the IL-10 levels (Figure 21c).

Effects of VNS on mitochondrial function

All VNS treated groups significantly decreased mitochondrial ROS production (Figure 22a) and prevented mitochondrial membrane depolarization (Figure 22b). However, only LC-VNS and LtVNX groups could prevent mitochondrial swelling after I/R (Figure 22c). Electron photomicrographs demonstrated that in the ischemic area of the I/R group, severe mitochondrial damage was observed as demonstrated by marked mitochondrial swelling accompanied by a disruption in membrane integrity (Figure 22d).

Effects of VNS on cardiac mitochondrial dynamics

The expression level of MFN2, OPA1, and DRP1 proteins were determined to evaluate mitochondrial dynamics (Figure 23). Both LC-VNS and LtVNX significantly increased the expression of MFN2 , OPA1, and the phosphorylation of DRP1 at Ser 637 as well as significantly decreased phosphorylation of DRP1 at Ser 616 compared with the I/R group. The RtVNX significantly increased OPA1 but not MFN2 and phosphorylation of DRP1 at Ser 637 compared with I/R group. In addition, the RtVNX significantly decreased phosphorylation of DRP1 at Ser 616 compared with the I/R group. The effects of VNS on cardiac mitochondrial dynamics were abolished by atropine.

Effects of VNS on cardiac mitochondrial biogenesis and fatty acid oxidation

The biogenesis of the cardiac mitochondria and fatty acid oxidation were studied by determining the key markers for cellular energy metabolism and fatty acid oxidation (AMPK phosphorylation, PGC1α and CPT1) (Figure 24). Both LC-VNS and LtVNX significantly increased the expression of AMPK phosphorylation, PGC1α and CPT1 compared with the I/R group. However, there was no statistically significant difference in AMPK phosphorylation and PGC1α levels in the RtVNX group. These effects were abolished by atropine.

Effects of VNS on cardiac mitochondrial oxidative phosphorylation

The expression of cardiac mitochondrial complex I, III and IV were significantly increased in all VNS treated groups when compared with the I/R group (Figure 25b, 25d and 25e). However, the level of cardiac mitochondrial complex II was significantly increased only in the LC-VNS when compared with the I/R group (Figure 25c). The level of mitochondrial complex V was not significantly different in all groups (Figure 25f). These effects were abolished by atropine.

อภิปรายผลการทดลอง

In this present study, we sought to investigate whether the cardioprotective effects against myocardial I/R injury of VNS were mainly due to direct ipsilateral efferent vagal fibers activation or indirect effects mediated by the afferent vagal fibers by using myocardial infarct size as the primary endpoint. Furthermore, roles of the contralateral efferent vagal fibers during VNS were also investigated. The major findings of this study are as followed: (1) VNS exerted cardioprotection against myocardial I/R injury via

attenuation of mitochondrial dysfunction, improved mitochondrial dynamics and shifted cardiac fatty acid metabolism toward beta oxidation; (2) LC-VNS and LtVNX produced more profound cardioprotection, particularly infarct size reduction, decreased arrhythmia score, oxidative stress and apoptosis and attenuated mitochondrial dysfunction compared to RtVNX; (3) VNS required both ipsilateral and contralateral efferent vagal activities to fully provide its cardioprotection. These findings suggest that selective efferent VNS may potentially be effective in attenuating myocardial I/R injury.

Impact of VNS during intact, after ipsilateral and contralateral vagus nerve transection on reperfusion arrhythmia and myocardial infarct size

Since both reperfusion arrhythmia[82] and infarct size[83] are the potential serious complications after myocardial reperfusion, strategies to limit these two components of I/R injury have significant therapeutic potential. In the present study, we found that LC-VNS prevented cardiac arrhythmias during I/R as indicated by decreasing the total number of PVC, VT/VF incidence, arrhythmia score, Tpe and preserving Tpe/QT ratio, suggesting that LC-VNS decreased heterogeneity of ventricular repolarization. It is well recognized that increased myocardial infarct size and decreased phosphorylation of connexin43 play an important role in the development of cardiac arrhythmias, including VT/VF, during I/R.[37] Thus, the anti-arrhythmic effects of VNS might be due to its potential to decrease the arrhythmogenic substrates during I/R by reducing myocardial infarct size and increasing the phosphorylation of connexin43 at the serine 368 residue. In the present study, we found that VNS significantly reduced myocardial infarct size and increased phosphorylation of connexin43 compared with the I/R group, which are consistent with our previous studies in swine model[65] and other studies in rat model.[37,

[84] Furthermore, LC-VNS also preserved LV function in the heart subjected to I/R injury when compared with baseline, which might be due to its ability to reduce myocardial infarct size. Because the vagal trunk consists primarily of afferent fibers (80%)[72], the role of these fibers, particularly during VNS, needs to be clearly assessed. Previous study reported that vagal afferent fibers are activated during VNS and decrease efferent parasympathetic electrophysiological and hemodynamic effects of electrical stimulation.[74] However, it is still remained unclear whether the cardioprotection against myocardial I/R injury of VNS is mainly due to direct vagal activation through its ipsilateral efferent vagal fibers (motor) or indirect effects mediated by the afferent vagal fibers (sensory). Thus, roles of the ipsitralateral afferent vagal fibers during VNS were also investigated by LtVNX 2 cm above the stimulation probe. Interestingly, we found that LtVNX exerted the anti-infarct and anti-arrhythmic effects similar to LC-VNS, suggesting that the anti-infarct effect of VNS were driven primarily through its efferent vagal fibers, rather than the indirect afferent vagal activation in the ipsilateral vagal trunk.

Although all VNS treated groups exerted cardioprotection against myocardial I/R injury, LC-VNS and LtVNX produced more profound cardioprotection, particularly infarct size reduction (by 89% for LC-VNS and 84% for LtVNX), compared to RtVNX (by 63% reduction). Moreover, for the anti-arrhythmic effect, RtVNX did not significantly decrease the total number of PVC and arrhythmia score, but significantly decreased VT/VF incidence when compared with I/R and atropine groups. Interestingly, RtVNX also preserved LV function similar to the LC-VNS and LtVNX groups. These beneficial effects of VNS were abolished by atropine. Previous study demonstrated that bilateral

cervical or subdiaphragmatic vagotomy can also abolish the infarct size reduction by remote ischemic conditioning with one cycle of ischemia (15 minutes) and reperfusion (10 minutes) on both hind limbs in rats[85], suggesting the important role of efferent vagal tone to the heart. Moreover, VNS can mimic the effect of remote ischemic conditioning in rabbits[86] and pigs[87] and reduced infarct size. Interestingly, a previous study using an optogenetic approach to recruit vagal efferent fibers clearly demonstrated that stimulation of vagal efferents exerted cardioprotection against I/R injury.[88] Thus, these findings suggest that selective efferent VNS may potentially be effective in attenuating myocardial I/R injury partly through mimicking the effect of remote ischemic conditioning.[71, 89] Moreover, our finding also suggested that VNS required the contralateral efferent vagal activities to fully provide its cardioprotection. A recent study in an in vivo rat model of acute myocardial I/R injury demonstrated that infarct size and serum cTnI and CK-MB levels were markedly lower in the combined vagal stimulation perconditioning (VSPerC) and limb remote ischemic perconditioning (LRIPerC) group compared to the use of either treatment alone[90], which indicated that the combination of the two interventions significantly improved cardioprotection compared to the use of either treatment alone.

Impact of VNS during intact, after ipsilateral and contralateral vagus nerve transection on myocardial apoptosis during I/R

In the present study, we found that, all VNS treated groups markedly decreased the expression of Bax (a pro-apoptotic protein), Claved caspase-3/Pro caspase-3 ratio, and % TUNEL positive cells. However, only LC-VNS and LtVNX significantly increased

the expression of Bcl-2 (an anti-apoptotic protein) when compared with other groups. Moreover, % TUNEL positive cell of RtVNX significantly increased when compared with LC-VNS (both intact and LtVNX), which also consistent with myocardial infract size. Myocardial I/R injury leads to the activation of program cell deaths, including cell apoptosis and necrosis.[91] Specifically, the decrease in an anti-apoptosis Bcl-2 and the increase in a pro-apoptosis Bax expression are the underling of myocardial ischemia-induced apoptosis.[33] Moreover, a recent study demonstrated that overexpression of cardiac specific caspase 3, a key molecule in the execution of apoptosis, decreased cardiac function and caused abnormality of ultrastructural damage to the nucleus as measured by the TUNEL staining method.[81] These results suggested that the anti-apoptosis Bcl-2 molecule could be responsible for the reduction of % TUNEL positive cells observed in both LC-VNS and LtVNX groups. Previous study in rat model demonstrated that VNS prevents downregulation of the anti-apoptotic protein Bcl-2.[33] which consistent with our finding. Thus, our results suggested that VNS reduced myocardial infarct size through the anti-apoptotic effect. Moreover, VNS also required the contralateral efferent vagal activities to fully provide its cardioprotection.

