รายงานวิจัยฉบับสมบูรณ์

โครงการ: ผลของยาซิลเดนาฟิลซิเตรท และผลร่วมของไนตริกออกไซด์โดเนอร์กับซิลเดนาฟิลซิเตรทต่อความสามารถในการทำดีฟิบิเลชั่นในหัวใจ รวมทั้งผลต่อภาวะการเกิดการเต้นผิดจังหวะของหัวใจชนิดร้ายแรง

โดย

รองศาสตราจารย์ ดร. นายแพทย์ นิพนธ์ ฉัตรทิพากร

พฤษภาคม พ.ศ. 2552

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ผู้วิจัย

รองศาสตราจารย์ ดร. นายแพทย์ นิพนธ์ ฉัตรทิพากร
ศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ
ภาควิชาสรีรวิทยา คณะแพทยศาสตร์
มหาวิทยาลัยเชียงใหม่

สนับสุนนโดยสำนักงานกองทุนสนับสนุนการวิจัย

คำนำ

การเสียชีวิตโดยเฉียบพลันจากหัวใจ (Sudden Cardiac Death) เป็นสาเหตุหลักของการเสียชีวิตของประชากรในกลุ่มประเทศอุตสาหกรรมรวมทั้งประเทศไทย โดยที่สาเหตุหลักของการเสียชีวิต จะเกิดจากการเต้นของหัวใจห้องล่างที่ผิดจังหวะแบบร้ายแรงชนิดที่เรียกว่า Ventricular Fibrillation (VF) เป็นส่วนใหญ่ ในปัจจุบันนี้ วิธีการรักษา VF อย่างมีประสิทธิภาพมีอยู่เพียงวิธีเดียว คือ การใช้ไฟฟ้าแรงสูงเข้าไปช็อคหัวใจ หรือที่เรียกว่าการทำ ดีฟิบริลเลชั่น (Defibrillation) การรักษาแบบนี้มีผลข้างเคียงที่ไม่พึงประสงค์คือ การบาดเจ็บของกล้ามเนื้อหัวใจจากการได้รับไฟฟ้าแรงสูง ซึ่งอาจก่อให้เกิดภาวะหัวใจเต้นผิดจังหวะตามมาในภายหลังได้ ดังนั้น จึงมีการศึกษาวิจัยเพื่อที่จะพยายามลด หรือ กำจัดภาวะอันไม่พึงประสงค์ดังกล่าว โดยการใช้ยาหรือสารเคมีที่สังเคราะห์ขึ้น อย่างไรก็ตามยาบางตัวถึงแม้ว่าจะใช้ในการรักษาโรคบางชนิดได้อย่างมีประสิทธิภาพ แต่มีการค้นพบว่ากลับมีผลในการลดประสิทธิภาพในการทำ Defibrillation ไปด้วยในขณะเดียวกัน ซึ่งในปัจจุบันนี้มียาตัวหนึ่งที่มีรายงานว่าพบผลข้างเคียงดังกล่าว คือยากลุ่มที่ยับยั้ง Phosphodiesterase-5 enzyme

งานวิจัยในฉบับนี้เป็นการศึกษาถึงความสัมพันธ์ของยา Phosphodiesterase-5 inhibitor ที่ชื่อ sildenafil citrate ต่อการทำ Defibrillation ทั้งนี้เนื่องจากปัจจุบันมีการใช้ยาตัวนี้กันอย่างแพร่หลาย การศึกษาถึงผลของยาตัวนี้ต่อประสิทธิภาพในการทำ Defibrillation จะทำให้เกิดความเข้าใจต่อการใช้ยาเพื่อประโยชน์สูงสุดและเกิดผลอันไม่พึงประสงค์น้อยที่สุดต่อผู้ใช้ ซึ่งนับวันจะมีจำนวนเพิ่มมากขึ้น

สำหรับงานวิจัยในครั้งนี้ผู้วิจัยขอขอบคุณ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) (RMU4980001) และ คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ให้การสนับสนุนงานวิจัยในครั้งนี้เป็นอย่างดี ทำให้งานวิจัยสำเร็จลุล่วงได้

นิพนธ์ ฉัตรทิพากร

พฤษภาคม พ.ศ. 2552

สารบัญ

สารบัญรูปภาพ

สารบัญตาราง

บทคัดย่อภาษาไทย

แม้ว่าภาวะหัวใจเต้นผิดจังหวะชนิดร้ายแรงถึงขั้นเสียชีวิต จะถูกรายงานในผู้ป่วยที่ได้รับยาซิลเดนาฟิลซิเตรท ทว่าผลของยาชนิดนี้ต่อประสิทธิผลของการทำดีฟิบริลเลชั่นยังไม่เคยมีการวิจัยมาก่อน จุดมุ่งหมายของการศึกษาในครั้งนี้เพื่อทดสอบสมมุติฐานที่ว่าซิลเดนาฟิลซิเตรทสามารถ (1) เพิ่มความแรงของช็อคที่จำเป็นต้องใช้ เพื่อการทำดีฟิบริลเลชั่นให้ประสบความสำเร็จในขณะเกิดภาวะฟิบริลเลชั่นของหัวใจห้องล่าง (Ventricular fibrillation, VF) (2) เพิ่มค่าอับเปอร์ลิมิทออฟวัลเนอราบิลลิตี้ (upper limit of vulnerability, ULV) (3) ลดระดับค่าความแรงไฟฟ้าในการเหนี่ยวนำให้หัวใจเกิดการเต้นผิดจังหวะชนิดร้ายแรง (VF threshold, VFT) และ (4) ไนตริคอ็อกไซด์เป็นตัวเพิ่มความรุนแรงของซิลเดนาฟิล **วิธีการทดลอง** งานวิจัยนี้ใช้สุกรจำนวน 63 ตัว (น้ำหนักระหว่าง 25-30 กิโลกรัม) ในกลุ่มที่ได้รับยาจะถูกทดสอบที่ 2 ความเข้มข้นได้แก่ 100 มิลลิกรัม (ขนาดสูงกว่าที่ใช้ในการรักษาปรกติ) 50 (ขนาดปกติที่ใช้ในการรักษา) ส่วนกลุ่มควบคุมใช้ saline แทนซิลเดนาฟิล การวิจัยแบ่งออกเป็น 3 ส่วนเพื่อทดสอบหาค่าของความแรงไฟฟ้าต่ำสุดที่หยุด VF ได้ที่เรียกว่า ดีฟิบริลเลชั่นเทรชโฮล (defibrillation threshold, DFT, ส่วนที่ 1, n=18), ULV และ VFT (ส่วนที่ 2, n=24) ขณะที่การศึกษาผลของไนตริคอ็อกไซด์ร่วมกับซิลเดนาฟิลก็จะทดสอบหาค่าต่างๆ เหล่านี้เช่นกัน (ส่วนที่ 3, n=21) ในส่วนที่ 1 และส่วนที่ 2 จะแบ่งสัตว์ทดลองออกเป็น 3 กลุ่มโดยได้รับซิลเดนาฟิลขนาด 50, 100 mg หรือ saline ทว่าส่วนที่ 3 จะได้รับไนโตรกรีเซอรีนร่วมด้วยโดยการแบ่งกลุ่มจะเหมือนกับการทดลองทั้งส่วนที่ 1 และ 2 **ผลการทดลอง** ในส่วนที่ 1 ค่า DFT เพิ่มขึ้นภายหลังจากได้รับซิลเดนาฟิลขนาด 100 mg เข้าทางเส้นเลือดดำ โดยคิดเป็นค่าความต่างศักย์เพิ่มขึ้น ~19% และค่าพลังงานรวมเพิ่มขึ้น ~38% ในส่วนที่ 2 ภายหลังจากได้รับซิลเดนาฟิลขนาด 100 mg พบว่าค่า ULV เพิ่มขึ้นเช่นกัน (ค่าความต่างศักย์เพิ่มขึ้น ~28% และค่าพลังงานรวมเพิ่มขึ้น ~56%) ขณะที่ค่า VFT กลับลดลง (ค่าความต่างศักย์ลดลง ~36% และค่าพลังงานรวมลดลง ~52%) ในส่วนที่ 3 พบว่าไนโตรกลีเซอรีนไม่ได้ส่งผลกระทบใดๆ กับค่าพารามิเตอร์ต่างๆ **สรุปผลการทดลอง** ซิลเดนาฟิลซิเตรทขนาดสูงกว่าที่ใช้การรักษาสามารถเพิ่มระดับของ DFT, ULV และลดค่า VFT ได้ การค้นพบเหล่านี้บ่งชี้ให้เห็นว่าที่ความเข้มข้นของซิลเดนาฟิลสูงๆ สามารถลดประสิทธิผลของการทำดีฟิบริลเลชั่นและเพิ่มความอ่อนแอของเนื้อเยื่อหัวใจต่อการเกิดฟิบริลเลชั่นได้มากขึ้น

ABSTRACT

Although fatal arrhythmia and sudden death have been reported in patients taking sildenafil citrate, its effects on defibrillation efficacy as well as arrhythmia induction have not been investigated. The aim of this study was to test the hypotheses that sildenafil citrate (1) increases the shock strength required to successfully defibrillate during ventricular fibrillation (VF), (2) increases the upper limit of vulnerability (ULV), (3) decreases the VF threshold (VFT) and (4) nitric oxide augments these effects of sildenafil. **Methods**: Sixty-three pigs (25-30 kg) were used in this study. For drug tests, 2 concentrations of sildenafil were used: 100 mg (supratherapeutic level) and 50 mg (therapeutic level). Saline was used as a vehicle. Three experimental series were designed to test the defibrillation thresholds (DFT, series 1, n=18), the ULV and the VFT (series 2, n=24) as well as the effects of combined nitric oxide and sildenafil on these parameters (series 3, n=21). In series 1 and 2, pigs were divided into groups which received either 50 mg or 100 mg sildenafil or saline. In series 3, pigs were divided into groups similar to series 1 with an addition of nitroglycerine in all groups. **Results**: In series 1, the DFT was increased after 100-mg sildenafil infusion, accounting for a reduction of ~19% by peak voltage and ~38% by total energy. In series 2, the ULV was increased (~28% by voltage and ~56% by total energy), whereas the VFT was decreased (~36% by voltage and ~52% by total energy) after 100-mg sildenafil infusion. In series 3, nitroglycerine did not alter the effects of 100-mg sildenafil on the DFT. Both 50-mg sildenafil and saline did not alter any measured parameters. **Conclusion**: Supra-therapeutic concentration of sildenafil citrate significantly increased the DFT and the ULV and decreased the VFT. These findings indicate that this concentration of sildenafil can decrease the defibrillation efficacy and increase myocardial vulnerability to VF.

EXECUTIVE SUMMARY

ชื่อโครงการ:

ผลของยาซิลเดนาฟิลซิเตรท และผลร่วมของ ไนตริกออกไซด์โดเนอร์กับซิลเดนาฟิลซิเตรทต่อความสามารถในการทำดีฟิบิเลชั่นในหัวใจ รวมทั้งผลต่อภาวะการเกิดการเต้นผิดจังหวะของหัวใจชนิดร้ายแรง

Effect of Sildenafil Citrate and the Combined Nitric Oxide Donor and Sildenafil on the Defibrillation Efficacy and the Arrhythmogenesis of Ventricular Fibrillation

ชื่อหัวหน้าโครงการ หน่วยงานที่สังกัด ที่อยู่ หมายเลขโทรศัพท์ โทรสาร และ e-mail

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สาขาวิชาที่ทำการวิจัย: Cardiac Electrophysiology

ระยะเวลาดำเนินงาน: 3 ปี

ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

Ventricular fibrillation (VF) kills thousands of victims and is mainly responsible for sudden cardiac death. Electrical defibrillation is the only effective clinical treatment of this fatal arrhythmia. It has been demonstrated that a number of pharmacological therapies have significantly affected the therapeutic strategies during defibrillation. Therefore, extensive investigation has been done in the past to understand the benefit as well as the adverse

effects of each drug on defibrillation efficacy. Among them, sildenafil citrate is of interesting since it is a fairly new drug and its use is growing around the world, including Thailand. This drug is primarily known to effectively treat erectile dysfunction (ED). In addition, many studies have suggested that high-dose sildenafil citrate may be of benefit as an effective treatment in patients with pulmonary hypertension. More reports have also suggested other possible benefits of sildenafil in treating various diseases such as cognitive impairment.

Although sildenafil citrate has been shown to be an effective treatment for ED and possibly other diseases, cases of sudden cardiac death have been reported in patients taking sildenafil citrate. These reports raised concerns that sildenafil citrate may increase the risk of cardiovascular events, leading to VF and sudden cardiac death. Recent reports already demonstrated its potential in causing fatal arrhythmogenic events. With an increasing use of sildenafil citrate in men with erectile dysfunction, more cases of sudden cardiac death caused mainly by VF in this same group have been reported.

Since the age group that uses sildenafil is also in the same age group of having a high-risk of cardiovascular diseases, it is important to know the effects of this drug on cardiac electrophysiology, especially its effects on the defibrillation efficacy. Due to the reports of proarrhythmic effects that could lead to VF of sildenafil citrate and that defibrillation is the only effective means of terminating VF, its effect on defibrillation efficacy must be investigated so that proper precaution and management in this group of patients can be made. The research proposed in this grant will be the first to study this electrophysiological effect of this drug. The findings from this study will provide valuable information regarding the defibrillation mechanisms as well as the effects on defibrillation efficacy and resuscitation.

In this study, the unavailable electrophysiological evidence regarding the arrhythmogenic effects as well as the effects of sildenafil on the

defibrillation efficacy were investigated using a well-accepted human-like animal model in a clinical setting laboratory. Also, since it is known that nitrate or any nitric oxide donor drugs cannot be used in conjunction with sildenafil due to its vasodilatory effect, the electrophysiological effects of these two drugs given together were investigated. This later issue is important since there are patients taking these two drugs together even though they are contraindicate to be used in combination. Therefore, in this study we investigated whether nitric oxide donor could worsen the electrophysiological effects caused by sildenafil citrate.

วัตถุประสงค์

To determined the effect of sildenafil citrate and the combined nitric oxide donor and sildenafil on the defibrillation efficacy as well as their effects on the arrhythmogenesis of VF. The following hypotheses will be tested.

Hypothesis #1:Acute sildenafil citrate administration significantly decreases the defibrillation efficacy.

Hypothesis #2: Acute administration of combined sildenafil citrate and nitric oxide donor significantly decreases the defibrillation efficacy more than the administration of sildenafil alone.

Hypothesis #3: Acute sildenafil citrate administration causes the heart to be vulnerable to VF.