Impact of VNS during intact, after ipsilateral and contralateral vagus nerve transection on cardiac mitochondrial function and inflammation during I/R

Furthermore, accumulating evidence has demonstrated that myocardial ischemia and post-ischemic reperfusion cause a wide array of functional and structural alterations of cardiac mitochondria.[15, 39, 65, 92, 93] Our previous studies have shown that one potential possible mechanism underlying the pronounced cardioprotection of VNS

against I/R injury is through the prevention of cardiac mitochondrial dysfunction.[15, 65] Increasing ROS production and the oscillation of mitochondrial membrane potential have been shown to play an important role in the genesis of cardiac arrhythmias and myocardial infarction.[39] In the present study, all VNS treated groups significantly reduced cardiac mitochondrial ROS production and prevented depolarization of mitochondrial membrane potential. These results could be responsible for the anti-infarct, anti-arrhythmia and the preservation of cardiac function of VNS in the heart subjected to myocardial I/R. Interestingly, we found that both LC-VNS and LtVNX, but not RtVNX, could prevent mitochondrial swelling after I/R injury. This result might explain why LC-VNS and LtVNX have higher efficiency on infarct size and prevention of cardiac arrhythmia than RtVNX.

In addition to cardiac mitochondrial dysfunction during I/R, inflammatory processes have been shown to play a critical role during myocardial I/R injury.[94] In the present study, only LC-VNS significantly decreased the expression level of myocardial TNF-α (a pro-inflammation marker) after I/R injury. However, the level of tissue TNF-α was not changed after vagus nerve transection, suggesting that VNS required both ipsilateral and contralateral efferent vagal activities to fully exert the anti-inflammation. Thus, it is reasonable to speculate that LC-VNS, both efferent vagal fibers are intact, provides the vagal tone that high enough to reach the activation threshold of the anti-inflammatory signaling pathway. However, the expression level of myocardial IL-10 (anti-inflammation) tended to increase in all VNS treatments after I/R injury, but did not reach the statistically significant level. Additionally, both LC-VNS and LtVNX significantly decreased the oxidative stress level as shown by the reduction in

myocardial MDA levels after I/R when compared with RtVNX and atropine groups. Again, this result suggests that both efferent vagal fibers are required for VNS to provide the vagal tone that high enough to reach the activation threshold of the anti-oxidative effect.

Impact of VNS during intact, after ipsilateral and contralateral vagus nerve transection on cardiac myocardial dynamics and fatty acid oxidation during I/R

Mitochondria are dynamic organelles that continually undergo fusion and fission.[64, 73, 81, 95, 96] Generally, mitochondrial outer and inner membrane fusion events are mediated by MFN1/2 and OPA1 protein.[97] In contrast, phosphorylation of DRP1 on Ser 616 promotes mitochondrial fission and phosphorylation of DRP1 on Ser 637 inhibits mitochondrial fission.[95] A growing body of literature has shown that enhancing mitochondrial dynamics and reducing mitochondrial oxidative stress have emerged as crucial therapeutic strategies to ameliorate myocardial I/R injury.[98, 99] In the present study, LC-VNS and LtVNX, but not RtVNX, significantly increased mitochondrial fusion protein (MFN2 and OPA1) expression, increased phosphorylation of DRP1 on Ser 637, and decreased the phosphorylation of DRP1 on Ser 616 when compared with I/R group.

Additionally, previous studies demonstrated that AMPK activation played an important role in ACh mediated cell survival via an AMPK induced cardiomyocyte autophagy pathway during cardiomyocyte hypoxia/reoxygenation injury.[100] Interestingly, AMPK activation can prevent mitochondrial fission by decreasing DRP1 and Fis1 levels in high glucose induced endothelial apoptosis.[101] In the present study, LC-VNS and

LtVNX, but not RtVNX, significantly increased the phosphorylation of AMPK when compared with I/R group, suggesting that AMPK was indeed involved in VNS mediated protection of mitochondrial dynamics and function. Furthermore, it has been shown that pathological stressors for the heart, such as ischemia, are associated with a downregulation of mitochondrial biogenesis via PGC1α activity[102], and the impairment of the PGC1α-mediated mitochondrial biogenesis increased heart vulnerability to myocardial I/R injury.[99] Accordingly, upregulation of the PGC1α pathway has been shown to confer protection against simulated I/R in cardiomyoblast cells.[98] Previous studies in skeletal muscle have demonstrated that pharmacological- or exercise-induced AMPK activation increased PGC1α to promote mitochondrial biogenesis.[103-105] In the present study, LC-VNS and LtVNX, but not RtVNX, significantly increased the expression level of AMPK phosphorylation and PGC1α. Moreover, VNS significantly increased CPT1 expression in the heart subjected to I/R injury and this effect was abolished after administration of atropine, suggesting that VNS exerts its cardioprotection against I/R injury through muscarinic receptors (mAChR). At cellular level, acetylcholine (ACh), a neurotransmitter of the cardiac vagus nerve has been shown to replicate the effect of cardiac ischemic conditioning, a therapeutic strategy for protecting organs or tissue against the detrimental effects of myocardial I/R injury. ACh, a non-selective ligand, initiates its downstream signal by activating G-protein-coupled mAChR or by binding to nicotinic receptors (nAChR) that are ligand-gated ion channels, and both receptors are present in the heart.[106-108] Interestingly, previous study demonstrated that both mAChR and nAChR significantly increased after I/R, suggesting the compensatory response to myocardial I/R injury.[108] Although the effects of ACh on

both electrical and mechanical properties of the heart are well known and have been attributed to mAChRs activation, including our present study, the effects of the action of ACh on α7nAChRs can not be excluded. In an isolated perfused rat heart, GTS21 (α7nAChR agonist) administration at the initiation of reperfusion provided therapeutic benefit by improving cardiac contractile function through stimulating prosurvival signaling pathways, leading to the preservation of mitochondrial function, maintaining intracellular ATP and reducing ROS production, thus limiting infarct size.[108] Moreover, during ischemia, VNS exhibited a significant reduction in the number of apoptotic cells.[12] Interestingly, this beneficial effect was abrogated by mecamylamine (MEC), a non-selective α7nAChR antagonist.[12] Additionally, VNS has been shown to protect against remote vascular dysfunction, through the cholinergic anti-inflammatory pathway which is dependent on α7nAChR.[109] These findings suggest that not only the activation of the mAChRs, but the activation of α7nAChRs can also trigger cardioprotective signaling cascades which are effective against I/R injury. Thus, the distinct role of mAChR versus nAChR on these mechanisms in the heart remains to be determined.

The observed elevation of AMPK phosphorylation, PGC1α and CPT1 expression suggests that cardiac fatty acid metabolism is shifted toward mitochondrial beta oxidation. Furthermore, increased levels of cardiac mitochondrial complex I, II (only in the LC-VNS), III and IV of the electron transport chain were significantly increased in VNS treated groups. Therefore, increased levels of cardiac mitochondrial complexes by VNS may also be responsible for the preservation of cardiac function in the heart subjected to I/R injury. In summary, we have demonstrated that the mechanism underlying the cardioprotection of VNS were associated with anti-apoptosis,

anti-oxidative stress, anti-inflammation, prevent cardiac mitochondrial dysfunction, improved mitochondrial dynamic (increased mitochondrial fusion and decreased mitochondrial fission), improved mitochondrial biogenesis, shifted cardiac fatty acid metabolism toward beta oxidation and increased levels of cardiac mitochondrial complexes. Finally, VNS required both ipsilateral and contralateral efferent vagal activities to fully provide its cardioprotection against myocardial I/R injury, suggesting the important role of maintaining cardiac vagal tone during I/R.

Conclusions and clinical implications

Our study has shown that VNS exerted cardioprotection against myocardial I/R injury via attenuation of mitochondrial dysfunction, increased mitochondrial fusion, decreased mitochondrial fission and shifted cardiac fatty acid metabolism toward beta oxidation. However, LC-VNS and LtVNX produced more profound cardioprotection, particularly infarct size reduction, decreased arrhythmia score and apoptosis and attenuated mitochondrial dysfunction compared to RtVNX. Our findings suggest that selective efferent VNS may potentially be effective in attenuating myocardial I/R injury. Moreover, VNS also required the contralateral efferent vagal activities to fully provide its cardioprotection. It is important to note that most of the current devices implanted in animal and clinical investigations, activate both afferent and efferent pathways.[8, 10, 44, 70, 110-112] Recently, Patel and colleagues have developed a kilohertz electrical stimulation (KES) nerve block technique to preferentially activate efferent pathways while blocking afferent pathways without the need to transect the vagus nerve.[113] Thus, by using KES nerve block that selectively stimulates the efferent vagal nerve fibers, VNS may

potentially be an attractive potential adjuvant therapy to limit reperfusion injury in patients with acute MI. However, further clinical studies are needed before we can conclude that VNS is a viable clinical treatment in the affected MI patients.

Table 3 Effects of VNS and Atropine on pressure-volume loop-derived functional parameters

	I/R			LC-VNS			LtVNX			RtVNX			Atropine		
Parameter	Baseline	Ischemia	Reperfusion	Baseline	Ischemia	Reperfusion	Baseline	Ischemia	Reperfusion	Baseline	Ischemia	Reperfusion	Baseline	Ischemia	Reperfusion
SV (mL)	27 ± 3	16 ± 3*	14 ± 3*	26 ± 1	25 ± 3	26 ± 3	29 ± 4	32 ± 5	20 ± 3	34 ± 7	37 ± 5	39 ± 6	26 ± 1	15 ± 1*	15 ± 1*
EF (%)	49 ± 5	28 ± 5*	38 ± 3*	49 ± 4	46 ± 3	49 ± 7	40 ± 4	41 ± 5	44 ± 3	42 ± 7	39 ± 7	43 ± 6	48 ± 3	33 ± 3*	36 ± 2*
ESP (mm Hg)	83 ± 6	75 ± 6	86 ± 7	82 ± 9	70 ± 4	65 ± 4	65 ± 3	64 ± 3	67 ± 2	69 ± 3	65 ± 2	62 ± 3	77 ± 4	71 ± 6	74 ± 3
EDP (mm Hg)	9 ± 2	18 ± 3*	21 ± 5*	11 ± 3	14 ± 4	13 ± 2	16 ± 2	20 ± 3	21 ± 2	11 ± 1	13 ± 2	11 ± 2	10 ± 1	18 ± 1*	18 ± 2*
SW (mmHg·mL)	2126 ± 188	906 ± 259*	879 ± 267*	1811 ± 168	±1824 298	±1744 ± 285	1883 ± 285	±2176 285	±1667 ± 504	2019 ± 381	±1319 297	±1288 ± 210	1687 ± 218	941 ± 120*	1035 ± 134*

Summary of left ventricular (LV) functional parameters at baseline, at 5 min before the end of ischemia, and at 5 min before end of reperfusion (n = 6 per group). Values are presented as mean ± SE. * $p < 0.05$ vs baseline within group. EDP = end-diastolic pressure; EF = ejection fraction; ESP = end-systolic pressure; I/R = ischemia reperfusion; LC-VNS = vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection; SV= stroke volume; SW = stroke work.

Figure 14: Schematic representation of the study protocols. a: Diagram of the I/R induction surgery. The ischemic period (60 minutes) was induced by complete LAD coronary artery ligation, followed by 120 minutes of reperfusion. VNS was applied at the onset of the ischemic period. b: Diagram of the experimental protocol. I/R = ischemia/reperfusion; LAD = left anterior descending; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

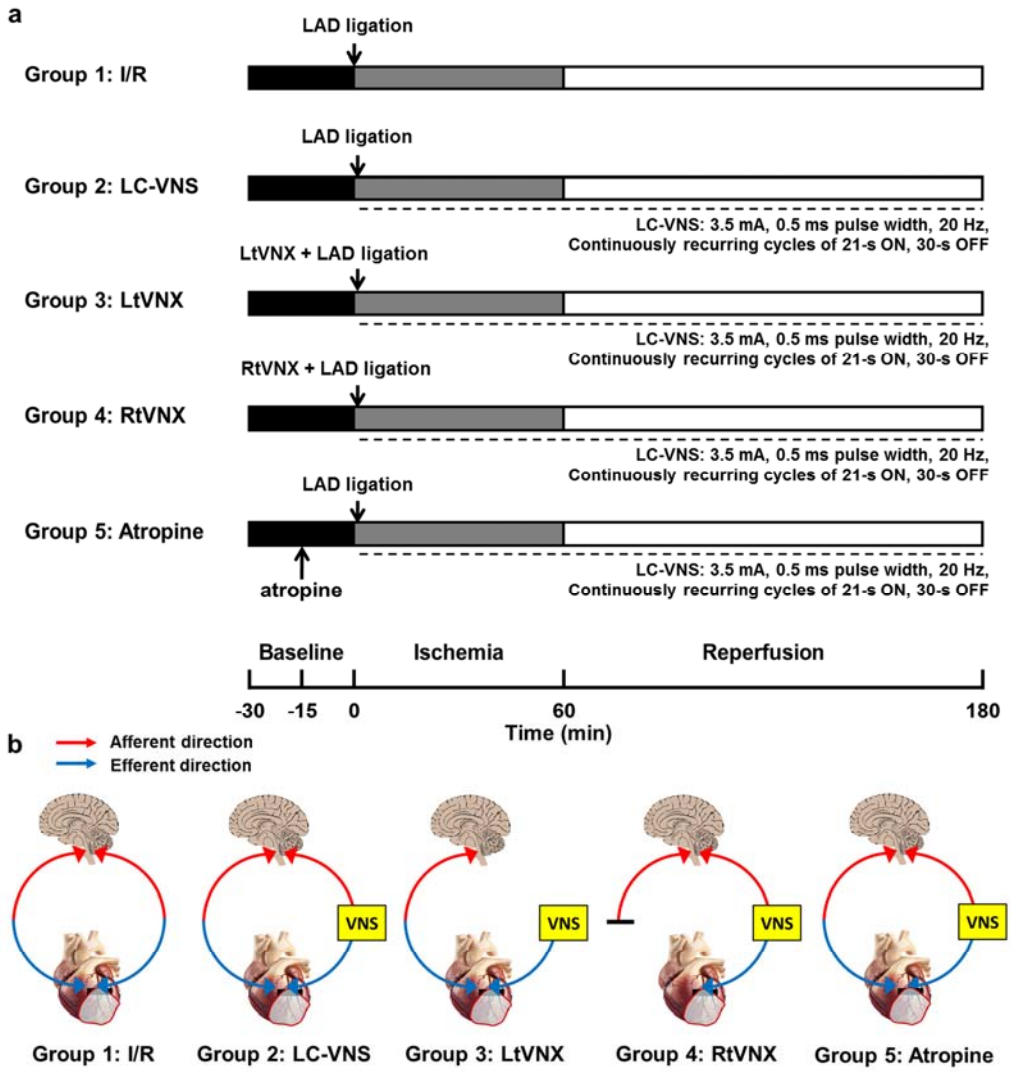


Figure 15: The electrocardiographic parameters during the ischemic and the reperfusion periods. a: Representative ECG at the baseline, 5 min after LAD ligation and 5 min before end of reperfusion. b: Effects of VNS on the heart rate. c: Effects of VNS on the mean PR interval. d: Effects of VNS on the mean QRS duration. Data are presented as mean±SE. *p < 0.05 vs baseline within group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

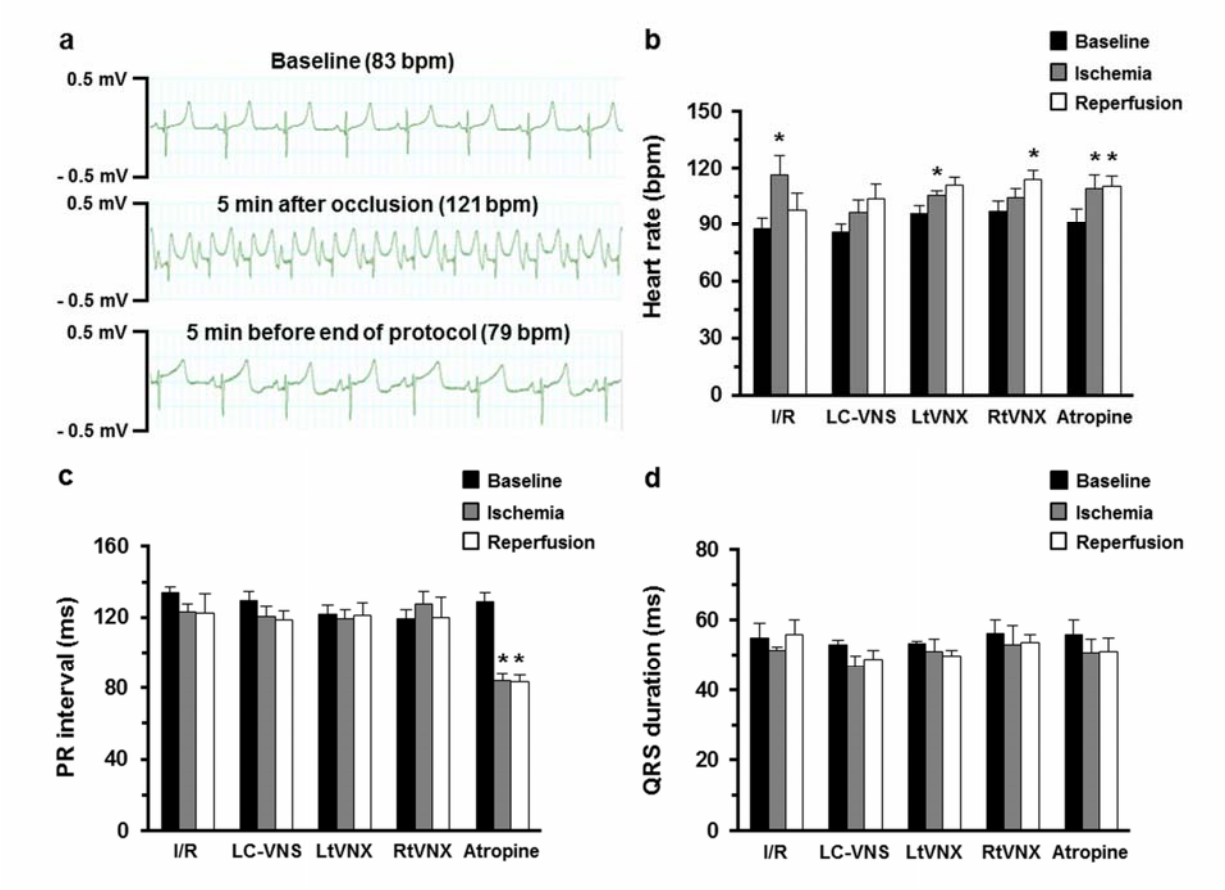


Figure 16: The electrocardiographic parameters during the ischemic and the reperfusion periods. a: Representative of the Tpe interval in LC-VNS group. b: Effects of VNS on the QT interval. c: Effects of VNS on the Tpe interval. d: Effects of VNS on the Tpe/QT ratio. Data are presented as mean±SE. *p < 0.05 vs baseline within group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; QT = QT interval; RtVNX = right vagus nerve transection; Tpe = T-wave peak to end.