ระเบียบวิธีวิจัย

The effect of sildenafil citrate and the combined sildenafil and nitric oxide donor on the defibrillation efficacy as well as on the fatal arrhythmic induction were investigated using a well-accepted human-like animal model. All of the cardiac electrophysiologic parameters such as defibrillation threshold, the VF threshold, the upper limit of vulnerability, the diastolic pacing threshold, and the effective refractory period were tested before (i.e. control) and after drug administration. Other vital parameters including heart

rate and arterial blood pressure were recorded and compared between two groups.

ผลการวิจัย

Effects of Sildenafil Citrate on Defibrillation Efficacy

Although fatal arrhythmia and sudden death have occasionally been reported in patients taking sildenafil citrate, its effect on defibrillation efficacy has not been investigated. The aim of this study was to test the hypothesis that sildenafil citrate increases the shock strength required to successfully defibrillate during ventricular fibrillation (VF). **Methods**: Eighteen pigs (20-25 kg) were divided into three groups (n=6 in each group). In each group, the defibrillation threshold (DFT) was determined at the beginning of the study using a three-reversal up/down protocol. Each shock (RV-SVC, biphasic) was delivered after 10 seconds of VF. Group 1 received 50-mg and Group 2 received 100-mg of sildenafil citrate intravenously at a rate of 2 ml/min for 50 min. Group 3 received 100 ml of saline intravenously at the same rate as in group 1 and 2. The DFT was determined again after the drug (drug-DFT) and saline (saline-DFT) administration. **Results:** For 100-mg sildenafil citrate infusion, the DFT (478$\pm$162 V, 19$\pm$12 J) was significantly ($P<0.003$ and $P<0.01$, respectively) higher than the control-DFT (371$\pm$147 V, 12$\pm$9 J). This sildenafil citrate infusion increased the DFT ~29 % by voltage, and ~58 % by total energy. After 50-mg sildenafil citrate infusion, the DFT (402$\pm$21 V, 12$\pm$1 J) was not different than the control DFT (394$\pm$30 V, 12$\pm$2 J). Saline infusion did not alter the DFT. **Conclusion:** The 100-mg sildenafil citrate infusion, representing a supra-therapeutic plasma level, significantly increased the DFT. This finding indicates that VF occurring during supra-therapeutic sildenafil citrate treatment would require a much stronger shock to successfully defibrillate.

Effects of Combined Sildenafil-Nitric Oxide Donor on Defibrillation Efficacy

Previous study demonstrated that supra-therapeutic concentration of sildenafil citrate attenuates defibrillation efficacy. However, the effect of combined sildenafil and NTG administration on defibrillation efficacy is not known. The present study investigated whether sildenafil administration at the therapeutic level increases the defibrillation threshold (DFT) when combined with NTG. **Method:** Twenty-four pigs (20-25 kg) were assigned into four groups. After the control DFT was obtained, a stock solution of 50-mg (group 1, therapeutic concentration) and 100-mg (group 2, supra-therapeutic concentration) of sildenafil, and 100-mL of saline (groups 3 and 4) were infused at 2 mL/min. Then, NTG was administered in groups 1-3 at 5 μg/min, with an increment of 5 μg/min every 5 min. The DFT was determined again after NTG was infused for 20 minutes. **Results:** In group 1, the DFT (402±33V, 11±2J) was not different from the control-DFT (404±28V, 11±2J). In group 2, the DFT (521±18V, 19±1J) was higher (P<0.004) than that in the control (444±31V, 14±2J). Saline did not alter the DFT, either individually or in combination with NTG. **Conclusion:** Supratherapeutic dose of sildenafil-NTG combination significantly increased the DFT (17% of peak voltage, 37% of total energy). This effect on DFT appears to be driven by sildenafil and not NTG.

Effects of Sildenafil Citrate on the Induction of Ventricular Fibrillation and Upper Limit of Vulnerability

Sildenafil citrate at supra-therapeutic level has been reported to decrease defibrillation efficacy. However, its effects on the inducibility of ventricular fibrillation and the upper limit of vulnerability (ULV) have never been investigated. We tested the hypothesis that sildenafil citrate reduces the ventricular fibrillation threshold (VFT) and increases the ULV. **Methods**: Twenty-one pigs (25-30 kg) were randomly assigned into three groups (n=7

each). A solution containing 100 mg (group I) or 50 mg (group II) sildenafil citrate or 100 cc saline (group III) was infused intravenously in each pig. A train of 10 S1s was delivered from an RV electrode and an S2 stimulus was delivered at the peak of the T-wave of the last S1 activation to determine the VFT and ULV, before and after drug administration. **Results:** The 100 mg sildenafil citrate significantly ($P < 0.03$) decreased VFT, accounting for ~36% by peak voltage and ~52% by total energy, and significantly ($P < 0.009$) increased ULV, accounting for ~28% by peak voltage, and ~56% by total energy. **Conclusion**: Supra-therapeutic concentration of sildenafil citrate significantly decreased the VFT and increased the ULV, resulting in an expansion of the VF induction window during the vulnerable period.

เนื้อหางานวิจัย

บทนำ

Sudden cardiac death is a major health concern in most nations around the world, including Thailand.[1, 2] It is mainly caused by ventricular fibrillation (VF).[1-6] Currently, the only effective clinical treatment for this lethal arrhythmia is electrical defibrillation, i.e. a method that applies a high voltage electric shock to the heart. Although the use of an automatic external and internal defibrillator has been shown to decrease the mortality rate, many sudden death victims will still not be resuscitated, in some cases because the shock fails to defibrillate. Therefore, there is still a great need to improve defibrillation. At this time the shock strength that is needed to defibrillate patients with VF is still very high. A goal that investigators in this field try to achieve is to lower the defibrillation shock strength, i.e. the defibrillation threshold (DFT), that can still successfully defibrillate. If the DFT could be decreased, myocardial tissue will be less injured by the shock delivered to the heart. This is very important.

Currently, a number of pharmacological therapies have been used as a primary treatment or as a concommittent drug for therapeutic purposes. Among them, sildenafil citrate is of interesting since its use is growing based on the rising of prescription of this drug around the world, including Thailand. This drug is primarily known to treat erectile dysfunction (ED).[7] Although ED was once diagnosed and treated primarily by urologists, primary care physicians and other specialists such as cardiologists now write ~80% of the prescriptions for sildenafil citrate.[8] In addition, many studies have suggested that high-dose sildenafil citrate may be of benefit as an effective treatment in patients with pulmonary hypertension.[2, 9, 10] More reports have also suggested other possible benefits of sildenafil in treating various diseases such as cognitive impairment.[11, 12]

Although sildenafil citrate has been shown to be an effective treatment for ED and possibly other diseases, cases of sudden cardiac death have been reported in patients taking sildenafil citrate since the approve of the use of this drug from the FDA in 1998.[13] These reports raised concerns that sildenafil citrate may increase the risk of cardiovascular events in men with ED and cardiovascular disease. Recent reports on the adverse effects of this drug have been published concerning its potential fatal arrhythmogenic events.[13-15] With an increasing use of sildenafil citrate in men with erectile dysfunction, more cases of sudden cardiac death caused mainly by VF in this same group have been reported. Furthermore, growing evidence suggest that sildenafil citrate is an effective agent for treating pulmonary arterial hypertension, leading to an increased use of this drug.[16-21]

Extensive investigations are now in progress attempting to understand its mechanisms on the adverse effects of cardiovascular system and its relations to the cardiovascular mortality and sudden cardiac death. Despite these numerous investigations, the investigation on the effects of sildenafil citrate on the defibrillation efficacy has never been studied. Furthermore, its effects on the arrhythmogenesis of VF using a human-like model have never been investigated. Our preliminary data indicated that high concentration of sildenafil is harmful (see preliminary data section in this proposal).

Since previous study[13-15] indicate the possible arrhythmogenic events as well as VF caused by sildenafil and that defibrillation is the only effective clinical treatment for VF, it is also very important to know the effects of sildenafil on the defibrillation efficacy. The information gained from this study will be *novel* and will provide valuable information and benefits for both clinicians as well as patients. These new findings will lead to proper managements in patients treated with this drug (i.e. ED and/or pulmonary hypertension) and that appropriate therapeutic strategies can be provided.

In this study, the unavailable electrophysiological evidence regarding the arrhythmogenic effects as well as the effects of sildenafil on the

defibrillation efficacy were investigated using a well-accepted human-like animal model in a clinical setting laboratory. Also, since it is known that nitrate or any nitric oxide donor drugs cannot be used in conjunction with sildenafil due to its potent vasodilatory effect,[22] the electrophysiological effects of these two drugs given together were also investigated. This later issue is important since there are patients still taking them together even though they are contraindicate. Therefore, in this study we investigated whether nitric oxide donor could worsen the electrophysiological effects caused by sildenafil citrate.

วัตถุประสงค์ของโครงการ

To determined the effect of sildenafil citrate and the combined nitric oxide donor and sildenafil on the defibrillation efficacy as well as their effects on the arrhythmogenesis of VF. The following hypotheses will be tested.

Hypothesis #1:

Acute sildenafil citrate administration significantly decreases the defibrillation efficacy.

Previous studies have suggested that sildenafil citrate may have arrhythmogenic effects. Our preliminary data (see page 27) also indicated that sildenafil could significantly decrease the defibrillation efficacy. However, whether this effect is dose dependent is not known. Therefore, we will test the hypothesis that high-dose sildenafil citrate (100-mg infused intravenously) could deteriorate defibrillation efficacy more than lower-dose sildenafil (50-mg infusion).

Hypothesis #2:

Acute administration of combined sildenafil citrate and nitric oxide donor significantly decreases the defibrillation efficacy more than the administration of sildenafil alone.

Since nitric oxide donor is contraindicate to be used with sildenafil citrate. In this proposal, we will test the hypothesis that nitrate can worsen the defibrillation efficacy even at lower-dose of sildenafil citrate.

Hypothesis #3:

Acute sildenafil citrate administration causes the heart to be vulnerable to ventricular fibrillation.

Recent report in isolated swine right ventricle indicated that high-dose sildenafil caused fatal arrhythmias. However, no preclinical study of this effect has been investigated. In this proposal, we will use the human-like animal model to test this hypothesis.

ผลงานวิจัยที่เกี่ยวข้อง

Ventricular fibrillation (VF) is responsible for sudden cardiac death that kills thousands of people each year.[1-6] In the US, sudden cardiac death is responsible for over 400,000 deaths annually. In Thailand, over 23,000 cases of sudden cardiac death has been reported each year, most of them are caused by VF. Currently, electrical defibrillation is the only effective means for terminating VF. Pharmacological defibrillation is known to be ineffective.[6] Furthermore, some anti-arrhythmic drugs are now known to cause arrhythmias and can be responsible for VF and sudden cardiac death when used in some patients. Up til now, there are a number of pharmacological interventions that have been demonstrated to affect the efficacy of electrical defibrillation.[6] Some have been shown to improve defibrillation efficacy.[6] The others, however, have been shown to worsen the defibrillation efficacy. It is important that the later group has to be used with serious precautions since it will make the defibrillation much more difficult and that the chance of saving lives could be at stake. Therefore, the use of any drug, particularly the drug

known to affect cardiovascular system, has to be extensively investigated and be certain that it will not have adverse electrophysiological effects. The two most important adverse electrophysiological effcts caused by the drug are the worsening of defibrillation efficacy and an increase in vulnerability to fatal arrhythmias, especially VF. Therefore, the better we understand the fundamental mechanisms of the effects of drugs on defibrillation and fatal arrhythmia induction, the more likely it is that we will be able to devise strategies to prevent these proarrhythmogenic effects of any drug to occur in patients. Hence, more lives can be saved.

Why are we interested in sildenafil citrate and defibrillation?

Sildenafil citrate is a highly selective inhibitor of cGMP-specific phosphodiesterase type 5 (PDE-5) and has been widely used for the treatment of erectile dysfunction (ED).[23] The prevalence of moderate to complete ED has been reported to be >30% in men aged 40 to 70 years.[24] These age ranges are also in the same age group of cardiovascular disease patients. It has been shown that ED is a common health concern among patients with cardiovascular disease.[7] According to the Massachusetts Male Aging Study (MMAS), 34.8% of men aged 40 to 70 years have moderate to complete ED, and 15% of men aged 70 have complete ED.[24] As of today, sildenafil is one of several drugs of choice used to treat this abnormality. Furthermore, growing evidence suggest that sildenafil citrate is an effective agent for treating pulmonary arterial hypertension, leading to an increased use of this drug.[16-19] Reports by the U.S. Food and Drug Administration (FDA) have shown the incidence of significant cardiovascular events, including sudden cardiac death, associated with the use of sildenafil citrate.[8]

In the past few years, the cardiovascular effects of sildenafil citrate have been investigated extensively in both animal and clinical studies. These include a number of studies that investigated the effects of sildenafil citrate on cardiac electrophysiological alterations that could cause VT/VF and sudden

cardiac death.[25-27] A recent report by Swissa et al indicated that administration of sildenafil citrate at a supra-therapeutic level combined with a nitric oxide donor could cause arrhythmia in isolated swine right ventricles.[27] Despite these extensive investigation, the effect of sildenafil citrate on the defibrillation efficacy has never been reported to date. Furthermore, its effects on the arrhythmogenesis has never been tested in a human-like animal model using a clinical setting. It is well accepted that these two effects of any drug could change the perspective of the use of that drug and that this information could significantly affect the treatment strategies in patients being treated with the drug. With an increasing use of sildenafil citrate together with more cases of sudden cardiac death caused mainly by VF in this same group, it is essential to know how the drug affects defibrillation efficacy.

Erectile dysfunction and cardiovascular diseases

Erectile dysfunction is a common health concern among patients with cardiovascular disease.[7] According to the Massachusetts Male Aging Study (MMAS), 34.8% of men aged 40 to 70 years have moderate to complete ED, and 15% of men aged 70 have complete ED.[24] The risk of ED has been shown to markedly increase with age, with a high prevalence of ED found in patients with cardiovascular disease. Greenstein and colleagues have shown that there is a significant correlation between the severity of ED and the number of vessels involved in patients with coronary artery disease (CAD).[28] The age-adjusted prevalence of complete ED has been reported to be 1.5 times higher in men with hypertension than in the entire population studied.[24] Epidemiologic studies have reported that ED is commonly found in smokers, diabetics and patients with hypercholesterolemia; thus, ED shares important risk factors with CAD.[29] A report by Khan and colleagues also found that patients with CAD and peripheral vascular disease have an increased prevalence of ED.[30]

Use of sildenafil citrate in patients with cardiovascular diseases

Sildenafil citrate is a cGMP-specific PDE-5 inhibitor.[23] PDE-5, which is located primarily in the cavernous body, thrombocytes and vascular smooth muscle cells, degrades cGMP.[20] Thus, by inhibiting phosphodiesterase-5 (PDE-5), sildenafil citrate selectively increases cGMP levels.[31] It shows far less affinity for other phosphodiesterase isozymes, including PDE-1, which is abundant in ventricular myocytes.[32] However, concern about adverse cardiovascular effects remains since PDE-5 inhibitors promote vasodilation, and thus have the potential to cause hypotension.[33] This concern has been greatest for elderly patients with pre-existing cardiovascular disease. Although several studies indicated that the use of sildenafil citrate was safe and that a single oral dose had no significant hemodynamic effect in cardiovascular disease patients,[34, 35] the ACC/AHA consensus statement recommended that patients taking sildenafil citrate with combinations of antihypertensive drugs (such as calcium-channel blockers, ß-blockers, diuretics and angiotensin-converting enzyme inhibitors) be alerted to the possibility of hypotension, particularly patients with congestive heart failure.[36] The Princeton Consensus Panel concluded that patients with well-controlled hypertension can be safely managed with approved medical treatments for ED.[37] However, concomitant use of nitrates is considered to be an absolute contraindication for the use of sildenafil citrate.[22] Nitrates are prescribed in several different forms, including sublingual nitroglycerin, oral isosorbide mononitrate or dinitrate, nitropatch and nitropaste, all of which have been associated with a prolonged decrease in blood pressure when used concomitantly with sildenafil citrate.[22] Nitrates are metabolized in vessel walls, where they release nitric oxide. Sildenafil citrate prolongs the vasodilatory effects of nitrates by decreasing the breakdown of nitric oxide's main effector, cGMP. It is not known how much time must elapse between administration of sildenafil citrate and administration of nitrates to avoid significant hypotensive effects, but it has been suggested to assume an interval of at least 24

hours.[22] Nitroprusside also causes vasodilatation by nonenzymatic release of nitric oxide, and thus is predicted to have a synergistic hypotensive effect with sildenafil citrate.[22]

Effects of sildenafil citrate on cardiac contractility, blood pressure and heart rate

Sildenafil citrate belongs to a class of compounds called PDE inhibitors. PDEs comprise a diverse family of enzymes that hydrolyze cyclic nucleotides (cAMP and cGMP) and therefore play a critical role in the modulation of second messenger signaling pathways.[20] Sildenafil citrate is a highly selective (~4,000-fold) inhibitor of human PDE-5 over human PDE-3.[22] This is important because inhibitors of PDE-3 (the isozyme involved in the regulation of cardiac contractility), such as milrinone, vesnarinone and enoximone, which have been used in patients with heart failure, are generally associated with an increased incidence of cardiac arrhythmias and other serious side effects.[38] The cardiotoxic effects of PDE-3 inhibitors are thought to be related to an increase in intracellular cAMP in the myocardium.[39, 40] However, PDE-5 is thought to be absent in cardiac myocytes.[22] Corbin and colleagues demonstrated in both dog and human hearts that sildenafil citrate was unlikely to directly produce an inotropic effect on cardiac muscle.[41] However, recent electrophysiological studies have demonstrated significant cardiac cellular electrophysiologic alterations caused by sildenafil citrate (see details on the next topic).[25-27, 42]

Systemic and pulmonary arterial and venous smooth muscle cells contain PDE-5. However, sildenafil citrate causes only a mild and transient decrease in blood pressure (8-10 mmHg for systolic blood pressure and 5-6 mmHg for diastolic blood pressure).[22] The peak effects are evident 1 h after the dose is given and last for approximately 4 hours.[22] Heart rate and cardiac output are not significantly affected. Along with a mild decrease in systemic vascular resistance and afterload, there is also a mild decrease in preload

and stroke volume due to venous vasodilatation. These effects are not dependent upon age or dose (within the range of 25 to 800 mg).[22] In a study of patients with severe coronary artery disease, Herrmann and colleagues confirmed that the hemodynamic effects of sildenafil citrate (when taken alone) are not associated with clinically significant hypotension.[43]

Cardiac electrophysiological effects of sildenafil citrate

Previously, sildenafil citrate has been shown to have no effect on the hemodynamic responses to exercise or change the incidence of ventricular arrhythmias in men with cardiovascular disease and ED.[44] Recent studies have also demonstrated that oral administration of 50 mg of sildenafil citrate does not affect QT dynamic properties,[45] and may reduce arrhythmia severity during ischemia 24 hours after oral administration in dogs.[46] However, due to the increased use of sildenafil, together with the reports of sudden cardiac death related to the use of this drug, extensive basic investigations have been performed in the past few years to elucidate the cardiac electrophysiologic effects of sildenafil citrate.[25, 26, 42]

Geelen and colleagues demonstrated that sildenafil citrate induces a dose-dependent block of the rapid component of the delayed rectifier potassium current (I_{Kr}).[26] They also reported that sildenafil citrate can have an action similar to that of class III antiarrhythmic drugs.[26] It is known that this class of antiarrhythmic drugs can cause fatal arrhythmia and sudden cardiac death in some groups of patients. However, these effects are observed at concentrations that may be found in conditions of impaired drug elimination such as renal or hepatic insufficiency, during co-administration of another CYP3A4 inhibitor, or after drug overdose.[42] Prolonged cardiac repolarization caused by sildenafil citrate as demonstrated in this study could result in malignant ventricular arrhythmias and lead to sudden cardiac death in some of these patients.[42] Furthermore, Swissa and colleagues demonstrated that a combination of sildenafil citrate and a nitric oxide donor

significantly increases VT/VF vulnerability in the normal right ventricle of swine.[27] More recently, Chiang and colleagues demonstrated that sildenafil citrate dose-dependently blocks L-type Ca^{2+} currents ($I_{Ca,L}$), but has no effect on persistent Na^{+} currents.[25] They also demonstrated that in supra-therapeutic concentrations, sildenafil citrate accelerates cardiac repolarization, presumably via its blocking effect on $I_{Ca,L}$.[25] All of these findings suggest that sildenafil significantly affects cardiac electrophysiological properties and may facilitate arrhythmias and VF.

Effect of sildenafil citrate on defibrillation efficacy

Although a number of studies have been performed to investigate the electrophysiological effects of sildenafil citrate, its effects on the defibrillation efficacy is still unknown. It is important to note that growing number of evidence indicates the arrhythmogenic effect of sildenafil citrate, particularly that involves VF and sudden cardiac death. Since defibrillation is the only means to treat VF, it is essential to know the effect of this drug on the efficacy of defibrillation. If sildenafil improves defibrillation efficacy, this will provide an additional benefit to the existing ones that already have. However, if sildenafil worsen the efficacy, this information will provide important information for clinicians as well as patients to be aware of the fact that VF occurring in patients being treated with this drug may require an even higher strength shock to successfully defibrillate. Ultimately, the benefits will be to the patients as well as to the medical community.

Effect of sildenafil citrate on VF induction by T-wave shocks

It is known that VF can be induced by delivering a strong stimulus or a shock during the vulnerable period of a cardiac cycle, i.e. T-wave on the ECG.[47-49] It has been shown that there is the lower limit of a stimulus strength than can induce VF when delivered during the T-wave. This strength is known as the VF threshold (VFT). When the strength of a stimulus is

increased, VF can still be induced up until at the strength when VF is no longer induced when delivered during the T-wave. This strength is known as the upper limit of vulnerability (ULV). Figure 1 illustrates the relationship between the VFT and the ULV.

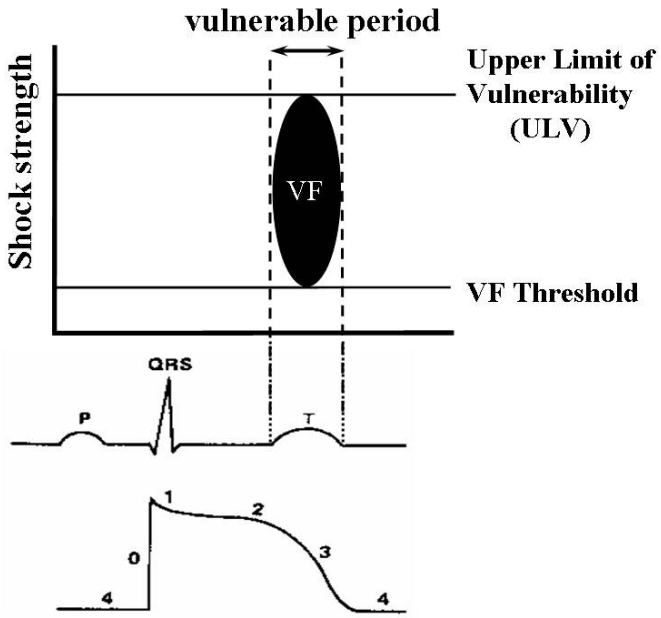

Figure 1: Illustration of the ULV and the VFT. (From: **Chattipakorn N,** Ideker RE. Mechanism of defibrillation. In: Aliot E, Clementy J, and Prystowsky EN, eds. *Fighting Sudden Cardiac Death: A Worldwide Challenge*.* New York: Futura Publ. Co., Inc. ISBN 0-87993-460-3)

The window of VF induction is important in the determination of arrhythmogenesis during the vulnerable period. It has been shown that the vulnerability to arrhythmias will be decreased if the VFT is raised and/or the ULV is reduced. Although previous study indicated the arrhythmogenic effect of sildenafil,[27, 50] this adverse effect has never been tested in the human-like animal model. Therefore, in addition to the investigation on the defibrillation efficacy, both the VFT and the ULV were determined in this study to investigate the effect of sildenafil citrate on the vulnerability to fatal arrhythmia.

วิธีการทดลอง

Because the ultimate goal is the clinical application of the findings from this research, it is necessary to use fairly large animals, such as pigs, to maintain similarities to human cardiac anatomy. Since pigs have been used in much research on arrhythmias and defibrillation, using this species also makes it possible to compare the results from the proposed research to other work.[3-6, 51, 52]

The number of animals was justified by performing a Chi square calculation based on previous results performed by the principle investigator. At least six pigs were to be used in each study group in order to justify statistical significant comparison. Healthy pigs weighing approximately 20-30 kg, of both sexes, were used. All animals were managed, monitored, and maintained under physiologic conditions by a veterinarian in the operating room at the Cardiac Electrophysiology Research and Training Center, Faculty of Medicine, Chiang Mai University.

General Methods

Each pig was pre-anesthetized with a combination of atropine (0.04 mg/kg), telazol (4.4 mg/kg), xylazine (2.2 mg/kg) injected intramuscularly. After a 15-minute waiting period, a suitable ear vein was cannulated. Normal saline was infused through a 18g. teflon catheter in the previously cannulated ear vein at a rate of 2-5 ml/kg throughout the experiment. The pig in a restrained dorsally recumbent position was then intubated with a cuffed endotracheal tube and mechanically ventilated (volume controlled, tidal volume = 12 ml/kg) at rate of 10-15 cycles/min. The pig was anesthetized with 1.5-3.0% isoflurane delivered in 100% oxygen. The animal was instrumented for electrocardiogram (ECG), arterial blood pressure, oxygen saturation and temperature monitoring. Water heated pad was used to maintain body temperature at 37 ± 1 degree celcius.

Arterial blood pressure from left femoral artery, ECG, blood gases, pH and electrolytes, oxygen saturation, end-tidal volume level of CO_2, and temperature were monitored and maintained within acceptable physiologic ranges by giving intravenous sodium bicarbonate or, preferably, by adjusting ventilation parameters as needed. Succinylcholine (2 mg/kg loading, 0.5 mg/kg/hr maintenance) was administered intravenously to minimize skeletal muscle contraction during defibrillation testing. At the end of the study, the animal was euthanized by VF.

Electrode Placements

The right ventricular (RV) coil and the superior vena cava (SVC) coil catheters (Guidant, Inc.) were inserted into the right and left external jugular vein respectively. Under fluoroscopic guidance, the electrodes were advanced to proper positions (RV apex and SVC). Then, the catheters at the venotomy site were secured to stabilize their positions. The electrode at the tip of the RV catheter was used as a pacing electrode for VF induction (Figure 2).

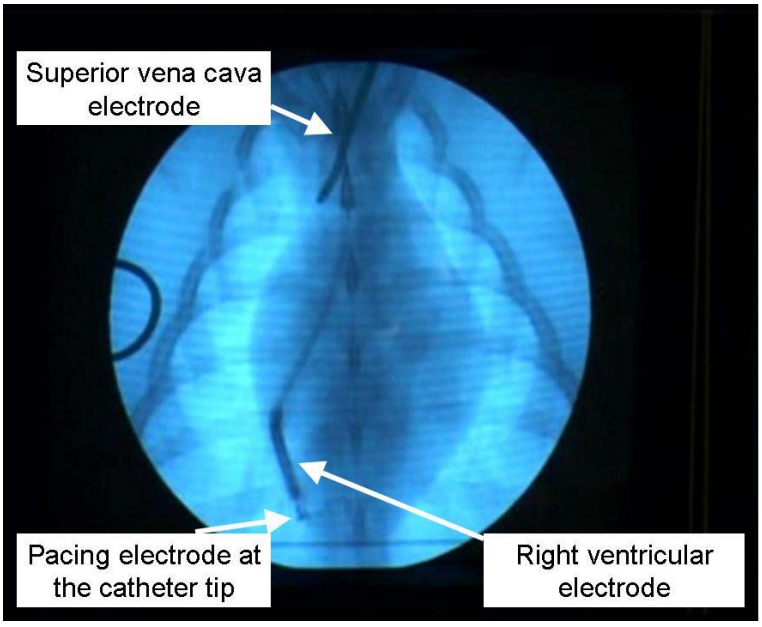

Figure 2: Fluoroscopic image of the heart with electrodes placed at designated sites.

Mehods used in the defibrillation protocol

In this protocol, we tested two hypotheses. We investigated whether acute sildenafil citrate administration significantly decreases the defibrillation efficacy. We also tested the hypothesis that acute administration of combined sildenafil citrate and nitric oxide donor significantly decreases the defibrillation efficacy more than the administration of sildenafil alone.

VF Induction

After all electrodes were inserted into the animal, we waited for the animal to return to normal physiological condition (~15 minutes). To begin the study protocol, VF was induced by delivering a 60-Hz alternating current via the electrode at the tip of the RV catheter for 3-5 seconds. This would lead the whole ventricles into VF. We then let the VF run for 10 seconds before the defibrillation shock was delivered to defibrillate.