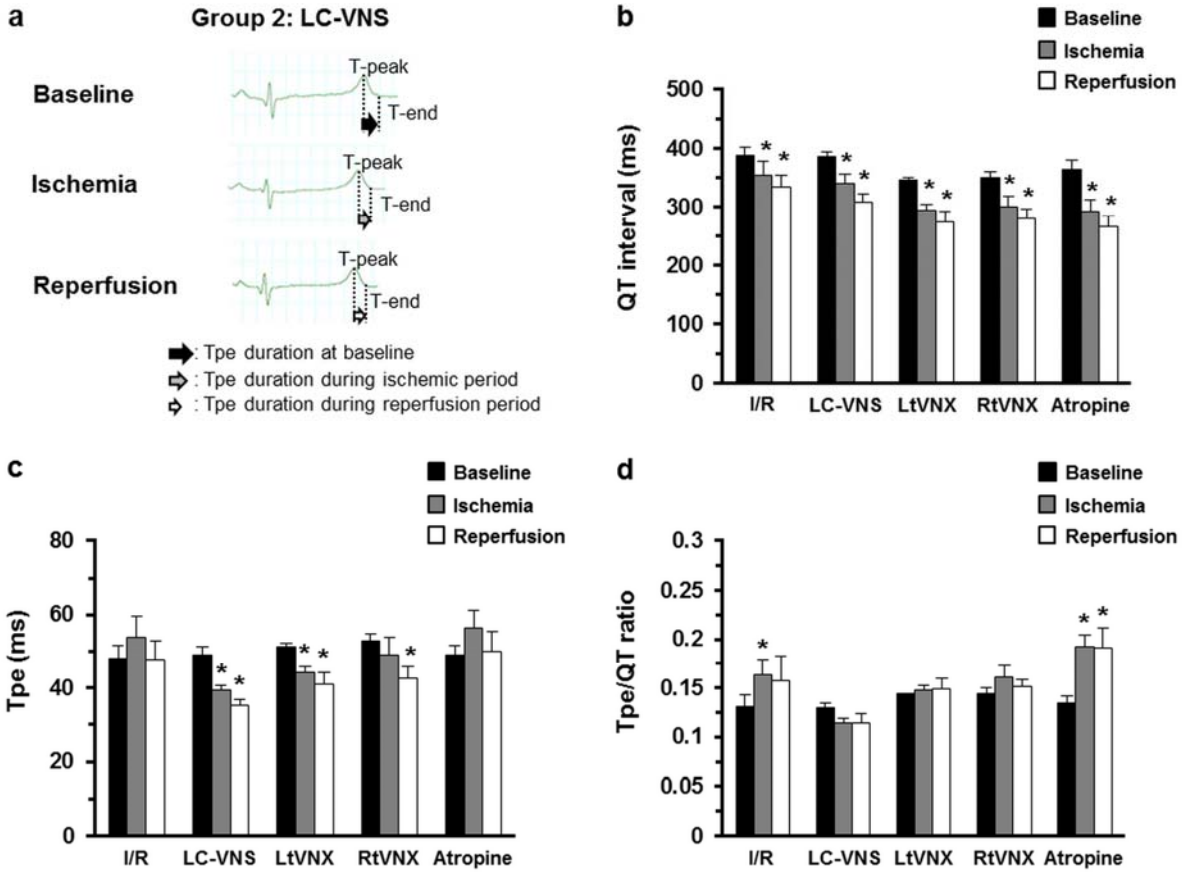


Figure 17: Effects of VNS on the incidence of cardiac arrhythmias. a: Representative morphology of the PVC, VT and VF. b: Effects of VNS on the total number of PVC. c: Effects of VNS on the total number of VT/VF. d: Effects of VNS on the time to 1st VT/VF. Data are presented as mean±SE. *p < 0.05 vs I/R group; †p < 0.05 vs RtVNX group; ‡p < 0.05 vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection; PVC = premature ventricular contraction; VF = ventricular fibrillation; VT = ventricular tachycardia.

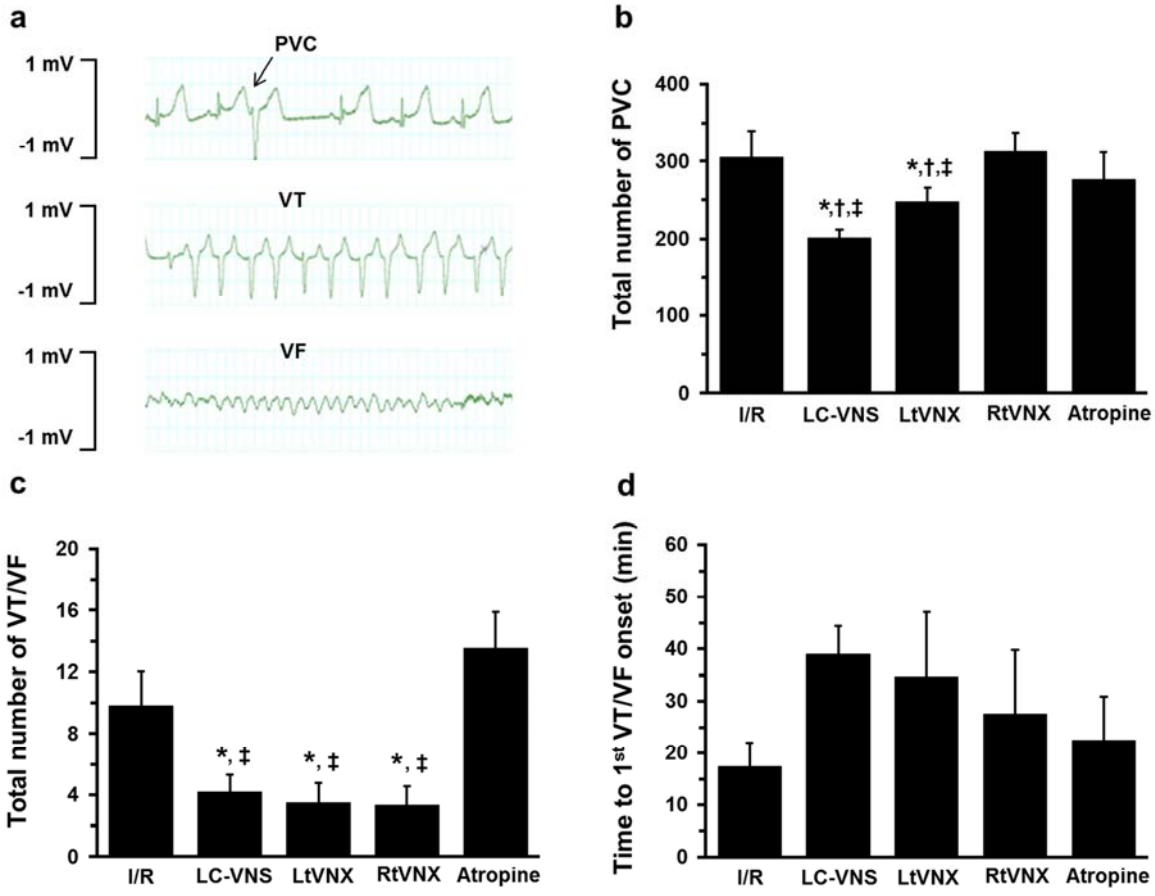


Figure 18: Effects of VNS on arrhythmia score, percent mortality during I/R induction and myocardial connexin43 expression. a: Effects of VNS on the arrhythmia score. b: Percent mortality during I/R induction. c: Effects of VNS on the phosphorylation of connexin43 at serine 368 in the ischemic myocardium. Data are presented as mean±SE. *p < 0.05 vs I/R group; †p < 0.05 vs RtVNX group; ‡p < 0.05 vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection; PVC = premature ventricular contraction; VF = ventricular fibrillation; VT = ventricular tachycardia.