The Determination of the Defibrillation Threshold

The defibrillation threshold (DFT) was determined by delivering a shock (biphasic) from a research defibrillator (Ventak ECD, Guidant, Inc.) after 10 seconds of VF using a modified up/down protocol as described previously.[6] Briefly, the initial shock strength was chosen at 400 V. In the event of successful defibrillation, the leading edge voltage was decreased in 80 V steps per defibrillation attempt until a first reversal from successful defibrillation to failure was achieved. If the initial shock was unsuccessful, the voltage would be increased in 80 V steps per defibrillation attempt until a reversal from failed to successful defibrillation is achieved. At each reversal point the algorithm would be iterated in the opposite direction except that after the first reversal the voltage step size would be diminished to 40 V and 20V for a total of three reversals. The DFT is defined as the lowest energy required for successful defibrillation of VF after three reversal points, when the next lower setting failed to defibrillate the heart.

During the DFT determination, if the shock failed to defibrillate, a rescue shock (600-700 Volts) would be delivered to successfully defibrillate the heart. A period of 4 minutes would be allowed between each VF induction so that the heart could return to physiologic conditions (i.e. normal blood pressure and heart rate).[6]

Drug Administration

Sildenafil citrate (Pfizer) was obtained in a tablet form (50 and 100 mg). Both sildenafil 50 mg and 100 mg would be glided and dissolved in 100-ml normal saline just before each experiment to form a stock solution. Therefore, two different concentrations of sildenafil were obtained to be injected intravenously according to the assigned group. The rate of infusion was 2 ml/min over 50 minutes. It has been shown that intravenous administration of 50-mg sildenafil citrate represents approximately 100-mg sildenafil taken orally as recommended dose, whereas 100-mg sildenafil infusion will be at supratherapeutic dose (~>200 mg taken orally).[23, 53]

For the groups that have the combination of sildenafil and nitric oxide donor, after the end of sildenafil infusion, nitroglycerine would be slowly drip intravenously beginning at 3 ml/hour (5 microgram/min) and would be increased in a 3-ml/hour step every 5 minutes. If the systolic blood pressure falls to 20 mmHg below the baseline systolic pressure, the increment would be ceased. Instead, the rate would be decreased at 3-ml/hr step every 5 minutes until the systolic blood pressure was within the accepted range (From Khan, M Gabriel: cardiac Drug Therapy, 5th ed. London, WB Sauders Co., 2000).

To confirm that the DFT alteration was caused by the drug(s), not by the solution used to dissolve the drug or the deterioation of the model, the same amount of normal saline would be infused into the animal for DFT determination in another group of pigs. Also, nitroglycerine alone would be

tested in another set of pigs to test if it has an effect on the defbrillation efficacy. And if so, we would see whether it has additive effect to the sildenafil.

Methods used in the arrhythmogenic study protocol

In this protocol, instead of the DFT, the upper limit of vulnerability (ULV) as well as the VF threshold (VFT) were be determined.

VF was induced by delivering a strong shock (S2) during the T-wave of a paced rhythm. This method has been prescribed elsewhere.[47-49] In brief, the ventricles were paced by delivering a train of a 5-ms square pulse (S1) via the electrode at the tip of the RV catheter at a 300-ms interval. The strength of the pulse would be 2x diastolic pacing threshold. The interval between the last S1 and the mid T-wave would be determined and used as the coupling interval between the last S1 and an S2 shock. The strength of the S2 shock would start at 100V. The increment of 20-V would be used for each successive shock until VF was induced. The lowest shock strength that can induce VF was called the VF threshold (VFT). When VF was induced, a period of 4 minutes would be allowed before the next S2 shock was delivered. After the first VF induction, the increment step would be 100V until VF was no longer induced. The S2 shock strength that did not induce VF would then be decreased in 40V steps until VF was induced again. Then, the S2 shock would be increased in 20-V steps until VF was no longer induced. The last S2 shock strength that did not induce VF was called the upper limit of vulnerability (ULV).

Basic cardiac electrophysiological measurements

In each pig, the electrophysiologic parameters such as the diastolic pacing threshold (DPT) and the effective refractory period (ERP) were determined at the beginning of the study and after the drug administration.

These parameters were measured at the left ventricular apex, where the pacing electrode was located.

Statistical Analysis

Comparisons of data (DFT, VFT, ULV, DPT, ERP, heart rate, systolic blood pressure) between different groups were performed using an analysis of variance. When statistical significance was found, individual differences were analyzed with a Fisher's post-hoc test. Differences was considered significant when $P < 0.05$.

ผลการทดลอง

Effects of Sildenafil Citrate on Defibrillation Efficacy

The weight of the pigs used in this study was 23.3±2 kg. The total number of shocks delivered to each animal was 13±1. The average number of shocks delivered to the animal before the DFT was obtained was 7±1 in the control group and 6±1 in the sildenafil citrate and saline injected group.

In group 1, the delivered leading-edge voltage and total energy for the control DFT was 394±30 V and 12±2 J, respectively. Drug-DFT (402±21 V and 12±1 J) was not different (Figure 3A) than the control DFT ($P = 0.6$ and 0.7 for leading-edge voltage and total energy, respectively). There were no significant changes in the impedance and pulse width between the two DFT groups (Figure 3B). The heart rate as well as the systolic blood pressure before and after drug infusion were not different ($P = 0.3$ and 0.1, respectively, Figure 3C).

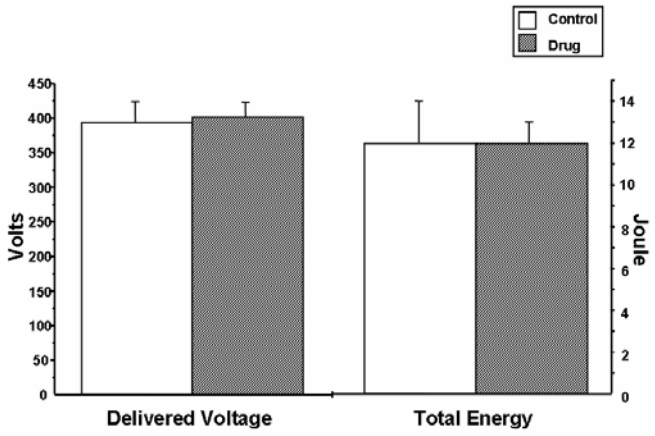

Figure 3A

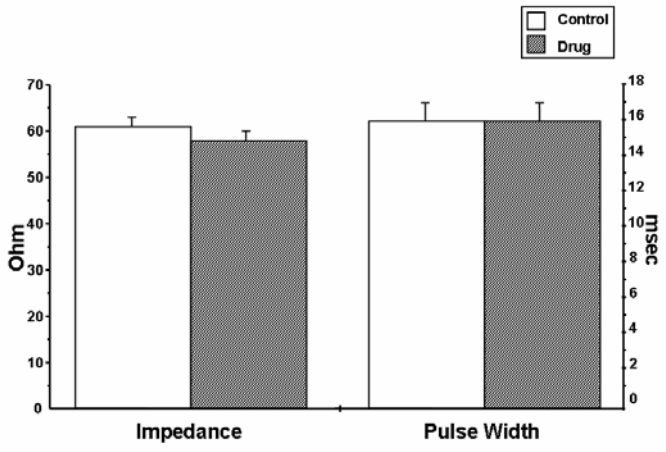

Figure 3B

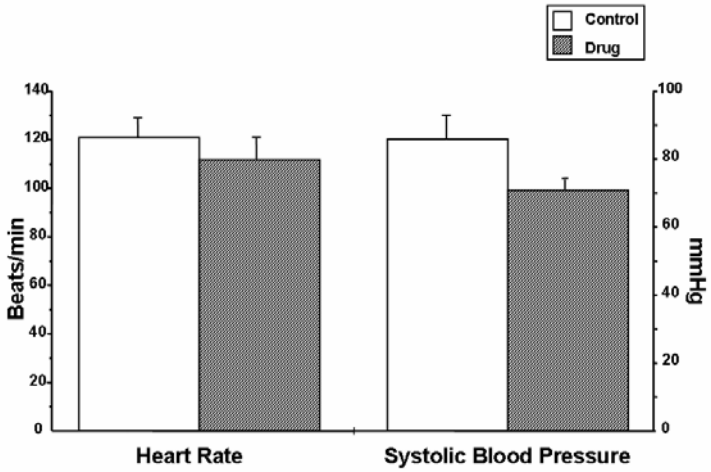

Figure 3C

Figure 3. The DFT, systolic blood pressure, and heart rate before and after a 50-mg sildenafil citrate infusion. Panel A: The delivered voltage and total energy for the DFT. The delivered voltage and total energy for the drug DFT was not different than the control DFT. Panel B: The impedance and pulse width measured in two groups. There was no difference in the impedance and pulse width between the two DFT groups. Panel C: The heart rate and systolic blood pressure measured before and after drug infusion. There was no difference in the heart rate and systolic blood pressure between the two groups.

In group 2, the delivered leading-edge voltage and total energy for the control DFT was 371±147 V and 12±9 J, respectively. Drug-DFT (478±162 V and 19±12 J) was significantly higher than the control DFT (P<0.003 and P<0.01 for leading-edge voltage and total energy, respectively, Figure 4A). A 100-mg sildenafil citrate infusion significantly increased the DFT ~29% by leading-edge voltage and ~58% by total energy. The impedance and pulse width of the drug-DFT group were not different (P = 0.8 and 0.4 for the impedance and pulse width, respectively) from the control group (Figure 4B). The heart rate as well as the systolic blood pressure before and after drug infusion were not different (P = 0.1 and 0.5, respectively, Figure 4C).

In group 3, the delivered leading-edge voltage and total energy for the control DFT was 404±28 V and 13±2 J, respectively. Saline DFT (406±21 V and 13±1 J) was not different (Figure 5A) than the control DFT (P = 0.9 and 0.9 for leading-edge voltage and total energy, respectively). There were no significant changes in the impedance and pulse width (P = 0.1 and 0.2 for the impedance and pulse width, respectively) between the two DFT groups (Figure 5B). The heart rate as well as the systolic blood pressure before and after saline infusion were not different (P = 0.4 and 0.1, respectively, Figure 5C).

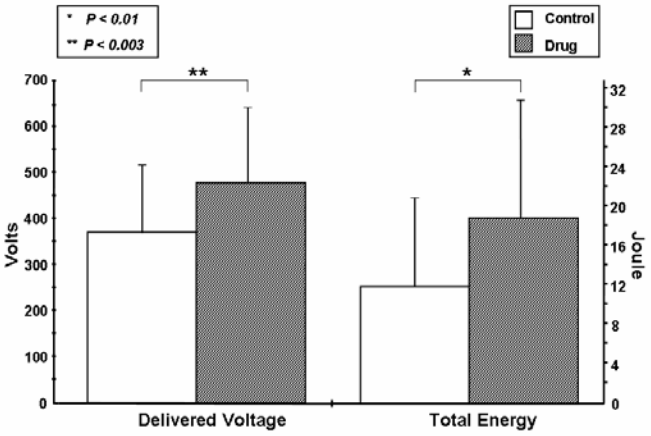

Figure 4A

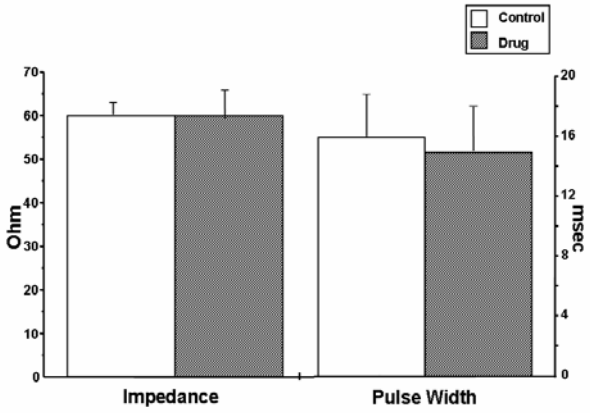

Figure 4B

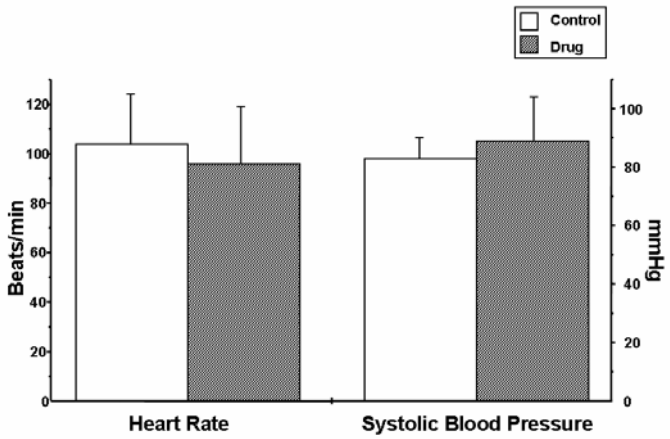

Figure 4C

Figure 4. The DFT, systolic blood pressure, and heart rate before and after a 100-mg sildenafil citrate infusion. Panel A: The delivered voltage and total energy for the DFT. The delivered voltage and total energy for the drug DFT was significantly higher than the control DFT. * $P < 0.003$ vs control DFT; ** $P < 0.01$ vs control DFT. Panel B: The impedance and pulse width measured in two groups. There was no difference in the impedance and pulse width between the two groups. Panel C: The heart rate and systolic blood pressure measured before and after drug infusion. There was no difference in the heart rate and systolic blood pressure between the two groups.

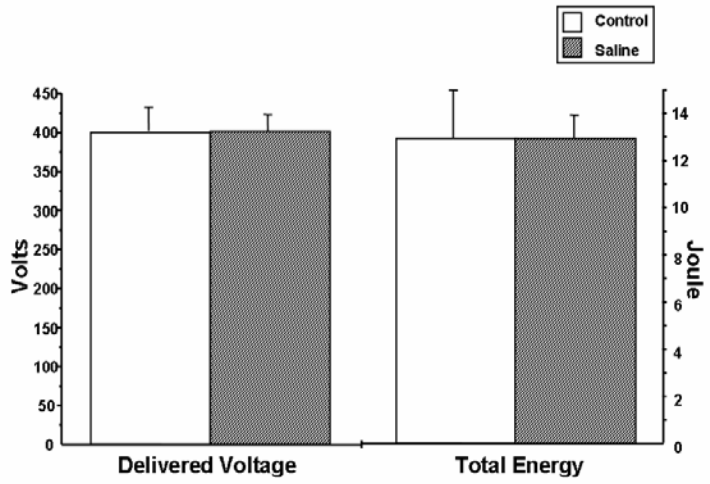

Figure 5A

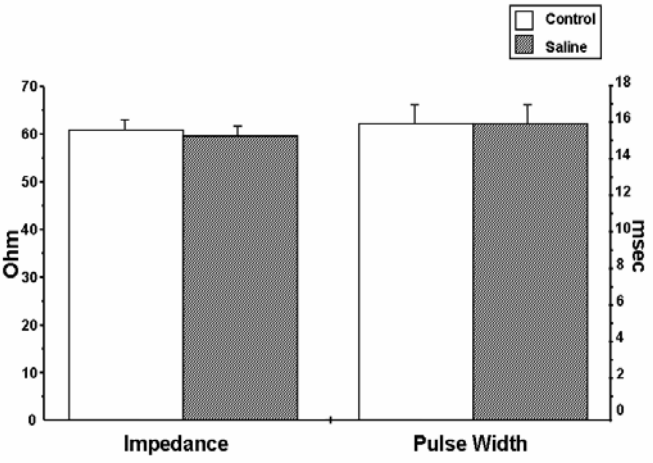

Figure 5B

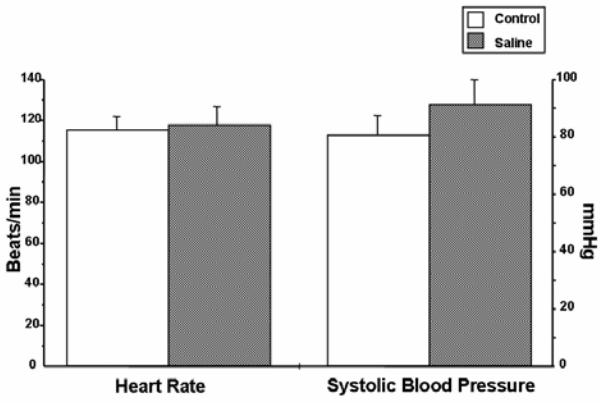

Figure 5C

Figure 5. The DFT, systolic blood pressure, and heart rate before and after a 100-ml saline infusion. Panel A: The delivered voltage and total energy for the DFT. The delivered voltage and total energy for the saline DFT was not different than the control DFT. Panel B: The impedance and pulse width measured in two groups. There was no difference in the impedance and pulse width between the two DFT groups. Panel C: The heart rate and systolic blood pressure measured before and after drug infusion. There was no difference in the heart rate and systolic blood pressure between the two groups.

Effects of Combined Sildenafil-Nitric Oxide Donor on Defibrillation Efficacy

The average weight of the pigs used in this study was 26 ± 1 kg. The total number of shocks delivered to each animal was 13 ± 1. The average number of shocks delivered to the animal before obtaining the DFT was 6 ± 1 in the control group and 6 ± 1 in the drug (sildenafil-NTG) or saline (either individually or in combination with NTG) injected groups.

In group 1, the peak voltage and total energy for the control DFT was 404 ± 28 V and 11 ± 2 J, respectively. After 50-mg of sildenafil-NTG infusion, the DFT (402 ± 33 V, 11 ± 2 J) was not different from the control DFT (P = 0.7 and P = 0.9, respectively, Table 1). Both the impedance and pulse width were not changed after drug administration. The systolic blood pressure after 50-mg of sildenafil-NTG infusion was significantly lower than that in the control (P < 0.05, Table 1). However, the heart rate before and after drug administration was not significantly different (Table 1).

In group 2, the peak voltage and total energy for the control DFT was 444 ± 31 V and 14 ± 2 J, respectively. After 100-mg of sildenafil-NTG infusion, the DFT (521 ± 18 V, 19 ± 1 J) was significantly higher than that in the control group (Table 1). The 100-mg of sildenafil-NTG infusion significantly increased the DFT by 17% of the leading-edge voltage and 37% of total energy. No changes in the impedance and pulse width were found in this group. The systolic blood pressure after 100-mg of sildenafil-NTG infusion was significantly lower than that in the control (P < 0.05, Table 1). The heart rate before and after drug administration was not different (P = 0.3).

TABLE 1. DFT and hemodynamic parameters measured before and after drug (sildenafil-NTG) or saline (either individually or in combination with NTG) administration.

Parameters	Group I		Group II		Group III		Group IV	
	Control	50-mg Sildenafil + NTG	Control	100-mg Sildenafil + NTG	Control	100-mL Saline + NTG	Control	100-mL Saline only
Delivered Voltage (Volts)	404 ± 28	402 ± 33	444 ± 31	521 ± 18*	424 ± 26	427 ± 34	439 ± 14	417 ± 17
Total Energy (Joules)	11 ± 2	11 ± 2	14 ± 2	19 ± 1†	12 ± 2	13 ± 2	14 ± 1	13 ± 1
Impedance (Ohm)	61 ± 2	61 ± 2	60 ± 2	59 ± 3	62 ± 2	62 ± 1	61 ± 3	58 ± 3
Pulse Width (msec)	15 ± 1	15 ± 1	15 ± 1	15 ± 1	15 ± 1	15 ± 1	16 ± 1	15 ± 1
Systolic BP (mmHg)	97 ± 3	84 ± 3‡	89 ± 4	76 ± 4‡	106 ± 3	95 ± 5‡	90 ± 3	102 ± 3‡
Heart Rate (bpm)	88 ± 4	94 ± 5	106 ± 4	95 ± 7	99 ± 4	100 ± 7	106 ± 2	116 ± 6

* = P<0.05 vs. control in the same group

In group 3, the peak voltage and total energy for the control DFT was 424 ± 26 V and 12 ± 2 J, respectively. Saline DFT (427 ± 34 V, 13 ± 2 J) was not different from that in the control group (Table 1). The systolic blood pressure after saline-NTG infusion was significantly lower than that in the control group ($P < 0.05$, Table 1). The impedance, pulse width, and heart rate before and after drug administration were not different ($P = 0.7$).

In group 4, the peak voltage and total energy for the control DFT was 439 ± 14 V and 14 ± 1 J, respectively. Saline DFT (417 ± 17 V, 13 ± 1 J) was not different from that in the control group (Table 1). The impedance and pulse width were not altered after drug infusion. The systolic blood pressure after saline infusion was significantly higher than that in the control group ($P < 0.05$, Table 1). However, the heart rate before and after drug administration was not different (P = 0.1).

Effects of Sildenafil Citrate on the Induction of Ventricular Fibrillation and Upper Limit of Vulnerability

The basic electrophysiological parameters such as DPT, S1-S2 coupling interval, and ERP were not different before and after sildenafil citrate or saline administration in each group (Table 2). For hemodynamic parameters, both concentrations of sildenafil citrate significantly reduced systolic blood pressure, diastolic blood pressure and mean arterial blood pressure (Table 2). However, heart rate (HR) was not changed in both groups.

For the VFT, 100 mg sildenafil citrate significantly decreased the VFT peak voltage and total energy compared to the control (Table 3). The percent reduction is ~ 36% by peak voltage and ~ 52% by total energy. However, neither 50 mg sildenafil citrate nor saline changed the VFT parameters compared to the control (Table 3).

For the ULV, 100 mg sildenafil citrate significantly increased ULV peak voltage and total energy compared to the control (Table 4), accounting for an

increment of ~28% by peak voltage and ~56% by total energy. The 50 mg sildenafil citrate and saline did not change the ULV compared to the control (Table 4).

The VF induction windows in each pig were calculated by subtracting both voltage and energy of the VFT from the ULV. The mean VF induction windows before and after sildenafil citrate and saline administration are shown in Table 5. The VF induction windows were expanded after 100 mg sildenafil citrate administration, compared to the control (Table 5). However, the VF induction window was not affected by 50 mg sildenafil citrate or saline administration (Table 5).

Table 2: Basic electrophysiological parameters, blood pressure and heart rate before and after sildenafil and saline administration.

Parameters	Group I		Group II		Group III	
	Control	100-mg Sildenafil	Control	50-mg Sildenafil	Control	100-mL Saline
Diastolic pacing threshold (mA)	1.1 ± 0.8	1.2 ± 0.8	0.6 ± 0.3	0.7 ± 0.2	0.5 ± 0.2	0.6 ± 0.2
S1-S2 coupling interval (ms)	317 ± 35	315 ± 28	283 ± 36	292 ± 36	256 ± 30	264 ± 32
Effective refractory period (ms)	251 ± 37	280 ± 39	247 ± 29	261 ± 32	229 ± 29	247 ± 24
Systolic BP (mmHg)	116 ± 18	94 ± 12 †	106 ± 9	86 ± 12 *	120 ± 12	129 ± 22
Diastolic BP (mmHg)	77 ± 17	59 ± 11 †	63 ± 6	51 ± 9 *	76 ± 12	82 ± 19
Mean arterial BP (mmHg)	90 ± 18	70 ± 12 †	78 ± 7	62 ± 9 *	70 ± 6	75 ± 12
Heart rate (bpm)	99 ± 33	90 ± 32	90 ± 16	98 ± 19	103 ± 14	106 ± 7

*** = $P < 0.01$ vs. control**

† = $P < 0.05$ vs. control

Table 3: Ventricular Fibrillation Threshold (VFT) before and after sildenafil and saline administration.

VFT	Group I		Group II		Group III	
	Control	100-mg Sildenafil	Control	50-mg Sildenafil	Control	100-mL Saline
Peak voltage (V)	107 ± 29	69 ± 32*	84 ± 44	74 ± 35	38 ± 17	41 ± 8
Total energy (J)	0.8 ± 0.3	0.4 ± 0.3*	0.6 ± 0.5	0.5 ± 0.4	0.1 ± 0.1	0.1 ± 0.0
Impedance (ohm)	71 ± 7	73 ± 6	60 ± 9	67 ± 11	76 ± 4	76 ± 10
Pulse width (ms)	18 ± 1	18 ± 1	17 ± 2	17 ± 2	16 ± 3	17 ± 2

*** = $P < 0.03$ vs. control**

Table 4: The Upper Limit of Vulnerability (ULV) before and after sildenafil and saline administration.

ULV	Group I		Group II		Group III	
	Control	100-mg Sildenafil	Control	50-mg Sildenafil	Control	100-mL Saline
Peak voltage (V)	342 ± 52	436 ± 59*	414 ± 147	446 ± 125	397 ± 61	403 ± 35
Total energy (J)	9 ± 2	14 ± 4*	14 ± 12	16 ± 11	12 ± 3	12 ± 2
Impedance (ohm)	66 ± 5	64 ± 5	65 ± 9	60 ± 7	68 ± 7	65 ± 6
Pulse width (ms)	17 ± 1	17 ± 1	16 ± 2	16 ± 1	18 ± 1	17 ± 1

*** = $P < 0.009$ vs. control**

Table 5: The mean ventricular induction window widths (ULV-VFT) among the three groups.

Parameter	**Group I**		**Group II**		**Group III**	
	Control	100-mg sildenafil	Control	50-mg sildenafil	Control	100-mL saline
Voltage (Volts)	235 ± 29	367 ± 40*	330 ± 152	372 ± 132	359 ± 69	362 ± 35
Energy (Joules)	8 ± 2	14 ± 4†	14 ± 12	16 ± 11	12 ± 3	12 ± 2

*** = *P* < 0.001 vs. control**

† = *P* < 0.004 vs. control

อภิปรายและวิเคราะห์ผล

Effects of Sildenafil Citrate on Defibrillation Efficacy

The major findings of the present study are as follows: (1) Intravenous administration of 100-mg sildenafil citrate significantly increased the DFT; (2) infusion of 50-mg sildenafil citrate and saline did not change the DFT; and (3) There were no changes in the heart rate or the systolic blood pressure after saline, 50-mg or 100-mg sildenafil citrate administration.

In the past few years, cardiac electrophysiological effects of sildenafil citrate have been investigated extensively.[4, 26, 45, 46, 52-54] These effects include its ability to decrease the threshold of ventricular tachyarrhythmias and prolong the cardiac repolarization.[27] However, its effect on the defibrillation efficacy has never been reported. To the best of our knowledge, the present study is the first to investigate the effect of sildenafil citrate on defibrillation efficacy. Our results demonstrate that intravenous administration of 50-mg sildenafil citrate (~100-mg taken orally[3]) does not affect the DFT. However, 100-mg sildenafil citrate administered intravenously (~ >200-mg taken orally[53]) significantly increased the DFT. These findings suggest that a dose-dependent effect on the DFT exists, in which the administration of the drug at a supra-therapeutic dosage, but not at a therapeutic dosage, increases the DFT. Supra-therapeutic concentrations of sildenafil citrate have been shown to have harmful effects in patients with conditions such as impaired drug elimination or drug overdose.[55] A recent study using isolated swine right ventricles demonstrated that the threshold for ventricular tachyarrhythmia was decreased with the administration of a high dose sildenafil citrate combined with a nitric oxide donor.[27] Geelen et al have demonstrated that sildenafil citrate induces a dose-dependent block of the rapid component (I_{Kr}) of the delayed rectifier potassium current.[26] Shakir et al reported that sildenafil citrate exerted direct cardiac electrophysiological effects similar to class III antiarrhythmic drugs at concentrations that may be found in conditions of impaired drug elimination, during co-administration of

CYP3A4 substrate/inhibitor, or after drug overdose, and suggested a potential explanation for sudden death during sildenafil treatment.[42] The present study indicates that sildenafil citrate at supra-therapeutic dosage can increase the DFT, a previously unreported adverse effect of this drug.

The increase in the DFT after supra-therapeutic sildenafil citrate administration could be due to its effects on cellular electrophysiological alterations in cardiomyocytes. Sildenafil citrate is known to inhibit PDE-5, causing a net increase in intracellular cGMP concentrations.[23, 56] Musialek et al demonstrated that an increase in cellular cGMP could stimulate the pacemaker current, which may promote an "automatic" tachycardia.[44] At a supra-therapeutic plasma level, sildenafil citrate could also prolong cardiac repolarization of ventricular myocardium by blocking I_{Kr} in a dose-dependent manner.[26] Furthermore, Chiang and colleagues have shown that at supra-therapeutic concentrations, sildenafil citrate accelerates cardiac repolarization in Purkinje fibers and papillary muscle by blocking $I_{Ca,L}$.[36] These electrophysiological changes could cause an increase in the dispersion of refractoriness of the myocardium, facilitating unidirectional block, leading to reentry and eventually VF.[26, 36] Future defibrillation studies such as cardiac mapping are necessary to elucidate the mechanism of increased DFT by supra-therapeutic doses of sildenafil citrate.

Effects of Combined Sildenafil-Nitric Oxide Donor on Defibrillation Efficacy

It was shown previously that sildenafil at the supra-therapeutic level in combination with nitric oxide donor promotes fatal arrhythmias.[27] In that study, sildenafil alone was not arrhythmogenic even at a supra-therapeutic concentration, suggesting that nitric oxide may play a role in the arrhythmogenicity of sildenafil. Recently, sildenafil demonstrated an increase in the DFT when a supra-therapeutic dose was given intravenously, but not when the therapeutic dose was administered.[57] However, the effect of the

sildenafil-nitric oxide donor combination on defibrillation efficacy has never been investigated. To the best of our knowledge, our study is the first to investigate the effect of sildenafil-nitric oxide donor on defibrillation efficacy. Our results demonstrated that intravenous administration of 50-mg of sildenafil (i.e. representing the therapeutic plasma level[53] in combination with NTG does not affect the DFT.