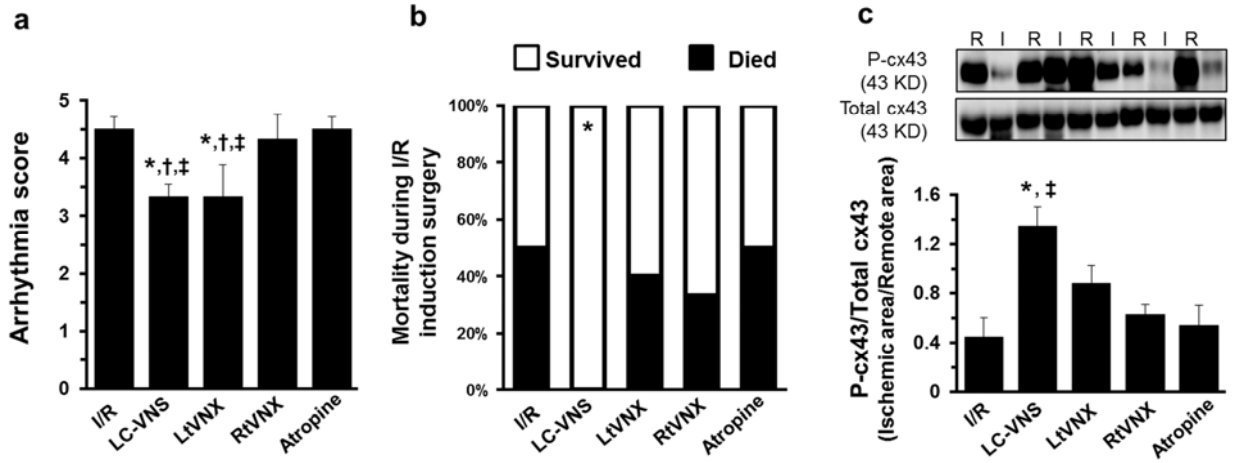


Figure 19: Effects of VNS on infarct size and the TUNEL positive cells. a: The total of area at risk. b: Effects of VNS on the myocardial infarct size per the area at risk (AAR). The inset shows representative photographs obtained after Evan Blue and triphenyltetrazolium chloride staining. Blue indicates the non-threatened myocardium, red indicates the non-infarcted area within the AAR, and white indicates myocardial infarction. c: Effects of VNS on the TUNEL positive cells. d: Representative Tunel assay. Arrow head represented Tunel positive cell. Data are presented as mean±SE. **p* < 0.05 vs I/R group; †*p* < 0.05 vs RtVNX group. ‡*p* < 0.05 vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

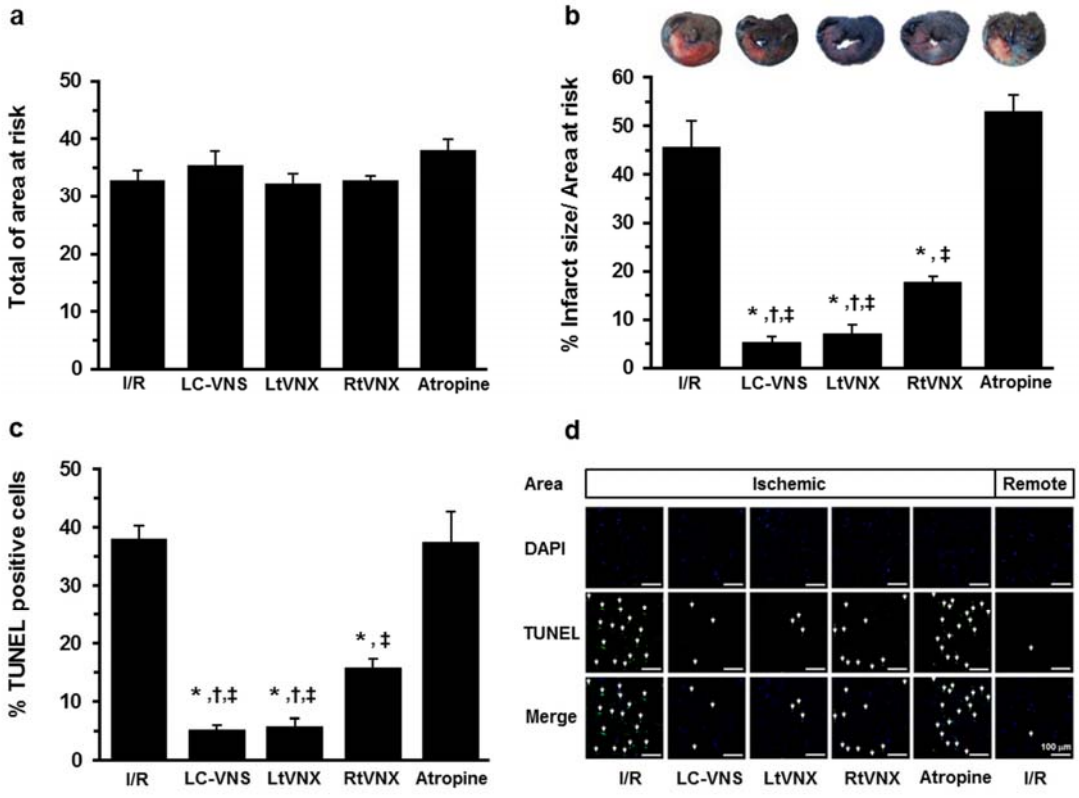


Figure 20: Effects of VNS on cardiomyocyte apoptosis. a: Representative bands for Bax, Bcl-2, Cleaved caspase-3 and Pro caspase-3. Actin was used as a loading control. b and c: Western blot analysis of Bax and Bcl-2. d: The ratio between cleaved caspase-3 and Pro caspase-3 expression. Data are presented as mean±SE. *p < 0.05 vs I/R group; †p < 0.05 vs RtVNX group; ‡p < 0.05 vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

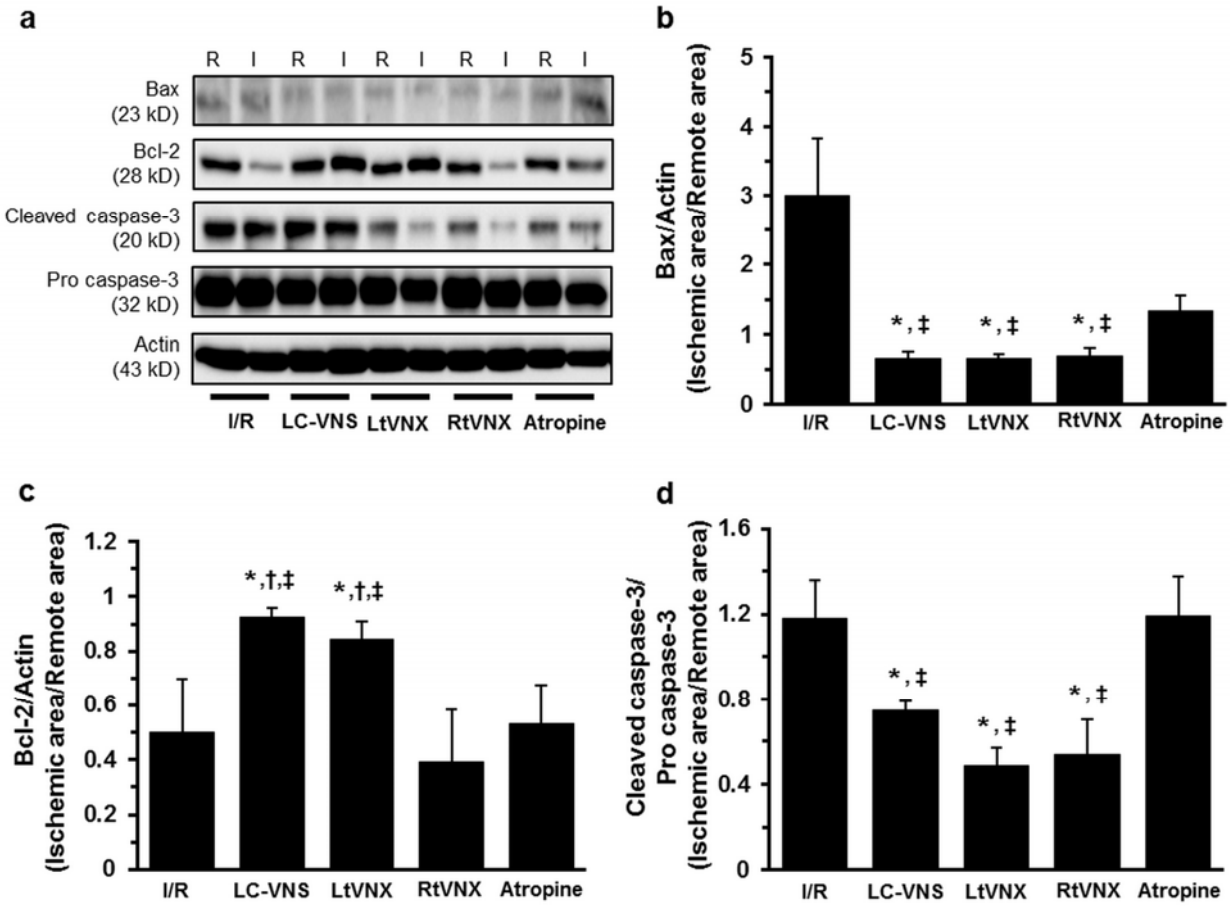


Figure 21: Effects of VNS on oxidative stress biomarker in myocardium. a: Myocardium MDA ratios between ischemic and remote areas. b: Myocardium TNF-α ratios between ischemic and remote areas. c: Myocardium IL-10 ratios between ischemic and remote areas. Data are presented as mean±SE. *p < 0.05 vs I/R group; †p < 0.05 vs RtVNX group; ‡p < 0.05 vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