Since our previous study demonstrated that 50-mg of sildenafil alone does not affect the DFT when administered intravenously, the results of this study indicate no detrimental effect of added NTG on defibrillation efficacy. Furthermore, this study demonstrated that the combination of 100-mg of sildenafil (i.e. a supra-therapeutic plasma level[53] and NTG significantly increased the DFT when administered intravenously. It was shown previously that the intravenous administration of 100-mg of sildenafil alone significantly increased the DFT by 19% of voltage and 38% of total energy.[57] In this study, a combined 100-mg of sildenafil-NTG raised the DFT by 17% of voltage and 37% of total energy. Since the results from using 100-mg of sildenafil alone or 100-mg of sildenafil-NTG combination demonstrated a similar percentage of DFT increment by both voltage and total energy, these findings indicate no beneficial or worsening effect of added NTG on defibrillation efficacy over sildenafil administered in a supra-therapeutic dose.

Many studies have been carried out to validate the cardiac electrophysiological effects of sildenafil.[26, 44-46, 54, 56] By using human *ether-a-go*-go-related gene (HERG)-transfected HEK293 cells, Geelen and colleagues [26]demonstrated that sildenafil at the supra-therapeutic level prolongs cardiac repolarization by blocking the rapid component of the delayed rectifier potassium current (I_{Kr}). Accordingly, sildenafil should be expected to improve defibrillation efficacy by lowering the DFT, as do most other I_{Kr} blockers, such as sotalol and dofetilide.[58, 59] However, a recent study performed by Chiang and colleagues[25] showed different findings. They demonstrated that sildenafil at a therapeutic concentration neither blocked I_{Kr} and I_{Ks} in guinea pig

ventricular myocytes, nor prolonged cardiac repolarization in guinea pig papillary muscles and canine purkinje fibers. Moreover, they also found that sildenafil at supra-therapeutic concentrations caused shortening of cardiac repolarization, presumably through its blocking effect on $I_{Ca,L}$[25]. In an *in vivo* study using a canine model, Sugiyama and colleagues demonstrated that intravenous administration of sildenafil at therapeutic to moderate supratherapeutic concentrations did not affect the action potential duration.[56] Therefore, the definite effect of sildenafil on cardiac ion channels must be investigated further and the discrepancy of results verified. Nevertheless, our previous study,[57] and this present study, demonstrated the undesired effect on defibrillation efficacy of the supra-therapeutic plasma level of sildenafil.

In this study, the increase in the DFT after the combination of 100-mg of sildenafil and NTG administration could be due to its effects on cellular electrophysiological alterations in cardiomyocytes. Sildenafil is known to inhibit PDE-5, causing a net increase in intracellular cGMP concentrations.[23, 60] Musialek and colleagues[61] found that an increase in cellular cGMP could stimulate the hyperpolarization-activated inward current (I_f), which may promote an “automatic” tachycardia.[61] However, the constant heart rate after sildenafil administration at both therapeutic and supratherapeutic concentrations reported in the present study suggests that this mechanism should not be the cause of a high DFT. At supra-therapeutic concentrations, sildenafil blocks $I_{Ca,L}$ and accelerates cardiac repolarization[25], which may possibly lead to a shortening of the action potential duration (APD) and effective refractory period (ERP). These electrophysiological changes could be responsible for an attenuation of defibrillation efficacy, resulting in the increase in the DFT found in this study. As for the sildenafil-NTG combination, Yoo and colleagues recently reported that sildenafil did not augment NTG to increase the plasma cGMP concentration in dogs.[62] This might explain no augmentative effect of NTG on defibrillation efficacy when given at either 50-mg or 100-mg of sildenafil, as reported in this study.

Further defibrillation studies such as cardiac mapping are necessary to elucidate the mechanism of increased DFT by supra-therapeutic doses of sildenafil.

Effects of Sildenafil-NTG Combination on Arterial Blood Pressure and Heart Rate

PDE-5 is not only found in the vasculature of the corpus cavernosum, but also the systemic arteries and veins throughout the body.[8] Accordingly, all PDE-5 inhibitors act as mild vasodilators. It has been shown that the blood pressure-lowering effects of sildenafil are dependent on the degree of the drug-activated nitric oxide-guanylate cyclase pathway.[22, 63] In a previous clinical study, when sildenafil was administered orally at doses of 100, 150, and 200 mg in healthy volunteers, the mean maximum decrease in upright SBP was ~10 mmHg, with the maximum change occurring 3 hours after dosing.[53] Although the SBP was significantly decreased, the heart rate was not affected.

In this study, 50-mg of sildenafil-NTG, 100-mg of sildenafil-NTG and saline-NTG administration significantly decreased systolic blood pressure (~12 + 2 mmHg reduction). The heart rate was not altered after 50-mg and 100-mg of sildenafil-NTG and saline (either individually or in combination with NTG) administration. These results on the hemodynamic effects of sildenafil are consistent with previous reports.[51, 57, 64]

Effects of Sildenafil Citrate on the Induction of Ventricular Fibrillation and Upper Limit of Vulnerability

In this study, VF inducibility was obviously facilitated as indicated by the reduction in the VFT after an administration of a supra-therapeutic concentration of sildenafil citrate. Moreover, the ULV was increased after administering a supra-therapeutic concentration of sildenafil citrate, indicating that for a delivered shock, a higher shock strength above the VFT is required

to reach the level at which VF is no longer induced during the vulnerable period of the cardiac cycle. Furthermore, both VFT reduction and an increased ULV evidently indicated that the VF induction window during the vulnerable period was widened by administering a supra-therapeutic concentration of sildenafil citrate.

Our findings slightly differed from Swissa et al.[27], which tested the effects of sildenafil citrate in a slab of pig's right ventricle and reported that VT/VF was promoted only by administration of a high concentration of sildenafil citrate together with nitric oxide (NO). Using a swine model, it has been shown that a supra-therapeutic concentration of sildenafil citrate alone can decrease defibrillation efficacy.[57] Furthermore, the present study also demonstrated that VFT is decreased and the ULV is increased after administration of a supra-therapeutic concentration of sildenafil citrate alone.[27] also found that neither the action potential duration (APD) nor the ERP were affected by any concentration of sildenafil citrate used in their study (0.2-2μg/ml). In the present study, we found no change in the ERP after either concentration of sildenafil citrate administration, a finding consistent with previous reports from clinical studies that QT interval is not changed after sildenafil citrate administration.[45, 54, 65]

A previous study demonstrated that a supra-therapeutic concentration of sildenafil citrate decreased defibrillation efficacy by increasing the DFT.[57] Similar to that report, only supra-therapeutic concentration of sildenafil citrate affected the VFT and the ULV in the present study. From this finding, it is revealed that only a supra-therapeutic concentration of this drug could facilitate VFT induction and decrease defibrillation efficacy. It is possible that supra-therapeutic concentration of sildenafil citrate has different effects on cardiac repolarization in various parts of the heart, as previously demonstrated by Geelen and coworkers and Chiang and colleagues.[25, 26] A study from Geelen group demonstrated that cardiac repolarization was prolonged by supra-therapeutic concentration of sildenafil citrate in isolated

guinea pig hearts.[26] Rapid component (I_{Kr}) of the delayed rectifier potassium current was inhibited by supra-therapeutic concentration of sildenafil citrate in HERG-transfected HEK 293 cells.[26] However, in guinea pig papillary muscles and canine Purkinje fibers, Chiang et al. demonstrated that cardiac repolarization was shortened by high concentrations of sildenafil citrate without any effect on I_K current.[25] Instead, they found that supra-therapeutic concentrations inhibited calcium current ($I_{Ca,L}$) in guinea pig ventricular myocytes.[25] Additionally, Chiang et al. have demonstrated that sildenafil citrate did not affect I_{Na} current and did not change the QRS duration, suggesting that the drug did not influence the conduction velocity.

All of these findings suggest that sildenafil citrate could cause myocardial substrate in the heart to be more vulnerable to arrhythmia due to its different electrophysiological effects on different myocardial cell types. Since QT analysis before and after sildenafil citrate administration did not show significant alteration,[45] future studies are needed to investigate the effects of sildenafil on different myocardial cells (i.e. papillary muscle, Purkinje fibers and myocardial layers) as substrates for arrhythmia induction. Furthermore, significant reduction in blood pressure could be responsible for promoting arrhythmia after sildenafil citrate administration since hypotension could increase sympathetic activity, leading to decreasing VFT and increasing ULV, independent of reflex tachycardia.[66] However, neither VFT and ULV were changed after 50 mg sildenafil citrate administration, suggesting that hypotension may not be the main mechanism for ventricular tachyarrhythmia induction by sildenafil citrate.

CONCLUSION

Sildenafil citrate, at supra-therapeutic concentration, markedly decreases the defibrillation efficacy by increasing the DFT. Furthermore, this high concentration also increases the inducibility of ventricular arrhythmia by decreasing the VFT as well as increasing the ULV, resulting in an expansion

of the VF induction window. Although the mechanism is still unclear, the findings from this study indicate that myocardial substrate is more vulnerable to tachyarrhythmias after supra-therapeutic sildenafil citrate administration. These findings could implicate that the use of this drug in patients with cardiovascular defects such as Brugada's syndrome, MI and heart failure should be cautious since arrhythmia could be facilitated and will be more difficult to defibrillate once VF occurs, particularly in those with conditions such as impaired drug elimination, as well as co-administration with CYP 3A inhibitors since the concentration of sildenafil could be elevated.

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OUTPUT

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(ดังเอกสารแนบหมายเลข 1)

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Sildenafil citrate on the inducibility of ventricular fibrillation and upper limit of vulnerability in swine

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C Statistical Analysis
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G Funds Collection

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Summary

Background: Sildenafil citrate at supratherapeutic levels has been reported to decrease defibrillation efficacy. However, its effects on ventricular fibrillation induction and the upper limit of vulnerability (ULV) have not been investigated. We tested the hypothesis that sildenafil citrate reduces the ventricular fibrillation threshold (VFT) and increases the ULV.

Material/Methods: Twenty-one pigs (25–30 kg) were randomly assigned into 3 groups of 7 pigs each. A solution containing 100 mg (group 100) or 50 mg (group 50) sildenafil citrate or 100 cc saline (group control) was infused intravenously in each pig. A train of 10 S1s was delivered from an RV electrode, and an S2 stimulus was delivered at the peak of the T wave of the last S1 activation to determine the VFT and ULV, before and after drug administration.

Results: The 100 mg sildenafil citrate significantly ($P<0.03$) decreased VFT, accounting for ~36% by peak voltage and ~52% by total energy, and significantly ($P<0.009$) increased ULV, accounting for ~28% by peak voltage, and ~56% by total energy.

Conclusions: Supratherapeutic concentrations of sildenafil citrate significantly decreased the VFT and increased the ULV, resulting in an expansion of the VF induction window during the vulnerable period.

key words: **sildenafil citrate • ventricular fibrillation • upper limit of vulnerability • sudden cardiac death**

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BACKGROUND

Sildenafil citrate is a highly selective phosphodiesterase type 5 (PDE5) inhibitor and has been used to treat erectile dysfunction. Although this drug has been used for many years, several authors have reported adverse cardiac effects associated with its use [1–5]. Although the effects of sildenafil citrate on cardiac electrophysiology have been investigated, the results of these investigations remain debatable [6–13].

In an *in vitro* study, Geelen and associates demonstrated that supratherapeutic concentrations of sildenafil citrate prolonged cardiac repolarization by blocking delayed rectifier potassium current (I_{Kr}) [9]. Chiang and associates, however, demonstrated that calcium current ($I_{Ca,L}$) was blocked, thus shortening cardiac repolarization by a supratherapeutic concentration of sildenafil citrate in isolated pig papillary muscle and canine Purkinje fiber [7]. Using a slab of the right ventricle, Swissa and associates reported that ventricular fibrillation (VF) was frequently induced when a high concentration of sildenafil citrate was administered together with nitric oxide, indicating its effect in inducing VF [13]. The QT interval can reflect the cardiac repolarization. However, clinical studies have shown that sildenafil citrate does not affect the QT interval [6,10,11].

Recently, the effect of sildenafil citrate on defibrillation efficacy has been investigated in a swine model. Shinlapawittayatorn and associates demonstrated that supratherapeutic concentrations of sildenafil citrate significantly decreased defibrillation efficacy by increasing the defibrillation threshold [12]. Because the effects of sildenafil citrate on arrhythmia induction have been done mostly in *in vitro* or *ex vivo* models, the purpose of the present study was to use a humanlike animal model to test the hypothesis that sildenafil citrate facilitates arrhythmia induction by decreasing the ventricular fibrillation threshold (VFT) and increasing the upper limit of vulnerability (ULV).

MATERIAL AND METHODS

Animal preparation and electrode placement

Twenty-one healthy pigs (25–30 kg) of both sexes were anesthetized and maintained under physiologic conditions as described previously [14–16]. All animals were cared for according to the *Guide for Care and Use of Laboratory Animals*, Faculty of Medicine, Chiang Mai University. A catheter with a 34-mm platinum-coated titanium coil electrode (Guidant Corp.) was inserted into the right ventricular apex. A 68-mm electrode catheter was positioned at the junction between the right atrium and the superior vena cava. A defibrillator (Ventak, Guidant Corp.) was used to deliver biphasic truncated exponential shocks (S2) via these 2 coils. An electrode at the tip of the RV catheter was used to deliver S_1 stimuli.

Diastolic pacing threshold and effective refractory period determination

The diastolic pacing threshold was determined by delivering a train of 10 S1 stimuli of a 5-ms square pulse at 500-ms intervals. The current strength began at 0.1 mA and was increased in 0.1-mA steps until all drive trains elicited a ventricular response (capture). The diastolic pacing threshold is defined as the minimum current strength necessary to capture the ventricle. The effective refractory period was determined by delivering an S2 stimulus (2–5× diastolic pacing threshold) in the late diastole of the last S1 paced beat. The coupling interval of the last S1-S2 was initially set at 350 ms. If an S2 could elicit a capture, decrements in 10-ms steps were done until S2 failed to elicit a capture. The effective refractory period is defined as the longest S1-S2 interval at which an S2 stimulus fails to elicit a ventricular response.

VFT determination

The interval between the last S1 and the peak T wave was determined 3 times from lead-2 electrocardiogram, and the average of the S1-peak T-wave interval was used as a coupling interval between the last S1 and S2 shock [17]. The VFT was determined by delivering an S2 shock starting at 100 V. If this shock induced VF, a decrement of 20 V was used for each successive shock until VF was no longer induced. If the 100 V did not induce VF, an increment of 20 V was used for each successive shock until VF was induced. The lowest shock strength that could induce VF was defined as the VFT. When VF was induced, a rescue shock was delivered immediately, and at least 4 minutes was allowed before the next S2 shock was delivered [12,17].

Determining the upper limit of vulnerability

The upper limit of vulnerability was determined after the VFT was obtained. The first shock strength was at 400 V, delivered at the S1-S2 coupling interval at which the VFT was obtained. In the event of successful VF induction, the leading edge voltage was increased in 80-V steps per shock attempt until a first reversal from successful VF induction to failure was achieved. If the initial shock was unsuccessful, the voltage was decreased in 80-V steps per shock attempt until a reversal from failed to successful VF induction was achieved. At each reversal point, the algorithm was iterated in the opposite direction, except that after the first reversal, the voltage step size was decreased to 40 V and 20 V for a total of 3 reversals [14,15]. The ULV is defined as the lowest shock strength (above the VFT) that cannot induce VF after 3 reversal points, when the next lower setting successfully induces VF.

Drug administration

The stock solution contained 50 and 100 mg sildenafil citrate in tablet form dissolved in 100 mL normal saline. Each concentration was injected intravenously over 50 minutes. The dosages used in this study were inferred from the pharmacokinetic study comparing intravenous and oral sildenafil citrate administration and had been used in a previous defibrillation study [12,18,19]. An intravenous dose of 50 mg sildenafil citrate has been shown to produce a therapeutic plasma level, whereas a 100-mg intravenous dose has been shown to be a supratherapeutic concentration [12,18,20].

Statistical analyses

Values are expressed as means ±SD. Comparisons of data were done using the paired 2-tailed *t* test. Values for *P* less than.05 were considered statistically significant.

Table 1. Basic electrophysiological variables, and blood pressure and heart rate before and after sildenafil and saline administration.

Parameters	Group I		Group II		Group III	
	Control	100-mg Sildenafil	Control	50-mg Sildenafil	Control	100-mL Saline
Diastolic pacing threshold (mA)	1.1±0.8	1.2±0.8	0.6±0.3	0.7±0.2	0.5±0.2	0.6±0.2
S1-S2 coupling interval (ms)	317±35	315±28	283±36	292±36	256±30	264±32
Effective refractory period (ms)	251±37	280±39	247±29	261±32	229±29	247±24
Systolic BP (mm Hg)	116±18	94±12**	106±9	86±12*	120±12	129±22
Diastolic BP (mm Hg)	77±17	59±11**	63±6	51±9*	76±12	82±19
Mean arterial BP (mm Hg)	90±18	70±12**	78±7	62±9*	70±6	75±12
Heart rate (bpm)	99±33	90±32	90±16	98±19	103±14	106±7

* P<0.01 vs control; ** P<0.05 vs control.

Table 2. Ventricular fibrillation threshold (VFT) before and after sildenafil and saline administration.

VFT	Group I		Group II		Group III	
	Control	100-mg Sildenafil	Control	50-mg Sildenafil	Control	100-mL Saline
Peak voltage (V)	107±29	69±32*	84±44	74±35	38±17	41±8
Total energy (J)	0.8±0.3	0.4±0.3*	0.6±0.5	0.5±0.4	0.1±0.1	0.1±0.0
Impedance (ohm)	71±7	73±6	60±9	67±11	76±4	76±10
Pulse width (ms)	18±1	18±1	17±2	17±2	16±3	17±2

* P<0.03 vs control.

Table 3. Upper limit of vulnerability (ULV) before and after sildenafil and saline administration.

ULV	Group I		Group II		Group III	
	Control	100-mg Sildenafil	Control	50-mg Sildenafil	Control	100-mL Saline
Peak voltage (V)	342±52	436±59*	414±147	446±125	397±61	403±35
Total energy (J)	9±2	14±4*	14±12	16±11	12±3	12±2
Impedance (ohm)	66±5	64±5	65±9	60±7	68±7	65±6
Pulse width (ms)	17±1	17±1	16±2	16±1	18±1	17±1

* P <.009 vs control.

Results

The basic electrophysiological parameters such as diastolic pacing threshold, S1-S2 coupling interval, and effective refractory period were not different before and after sildenafil citrate or saline administration in each group (Table 1). For hemodynamic variables, both concentrations of sildenafil citrate significantly reduced systolic blood pressure, diastolic blood pressure, and mean arterial blood pressure (Table 1). However, heart rate was unchanged in both groups.

For the VFT, 100 mg sildenafil citrate significantly decreased the VFT peak voltage and total energy compared with controls (Table 2). The percentage reduction is approximately 36% by peak voltage and approximately 52% by total energy. However, neither 50 mg sildenafil citrate nor saline changed the VFT variables compared with controls (Table 2).

For the ULV, 100 mg sildenafil citrate significantly increased ULV peak voltage and total energy compared with controls (Table 3), accounting for an increment of approximately 28% by peak voltage and approximately 56% by total energy. The 50-mg sildenafil citrate and saline did not change the ULV compared with controls (Table 3).

The VF induction windows in each pig were calculated by subtracting both voltage and energy of the VFT from the ULV. The mean VF induction windows before and after sildenafil citrate and saline administration are shown in Table 4. The VF induction windows were expanded after administration

Table 4. Mean VF induction window width (ULV-VFT) among the 3 groups.

Parameter	Group I		Group II		Group III	
	Control	100-mg Sildenafil	Control	50-mg Sildenafil	Control	100-mL Saline
Voltage (volts)	235±29	367±40*	330±152	372±132	359±69	362±35
Energy (joules)	8±2	14±4**	14±12	16±11	12±3	12±2

* P<0.001 vs control; **P<0.004 vs control.

of 100 mg sildenafil citrate compared with controls (Table 4). However, the VF induction window was not affected by administering 50 mg sildenafil citrate or saline (Table 4).

Discussion

Sildenafil citrate and VF induction

In this study, VF inducibility was facilitated, as indicated, by a reduction in the VFT after administering a supratherapeutic concentration of sildenafil citrate. Moreover, the ULV was increased after administering a supratherapeutic concentration of sildenafil citrate, indicating that for a delivered shock, a higher shock strength above the VFT is required to reach the level at which VF is no longer induced during the vulnerable period of the cardiac cycle. Furthermore, both VFT reduction and an increased ULV evidently indicated that the VF induction window during the vulnerable period was widened by administering a supratherapeutic concentration of sildenafil citrate.

Our findings differed slightly from those of Swissa and associates [13], which tested the effects of sildenafil citrate in a slab of pig's right ventricle. Those authors reported that VT/VF was promoted only by administering a high concentration of sildenafil citrate together with nitric oxide. Using a swine model, it has been shown that a supratherapeutic concentration of sildenafil citrate alone can decrease defibrillation efficacy [12]. Furthermore, the present study also demonstrated that VFT is decreased and the ULV is increased after administering a supratherapeutic concentration of sildenafil citrate alone. Swissa and associates [13] also found that neither the action potential duration nor the effective refractory period were affected by any concentration of sildenafil citrate used in their study (0.2–2 μg/mL). In the present study, we found no change in the effective refractory period after either concentration of sildenafil citrate, a finding consistent with previous reports from clinical studies that the QT interval is not changed after administering sildenafil citrate [6,10,11].

A previous study demonstrated that a supratherapeutic concentration of sildenafil citrate decreased defibrillation efficacy by increasing the DFT [12]. Similar to that report, only supratherapeutic concentration of sildenafil citrate affected the VFT and the ULV in the present study. From this finding, it is revealed that only a supratherapeutic concentration of this drug could facilitate VFT induction and decrease defibrillation efficacy. It is possible that a supratherapeutic concentration of sildenafil citrate has different effects on cardiac repolarization in various parts of the heart, as previously demonstrated by Chiang and associates and Geelen and associates [7,9]. Geelen and associates demonstrated that cardiac repolarization was prolonged by a supratherapeutic concentration of sildenafil citrate in isolated guinea pig hearts [9]. Rapid component (I_{Kr}) of the delayed rectifier potassium current was inhibited by a supratherapeutic concentration of sildenafil citrate in HERG-transfected HEK 293 cells [9]. However, in guinea pig papillary muscles and canine Purkinje fibers, Chiang and associates demonstrated that cardiac repolarization was shortened by high concentrations of sildenafil citrate without any effect on I_K current [7]. Instead, the authors found that supratherapeutic concentrations inhibited calcium current ($I_{Ca,L}$) in guinea pig ventricular myocytes [7]. Additionally, Chiang and associates demonstrated that sildenafil citrate does not affect I_{Na} current and does not change the QRS duration, suggesting that the drug does not influence conduction velocity.

All of these findings suggest that owing to its different electrophysiological effects on different myocardial cell types, sildenafil citrate could cause the myocardial substrate in the heart to be more vulnerable to arrhythmias. Because QT analyses before and after sildenafil citrate administration did not show significant alterations [10], future studies are needed to investigate the effects of sildenafil on different myocardial cells (i.e., papillary muscle, Purkinje fibers, and myocardial layers) as substrates for arrhythmia induction. Furthermore, significant reductions in blood pressure could be responsible for promoting arrhythmias after administration of sildenafil citrate, because hypotension could increase sympathetic activity, leading to decreasing VFT and increasing ULV, independent of reflex tachycardia [21]. However, neither VFT nor ULV was changed after 50-mg sildenafil citrate administration, suggesting that hypotension may not be the main mechanism for ventricular tachyarrhythmia induction by sildenafil citrate.

Clinical implications

Several studies suggest that a high concentration of sildenafil citrate might be present only in patients with renal impairment or in patients in whom CYP 3A inhibitors also have been administered [22–24]. However, recent reports have demonstrated arrhythmic events in a child as well as in a patient with an acute myocardial infarction who took oral, high-dose sildenafil citrate [25,26]. These reports support our finding that supratherapeutic concentrations of sildenafil citrate could cause the myocardium to be vulnerable to ventricular arrhythmias.

Study limitations

This study was done in normal pig hearts, and the results may be different in diseased hearts or in humans. Also, we

recorded the effective refractory period from a single site. The unaltered effective refractory period found in this study may not represent the effects of supratherapeutic levels of sildenafil citrate on various cardiac cell types such as papillary muscle or Purkinje fibers in the heart. Since QT analyses from a previous study did not show any significant change after sildenafil administration [10], it is necessary that recordings obtained in various cell types be determined in the future. Sildenafil citrate plasma concentrations were not measured, so the estimates of therapeutic and supratherapeutic levels are based on inferences from previous studies [19]. In the current experiment, we did not use the T-wave scanning method to determine timing of the shocks for measuring the VFT and the ULV. Therefore, the VFT and the ULV could be different from those determined by a T-wave scanning method.

Conclusions

Sildenafil citrate, at supratherapeutic concentrations, markedly increases the inducibility of ventricular arrhythmias by decreasing the VFT as well as increasing the ULV, resulting in an expansion of the VF induction window. Although the mechanism is still unclear, the findings from this study indicate that myocardial substrate is more vulnerable to tachyarrhythmias after supratherapeutic sildenafil citrate administration. These findings could indicate that use of this drug in patients with cardiovascular defects such as Brugada's syndrome, MI, and heart failure should be cautious because arrhythmias could be facilitated, particularly in those with conditions such as impaired drug elimination, as well as coadministration with CYP 3A inhibitors because the concentration of sildenafil could be elevated.

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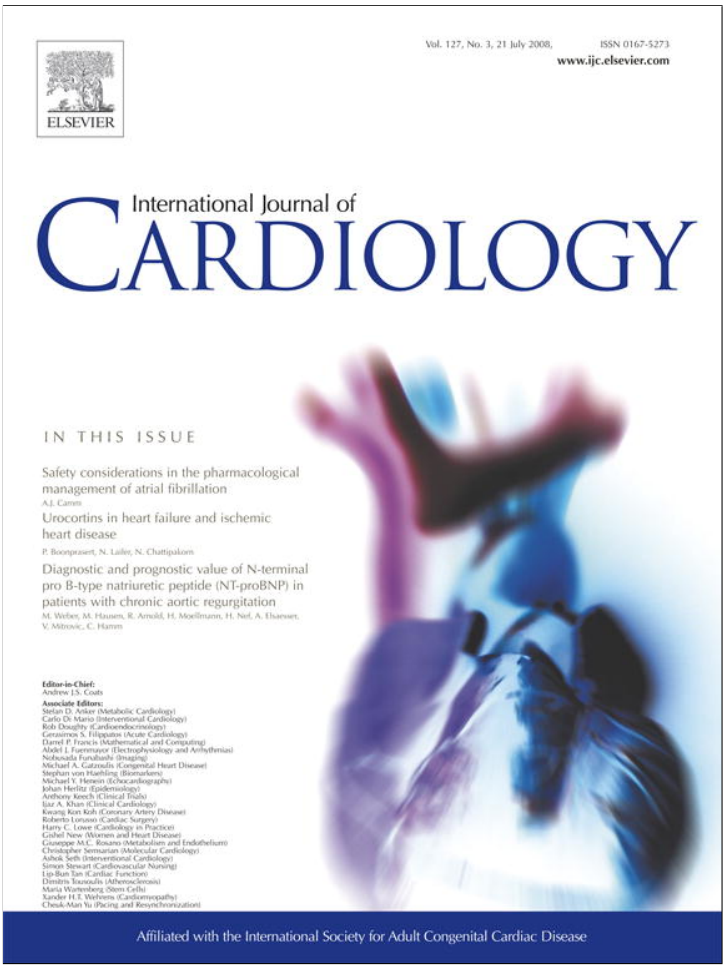

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Review

Urocortins in heart failure and ischemic heart disease ☆

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Abstract

Urocortins, a novel member of the corticotrophin-releasing factor (CRF) family, have been shown in animal and human studies to possess several beneficial effects in stress, cardiovascular and renal function, and inflammatory responses via CRF receptors. In the heart, urocortins have been demonstrated to produce cardioprotective effects during ischemia and reperfusion injury. Urocortins have also exerted effects on hemodynamic, endocrine and renal parameters in experimental animal heart failure models. In humans, plasma urocortin levels have been shown to significantly increase in systolic heart failure patients. This growing evidence suggests that urocortins may have a prognostic value as well as being a potential therapeutic treatment for heart failure and myocardial infarction patients. Currently, only a few clinical studies on urocortins are available. In this review article, the role of urocortins in the heart has been summarized. Their possible beneficial roles in heart failure and myocardial infarction have been discussed, based on relevant published articles from both basic and clinical studies available to date.