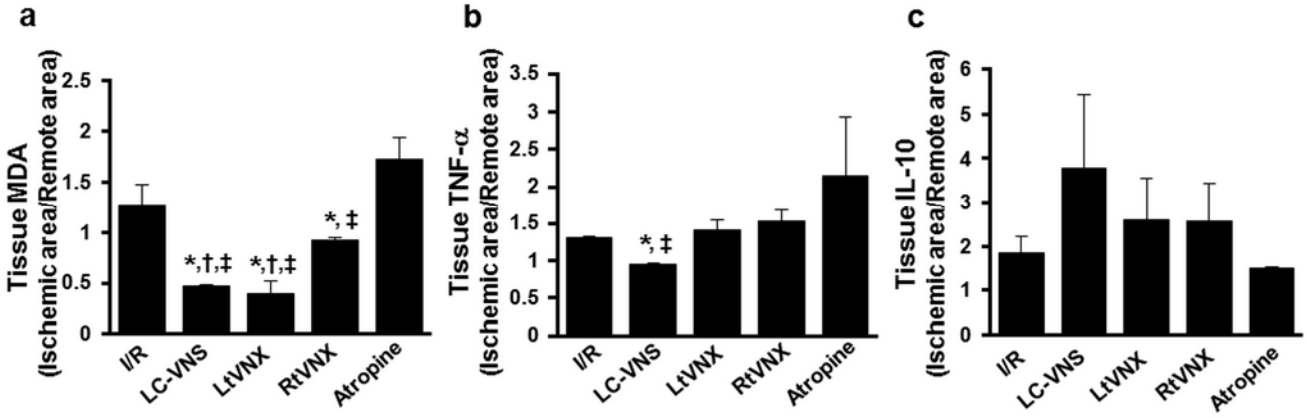


Figure 22: Effects of VNS on cardiac mitochondria function and morphology. a: Mitochondrial ROS production. b: Mitochondrial membrane depolarization. c: Mitochondrial swelling. d: Representative electron photomicrographs of a cardiac mitochondrial ultrastructure. Data are presented as mean±SE. #p < 0.05 vs remote area within group;*p < 0.05 vs I/R group; †p < 0.05 vs RtVNX group; ‡p < 0.05 vs Atropine group. I/R = ischemia reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

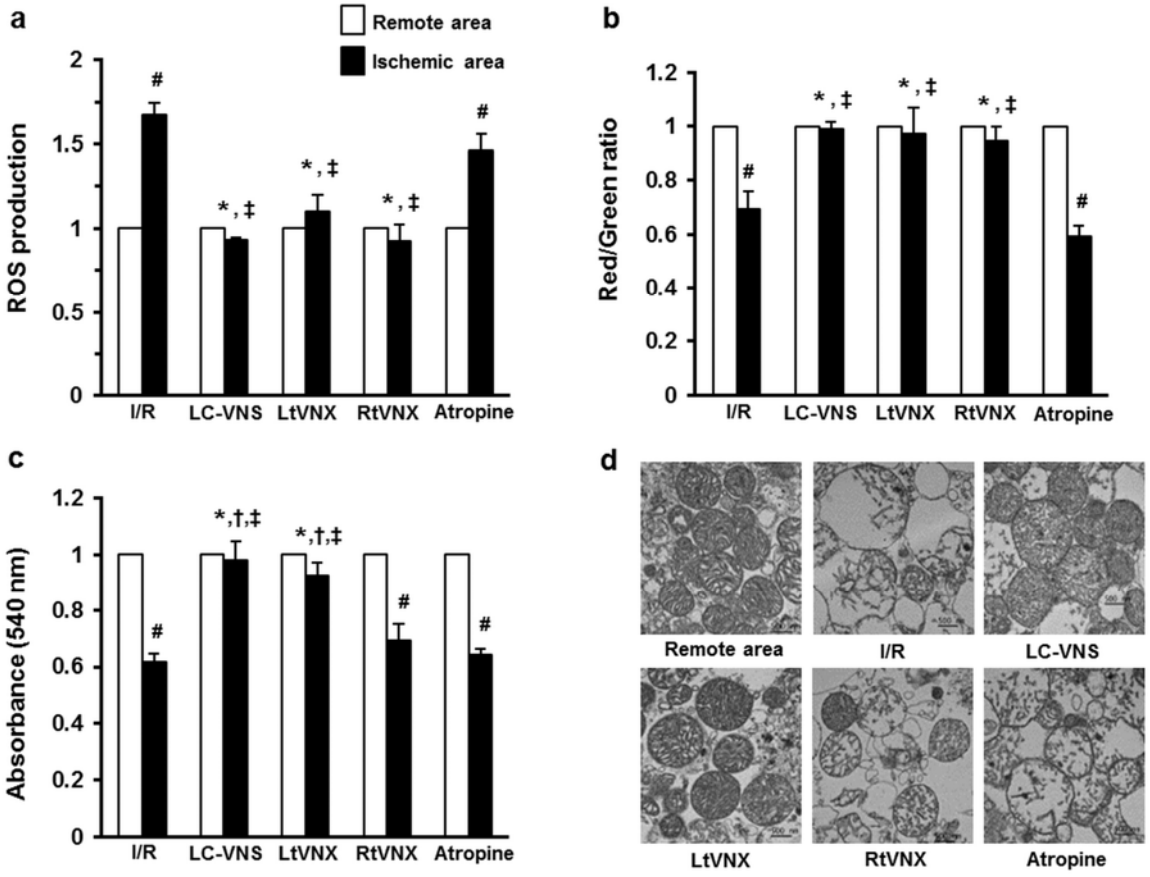


Figure 23: Effects of VNS on cardiac mitochondrial dynamics. a-d: Representative immunoblots (top) and densitometric analysis (bottom) of the mitochondrial dynamic proteins (OPA1, MFN2, P-DRP1 at Ser 616 and Ser 637). Data are presented as mean±SE. *p < 0.05 vs I/R group; †p < 0.05 vs RtVNX group; ‡p < 0.05 vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

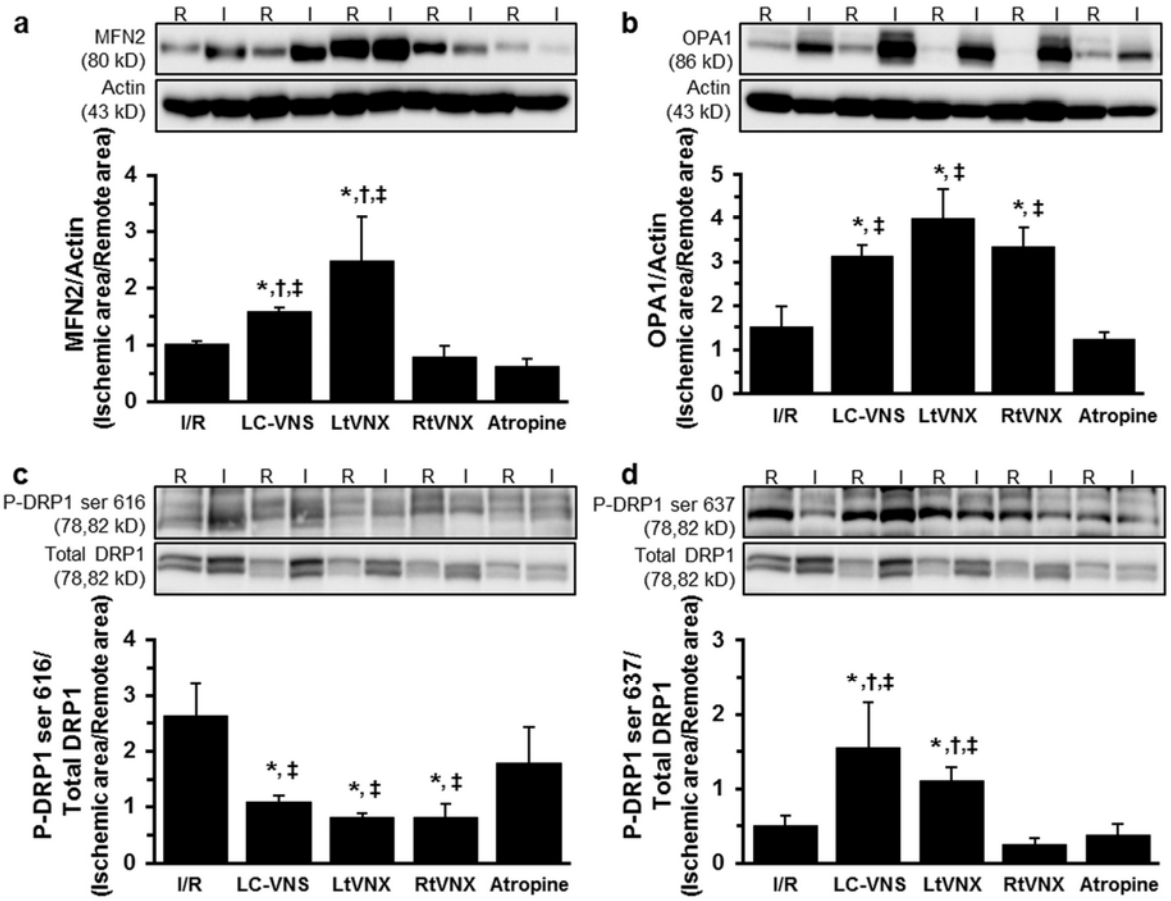


Figure 24: Effects of VNS on proteins related to cardiac metabolism. a: Representative immunoblots of key proteins involved in cardiac metabolism. b: Quantitative analysis of AMPK phosphorylation in the ischemic area normalized with that in the remote area, n = 6/group. c: Myocardial PGC1α expression in the ischemic area normalized with that in the remote area, n = 6/group. d: cardiac mitochondrial CPT1 expression in the ischemic area normalized with that in the remote area, n = 6/group. Data are presented as mean$\pm$SE. *p < 0.05 vs I/R group; †p < 0.05 vs RtVNX group; ‡p < 0.05 vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.

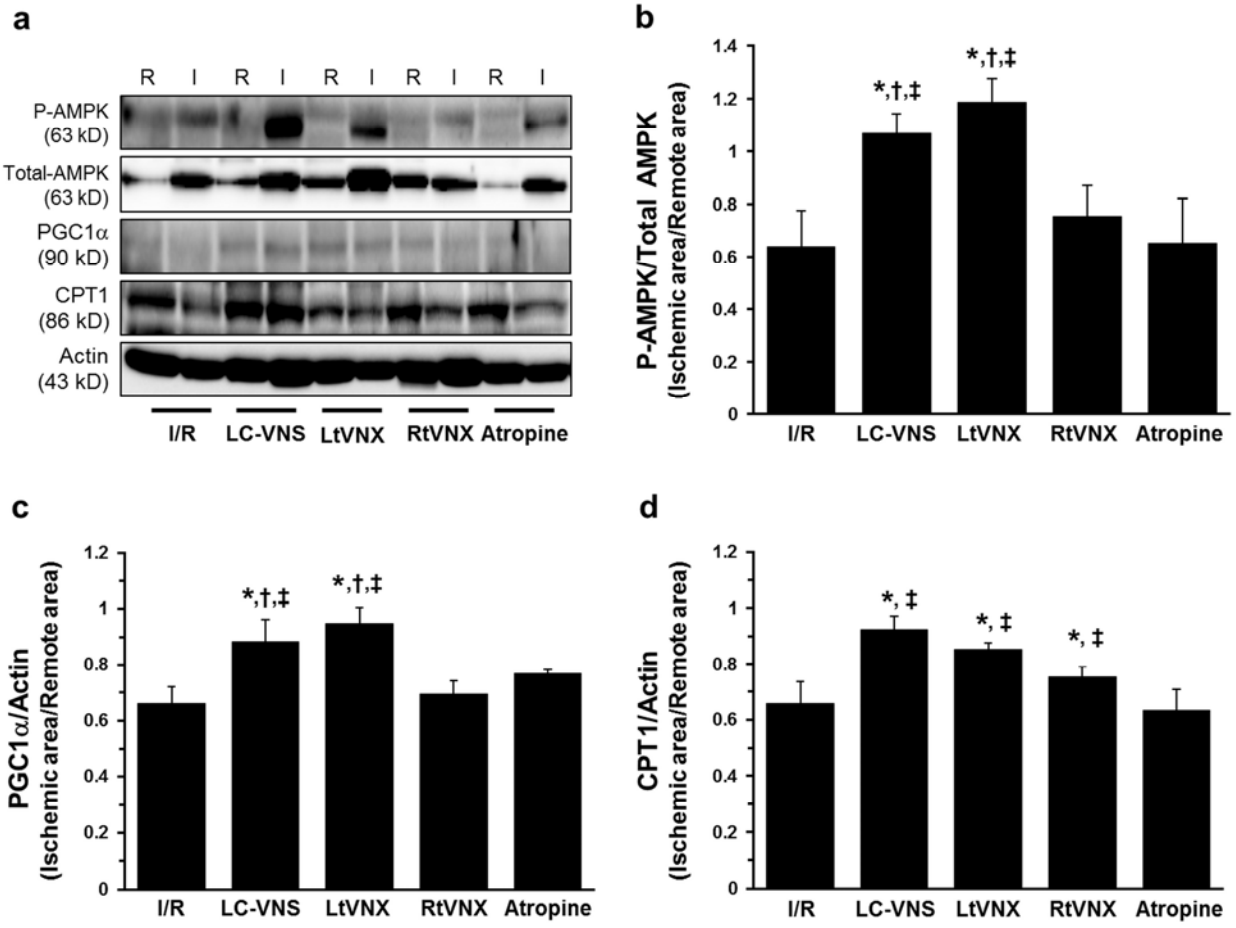
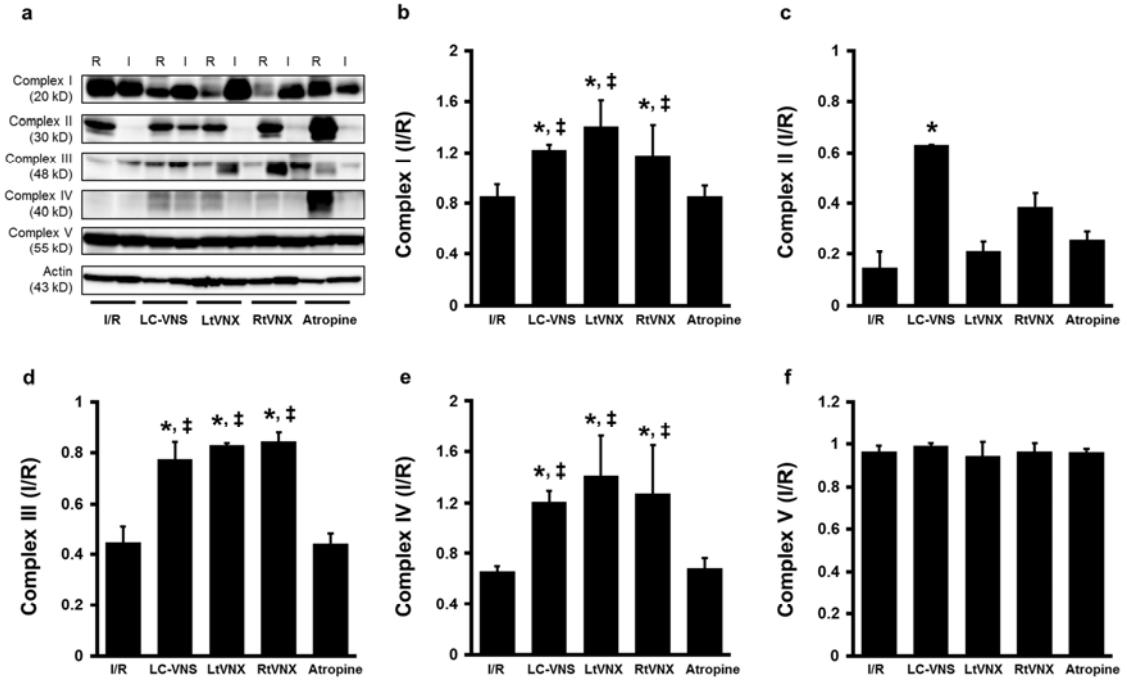


Figure 25: The effects of VNS on proteins related to cardiac mitochondrial oxidative phosphorylation in pigs with cardiac I/R injury. a: Representative Western blot bands of cardiac mitochondrial complex I, II, III, IV and V in the ischemic area and the remote area. b: cardiac mitochondrial complex I expression in the ischemic area normalized with that in the remote area, n = 6/group. c: cardiac mitochondrial complex II expression in the ischemic area normalized with that in the remote area, n = 6/group. d: cardiac mitochondrial complex III expression in the ischemic area normalized with that in the remote area, n = 6/group. e: cardiac mitochondrial complex IV expression in the ischemic area normalized with that in the remote area, n = 6/group. f: cardiac mitochondrial complex V expression in the ischemic area normalized with that in the remote area, n = 6/group. Data are presented as mean±SE. *p < 0.05 vs I/R group; †p < 0.05 vs RtVNX group; ‡p < 0.05 vs Atropine group. I/R = ischemia/reperfusion; LC-VNS = left cervical vagus nerve stimulation; LtVNX = left vagus nerve transection; RtVNX = right vagus nerve transection.



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OUTPUT

ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

1. Nuntaphum W, Pongkan W, Wongjaikam S, Thummasorn S, Tanajak P, Khamseekaew J, Intachai K, Chattipakorn S, Chattipakorn N, **Shinlapawittayatorn K**. Vagus Nerve Stimulation Exerts Cardioprotection Against Myocardial Ischemia/Reperfusion Injury Predominantly Through its Efferent Vagal Fibers. *Basic Res Cardiol* 2018 113(4):22. (Impact Factor = 5.723) **(เอกสารแนบหมายเลข 1)**

2. Charununtakorn ST, **Shinlapawittayatorn K**, Chattipakorn SC, Chattipakorn N. Humanin Directly Protects Cardiac Mitochondria Against Dysfunction Initiated by Oxidative Stress by Decreasing Complex I activity. *Mitochondrion*. 2018:38:31-40 (Impact Factor = 3.704) **(เอกสารแนบหมายเลข 2)**

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(เอกสารแนบหมายเลข 6)

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(เอกสารแนบหมายเลข 10)
*These authors contributed equally to this work.

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BOOK CHAPTERS

1. **Shinlapawittayatorn K**, Chattipakorn SC, Chattipakorn N. Vagus Nerve Stimulation: A Promising Cardioprotective Strategy Against Ischemia-Reperfusion Injury. In: Coronary Artery Disease - Research and Practice. iConcept Press. 2016 (ISBN 978-1-922227-98-0).
(เอกสารแนบหมายเลข 13)

การนำเสนอผลงานในงานประชุมทางวิชาการต่าง ๆ

1. **Shinlapawittayatorn K**, Chattipakorn S, Chattipakorn N. Vagus Nerve Stimulation Exerts Cardioprotection Against Myocardial Ischemia/Reperfusion Injury Predominantly Through its Efferent Vagal Fibers. J Physiol Sci 2018 (Impact Factor = 2.075)

นำเสนอที่ 95th Annual Meeting of the Physiological Society of Japan (JSP), Takamatsu, Kagawa, Japan

2. **Shinlapawittayatorn K**, NuntaphumW, Tanajak P, Thummasorn S, Khamseekaew J, Wongjaikam S, Chattipakorn S, Chattipakorn N. Vagus Nerve Stimulation Initiating Requires Both Ipsilateral and Contralateral Efferent Vagal Activity

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3. Charununtakorn ST, **Shinlapawittayatorn K**, Chattipakorn SC, Chattipakorn N. High-Dose Humanin Analogue Applied During Ischemia Provides Cardioprotection Against Ischemia-Reperfusion Injury Through Attenuating Mitochondrial Dysfunction. J Am Coll Cardiol 2017. (Impact Factor = 17.759)

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การผลิตนักศึกษาภายใต้การเป็นอาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก

1. นายวัฒนา นันต๊ะภูมิ (Mr. Watthana Nuntaphum)

นักศึกษาระดับปริญญาโท สาขาวิชาสรีรวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

สำเร็จการศึกษา 2/2560

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ผลงานวิจัย จำนวน 1 เรื่อง

"**Nuntaphum W**, Pongkan W, Wongjaikam S, Thummasorn S, Tanajak P, Khamseekaew J, Intachai K, Chattipakorn S, Chattipakorn N, **Shinlapawittayatorn K**. Vagus Nerve Stimulation Exerts Cardioprotection Against Myocardial Ischemia/Reperfusion Injury Predominantly Through its

Efferent Vagal Fibers. *Basic Res Cardiol* 2018 113(4):22. (Impact Factor = 5.723) **(เอกสารแนบหมายเลข 1)**

2. **นางสาวกรรณาภรณ์ อินทชัย (Miss Kannaporn Intachai)**
นักศึกษาระดับปริญญาเอก สาขาวิชาสรีรวิทยา คณะแพทยศาสตร์
มหาวิทยาลัยเชียงใหม่
อยู่ระหว่างการศึกษา คาดว่าจะจบปีการศึกษา 2563
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Intachai K, Chattipakorn S, Chattipakorn N, **Shinlapawittayatorn K.** Revisiting the Cardioprotective Effects of Acetylcholine Receptor Activation Against Myocardial Ischemia/Reperfusion Injury. *International Journal of Molecular Sciences* 2018 (Impact Factor = 3.687)

บทความสำหรับการเผยแพร่

ภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันเป็นปัญหาที่สำคัญอันดับต้นๆ ในหลายๆ ประเทศทั่วโลก อีกทั้งยังเป็นสาเหตุหลักของการเสียชีวิตของประชากรในกลุ่มประเทศอุตสาหกรรมรวมทั้งประเทศไทย ปัจจุบันวิธีการรักษาผู้ป่วยที่มีภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลัน คือ การปล่อยให้เลือดกลับไปเลี้ยงกล้ามเนื้อหัวใจบริเวณที่ขาดเลือดโดยการใช้ยาหรือ การใส่สายสวนทางหลอดเลือดแดงเพื่อทำการขยายหลอดเลือดที่ไปเลี้ยงหัวใจบริเวณที่มีการอุดตันเพื่อให้เลือดสามารถไหลผ่านได้ อย่างไรก็ตามจากงานวิจัยก่อนหน้านี้พบว่าการปล่อยให้เลือดกลับไปเลี้ยงกล้ามเนื้อหัวใจบริเวณที่ขาดเลือด ยังส่งผลทำให้กล้ามเนื้อหัวใจบางส่วนได้รับบาดเจ็บหรือตายได้ โดยภาวะดังกล่าวเรียกว่า “ภาวะการบาดเจ็บของกล้ามเนื้อหัวใจในภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันที่ได้รับเลือดกลับมาเลี้ยงใหม่” ซึ่งผู้ป่วยที่รอดชีวิตมีโอกาสที่จะเกิดภาวะหัวใจล้มเหลวตามมาได้ในเวลาต่อมา ดังนั้นจึงมีความจำเป็นที่จะต้องทำการวิจัยเพื่อที่จะเข้าใจถึงกลไกในการเกิด “ภาวะการบาดเจ็บของกล้ามเนื้อหัวใจในภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันที่ได้รับเลือดกลับมาเลี้ยงใหม่” อีกทั้งยังมีความจำเป็นในการทำวิจัยเพื่อหาวิธีการรักษาแบบใหม่เพื่อนำมาใช้ในการป้องกัน การเกิดภาวะดังกล่าวจากงานวิจัยในสัตว์ทดลองก่อนหน้านี้พบว่าการกระตุ้นเส้นประสาทเวกัสบริเวณลำคอ สามารถป้องกันการบาดเจ็บของกล้ามเนื้อหัวใจจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลัน และมีเลือดกลับมาหล่อเลี้ยงใหม่ได้ แต่ทว่ากลไกการป้องกันการบาดเจ็บของกล้ามเนื้อหัวใจจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลัน และมีเลือดกลับมาหล่อเลี้ยงใหม่โดยการกระตุ้นเส้นประสาทเวกัสยังไม่เป็นที่ทราบแน่ชัด

งานวิจัยในฉบับนี้เป็นการศึกษาถึง ผลของการกระตุ้นเส้นประสาทเวกัสระดับลำคอ ต่อภาวะการบาดเจ็บของกล้ามเนื้อหัวใจในภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันที่ได้รับเลือดกลับมาเลี้ยงใหม่ ซึ่งทางทีมผู้วิจัยพบว่าการกระตุ้นเส้นประสาทเวกัสข้างซ้ายระดับลำคอแบบ intermittent ทั้งในช่วงที่หัวใจพึ่งถูกเหนี่ยวนำให้เกิดภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลัน หรือแม้กระทั่งทำการกระตุ้นเส้นประสาทเวกัสในช่วงที่เกิดภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันผ่านมาแล้วเป็นระยะเวลา 30 นาที สามารถป้องกันการบาดเจ็บของหัวใจจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลันและมีเลือดกลับมาหล่อเลี้ยงใหม่ โดยทางทีมผู้วิจัยพบว่าการกระตุ้นเส้นประสาทเวกัสทำให้การทำงานและการเปลี่ยนแปลงรูปร่างของไมโทคอนเดรียดีขึ้น อีกทั้งยังทำให้เกิดการเปลี่ยนแปลงกระบวนการเผาผลาญกรดไขมันในไมโทคอนเดรียไปสู่กระบวนการเบต้าออกซิเดชั่น โดยข้อมูลทั้งหมดที่ได้จากการศึกษานี้สามารถสรุปได้ว่าผลในการป้องกันกล้ามเนื้อหัวใจจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลันและมีเลือดกลับมาหล่อเลี้ยงใหม่ของการกระตุ้นเส้นประสาทเวกัส ผ่านทางการนำกระแสประสาทขาออกไปยังหัวใจ (efferent) เป็นหลัก และยิ่งไปกว่านั้นหากต้องการประสิทธิภาพสูงสุดในการป้องกันการเกิดการบาดเจ็บจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลันและมีเลือดกลับมาหล่อเลี้ยงใหม่

จากการกระตุ้นเส้นประสาทเวกัส จะต้องมีการส่งสัญญาณกระแสประสาทขาออกไปยังหัวใจ (efferent) ผ่านเส้นประสาทเวกัสทั้งสองข้าง ดั้งนั้นการกระตุ้นเส้นประสาทเวกัสมีความเป็นไปได้ที่จะนำมาใช้ในการป้องกันการเกิดการบาดเจ็บจากภาวะกล้ามเนื้อหัวใจขาดเลือดเฉลียบพลัน และมีเลือดกลับมาหล่อเลี้ยงใหม่ในผู้ป่วยที่มีภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลัน ซึ่งจะเป็นการช่วยลดโอกาสในการเกิดภาวะหัวใจล้มเหลว และเป็นการช่วยให้ผู้ป่วยมีคุณภาพชีวิตที่ดีขึ้น