Keywords: Urocortins; Corticotropin-releasing factor; CRF receptor; Heart failure; Myocardial infarction; Ischemia–reperfusion injury

1. Introduction

Heart failure and ischemic heart disease are common cardiac syndromes, in which an increased incidence has been shown in most nations around the world. The progress of these clinical syndromes can lead to left ventricular dysfunction; including systolic and diastolic dysfunction, and sudden cardiac death. To prevent the progression of the diseases, their identification at an early stage is crucial, since late stage heart failure has been shown to have poor prognosis with high mortality [1]. In past decades, several cardiac markers were shown to have a predictive value regarding the progression and prognosis in myocardial infarction and heart failure patients. These markers included NT-proBNP [2], cardiac troponin [3], hs-CRP [4], and creatine kinase [5]. Recently, the novel cardioprotective peptides, "urocortins", have been demonstrated to possess potential value as an early diagnostic marker in heart failure [6]. In this review article, the roles of urocortins as an early cardiac marker as well as their effects in heart failure and ischemic heart disease from animal and clinical studies are presented. In addition, the roles of urocortins, which point to a future clinical research and therapeutic application, are also discussed.

2. Urocortins

Urocortins, 40 amino acid peptides, are a member of the hypothalamic corticotropin-releasing factor (CRF) family. The CRF family consists of CRF, fish urotensin I, frog sauvagine, urocortin I, urocortin II (stresscopin-related peptide, SRP), and

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urocortin III (stresscopin, SCP) [7–10]. The actions of the CRF family peptides are mediated via CRF receptors that are derived from two distinct genes; CRF type1 receptor and CRF type2 receptor [11,12]. Rat urocortin I was first discovered in 1995 from a discrete midbrain region [9]. Its structure is related to urotensin (65% sequence identified) and corticotropin-releasing factor (CRF) (45% sequence identified) [9]. Human urocortin I was identified later by the molecular cloning technique [10]. Regarding its sequence, it was 63% identical to fish urotensin I and 43% identical to CRF at the amino acid level. In rat, the rank orders of mRNA expression of CRF and urocortin I as well as their receptors in each of the tissue types have been shown as follows: the majority of CRF mRNA was found in the brain, whereas the majority of urocortin I mRNA was found in both the heart and brain [13]. Recently, new members of this family, urocortin II and urocortin III, have been identified from human libraries and the genome database [23]. Urocortin II and urocortin III have approximately 20–40% homology with CRF and urocortin I. The homology between urocortin II and urocortin III is approximately 40%. In 2001, human versions of these novel urocortins were also isolated, including a stresscopin-related peptide, which is equivalent to urocortin II, and a stresscopin that is equivalent to urocortin III [7,8,14].

Although urocortins share moderate sequence identity with one another and with CRF, each urocortin has a unique anatomical distribution under the control of different genes. In humans, urocortin I is detected in the brain, placenta, gastrointestinal tract, synovial tissue, lymphocytes, adipose tissue, endothelial cells, immunological tissues, and heart [15–21]. In the brain, urocortin I was detectable at the Edinger–Wesphal nucleus, the lateral superior olive, the supraoptic nucleus (SON), the lateral hypothalamic area, and most caudal part of several brainstem and spinal cord motoneuron nuclei [12,22]. In the nervous system, urocortin I has been shown to enhance anxiety and suppress appetite via the CRF type1 receptor [23]. The finding of urocortin mRNA expression in synovium tissues in rheumatoid arthritis and osteoarthritis patients suggests that urocortin may also act as an immune-inflammatory mediator [19]. Urocortin I activates the pituitary–adrenal axis inducing the release of adrenocorticotropic hormone (ACTH), and increasing plasma cortisol, and atrial natriuretic peptide in animal models [24–27]. In humans, the biological effects of urocortin I infusion in healthy males include increased plasma level of ACTH and cortisol. These effects of urocortin I are mediated via the CRF type1 receptor [28]. Similar to urocortin I, urocortin II is expressed in the heart and discrete areas of the central nervous system of rats, including stress-related cell groups in the hypothalamus, brainstem and spinal cord [8,29]. Urocortin III is also expressed in the brain and peripheral tissues such as gastro-intestinal tract, pancreas, and skin. However, the expression of urocortin III is considerably lower than that of urocortin II [7,30–32]. In humans, urocortin III was present in both the ventricles and atria [33]. However, the highest levels were found in all regions of the pituitary and hypothalamus [32]. Urocortin III was also distributed in other tissues, such as the lung, pancreas, liver, spleen and skeletal muscle, and present in human plasma and urine [33].

3. CRF receptors for urocortins

CRF receptors consist of two types of seven-transmembrane-spanning G-protein receptors, which are coupled to adrenylyl cyclase: CRF type1 and CRF type2 receptors. The CRF type1 receptor is found predominantly in the pituitary and brain regions, whereas the CRF type2 receptor is highly expressed in peripheral tissues including the heart [8,13,29,34]. The CRF type1 receptor has a single functional form: CRF type1 (alpha) receptor, whereas the CRF type2 receptor has three isoforms: CRF type2 (alpha) receptor, CRF type2 (beta) receptor and CRF type2 (gamma) receptor [11,35,36]. The CRF type1 receptor implicates in adrenocorticotropic hormone (ACTH) release and anxiety-like effect, while the CRF type2 receptor contributes to anxiolysis, appetite-inhibition, vasodilatation, positive inotropic action on the myocardium, and dearousal effects [12]. CRF and urocortin I can bind to both CRF type1 and type2 receptors [9]. Urocortin II and urocortin III bind to only the CRF type2 receptor [7,8]. The CRF type2 receptor binds to urocortin I, urocortin II and urocortin III, with approximately 40-fold greater affinity than CRF [9].

CRF type2 receptors have different tissue distributions. In rats, the CRF type2 (alpha) receptor is highly expressed in the brain, while the CRF type2 (beta) receptor is expressed in the brain, heart, lung, gastrointestinal tract, and skeletal muscle [37]. The CRF type2 (gamma) receptor has only been detected in the brain [38]. The cDNA sequence in the protein-coding region of rat tissue is 94% identical to the CRF type2 (beta) receptor [39,40]. The CRF type2 (beta) receptor has 10-fold higher affinity to urocortin than CRF in rat models [9], whereas the CRF type1 receptor shows little ligand selectivity for urocortin [10]. In the rat's heart, only the CRF type2 (beta) receptor is found. The high level of urocortin mRNA detected in the heart was related to the high expression of CRF type2 (beta) receptor coupling in the heart tissue of rats [13]. In the human heart, however, only the alpha type of CRF type2 receptor is found [41,42].

4. Urocortins and the heart

Kimura et al. reported that urocortins and the CRF type2 (alpha) receptor were detected in all chambers of four human hearts, and detected with highest intensity in the left ventricle, whereas CRF was not detected in cardiac myocytes [17]. In their study, detection of the CRF type1 receptor was weak in the left atrium, left ventricle and right ventricle [17]. Since the CRF type2 (alpha) receptor was detected predominantly in the left ventricle, it has been suggested that urocortins, not CRF, may have an important role on cardiac function [17]. By using radioligand binding techniques, Wiley and Davenport

demonstrated that urocortins have a high affinity to the CRF type2 (alpha) receptor in human left ventricular myocardium, intramyocardial blood vessels and within the medial layer of internal mammary arteries [43].

Currently, urocortins are overwhelmingly shown to affect cardiovascular physiology in normal animals, and pacing-induced heart failure in animal models and clinical studies [26,27,44,45]. The cardiovascular effects of urocortins include a dose-dependent increase in heart rate, cardiac output, and coronary blood flow [26]. Interestingly, urocortins have been shown to possess an anti-apoptotic effect in myocardium that has undergone ischemia–reperfusion injury [26,44,45]. Urocortin and CRF also exerted coronary vasodilatation and positive inotropic effect. These physiological effects of urocortin have been shown to last longer than the action of CRF [46].

The high dose administration of urocortin I in rats has been reported to decrease arterial pressure via vasodilatation, as revealed by a decrease in total peripheral resistance and slightly increased cardiac output [47]. The dilator action of urocortin I is mostly manifested in the heart and stomach [48]. In mice, urocortin I increases cardiac contractility, heart rate, and vasodilatation [46]. However, these effects were not found in CRF type2 receptor-deficient mice [49,50]. These data indicate that actions of urocortin I in peripheral tissues are expressed via the CRF type2 receptor. Urocortin I has also been shown to enhance cardiac output, protect myocytes from ischemia–reperfusion injury, stimulate cardiac natriuretic peptide secretion, induce vasodilatation, especially in the coronary arteries of normal and heart failure models [26,27,44–46,51–54], and decrease left atrial pressure and peripheral vascular resistance [27,27,47].

Urocortin II exerts positive inotropic effect via CRF type2 receptor-mediated stimulation and enhances contractility of rabbit ventricular myocytes [55]. Urocortin III causes an increase in cardiac output and a decrease in peripheral resistance and left atrial pressure in heart failure sheep [56]. Since these cardiovascular effects are beneficial in reducing preload and afterload to the heart, researchers are currently investigating the roles of urocortins in heart failure using animal models as well as heart failure patients [6,27,47,56,57].

5. Urocortins and heart failure

Rademaker et al. investigated the effects of urocortin administration in both normal and pacing-induced heart failure sheep [27,47,56,57]. Urocortin I, urocortin II and urocortin III induced an immediate increase in cardiac output and a decrease in peripheral resistance and left atrial pressure. In normal sheep, however, only slight changes in peripheral resistance and atrial pressure were found. Interestingly, urocortin I, urocortin II and urocortin III caused a dose-dependent decrease in plasma vasopressin, endothelin-1, renin, aldosterone, epinephrine, plasma atrial and brain natriuretic peptide levels in heart failure sheep, whereas adrenocorticotropic hormone and cortisol levels were increased. These urocortins also increased urine output, sodium excretion, and creatinine clearance [47,56,57]. Urocortin infusion in normal sheep resulted in a similar rise in cortisol and vasopressin, a decrease in aldosterone, and no significant effects on plasma renin activity and natriuretic peptides. Nevertheless, long-term peripheral and central administration of urocortin caused an increase in arterial pressure and heart rate in 15 normal ewes [58]. Furthermore, Mackay et al. also demonstrated that urocortin II caused a reduction in arterial pressure and increased heart rate in fifty adult heart failure rats [59]. These reports, using animal models, support the role of urocortins in arterial pressure and intravascular volume homeostasis in heart failure.

Currently, there are only a few clinical studies of urocortin available. In a cross-sectional study by Ng et al., investigation was carried out on plasma urocortin in 119 patients with systolic heart failure and 212 healthy individuals (LVEF > 50%) [6]. They reported that urocortin was elevated in systolic heart failure (LVEF ≤ 45%), especially in NYHA functional classes I–II [6]. The relative increase in plasma urocortin level was greater in males than in females, but the level decreased with increasing age, especially in heart failure groups. The decreased urocortin levels, with increasing NYHA class, were reinforced by a significant correlation between urocortin and ejection fraction in heart failure patients. From these findings, it was suggested that the urocortin level complemented NT-proBNP in the early diagnosis of mild clinical heart failure [6]. An increased plasma urocortin level in mild stage systolic heart failure, and the reduced level when heart failure progresses, could have potential clinical benefits if warranted, since these findings may be used for the diagnosis of early heart failure and as a prognostic indicator for heart failure progression. Recently, Davis et al. investigated the effects of urocortin II infusion in 8 healthy unmedicated men [60]. They found that urocortin II induced an increase in cardiac output, heart rate, and left ventricular ejection fraction, while decreasing systemic vascular resistance. These effects also increased plasma renin activity, angiotensin II and norepinephrine [60]. Although the clinical benefits of urocortins are still unclear, due to the small number of clinical studies, the growing body of evidence indicates the need to warrant their clinical significance by performing more clinical studies in the future, including those that investigate the plasma profile of urocortin in heart failure patients with long-term follow up.

6. Urocortins and myocardial ischemia

Apoptosis is known to implicate the pathogenesis of cardiovascular diseases such as myocardial infarction [61]. It causes the loss of cardiac myocytes and worsens the cardiac function. While hypoxia induces both necrotic and apoptotic forms of cell death in cardiac myocytes, the degree of cardiac dysfunction after ischemia–reperfusion injury reflects the level of myocardial injury and cell death [62].

In experimental studies, the increased urocortin level had been expressed when cardiac cells were exposed to thermal shock and ischemia [63,64]. Brar et al. studied urocortin

against the damaging effects of ischemia–reperfusion injury in rat hearts [44]. They demonstrated that exogenous urocortin caused increased cardiac contractility and vasodilatation, especially in coronary arteries, and protected the heart during ischemia by reducing the number of cell deaths induced by hypoxia [44]. Urocortin prevented neonatal rat myocytes from cell death when it was administered prior to stimulated hypoxia/ischemia, and at the point of reoxygenation after stimulated hypoxia/ischemia [44]. It acted via a p42/p44 MAPK-dependent signaling pathway in *in vitro* and *in vivo* adult rat hearts with ischemia–reperfusion injury [54]. In cardiac myocytes of rats, Okosi et al. demonstrated that the urocortin mRNA level was increased by thermal shock and that exogenous urocortin protected cardiac myocytes from cell death induced by hypoxia [64]. Exogenous urocortin also protected cardiac myocytes against necrotic cell death from a reduction in LDH release [64]. Other protective roles of urocortin in ischemia–reperfusion injury include inhibiting free radical activities, preventing mitochondrial damage, and activating the phosphatidylinositol 3-OH kinase and PKC-epsilon in mice [65–69]. The infarction size and mean arterial pressure were significantly decreased in rat hearts that received urocortin [54,69].

Urocortin increased interleukin-6 levels from peripheral blood mononuclear cells [19]. In mice, it reduced lipopolysaccharide-induced serum TNF-alpha and interleukin-1 beta levels [19,70]. Honjo et al. demonstrated that urocortin mRNA expression is also upregulated by TNF-alpha and IFN-gamma [18], suggesting that urocortin could have an anti-oxidative effect against oxidative stress in inflammatory lesions [18]. Urocortin has also been shown to reduce necrotic and apoptotic cell death in isolated rat hearts exposed to ischemia–reperfusion injury, and partially prevent the depletion of cellular energy stores, with enhancement of ventricular function [62].

In murine hearts, Brar et al. demonstrated that urocortin II and urocortin III protected murine cardiomyocytes from ischemia–reperfusion injury and reduced the percentage of infarct size [29]. These effects have been demonstrated to act via the ERK1/2-p42 signaling pathway and CRF type2 receptor mediated in the heart [29].

7. Future role and clinical application of urocortins

Previous studies have demonstrated the beneficial effects of urocortin, including coronary vasodilatation, increased cardiac contractility, coronary blood flow and conductance, cardiac output, and heart rate as well as protection against ischemia–reperfusion injury [26,27,29,44,45,47,56,57,63,64]. Theoretically, these effects are useful in the treatment of cardiovascular disorders, particularly heart failure and conditions associated with ischemia–reperfusion such as myocardial infarction. Practically, the role of urocortins in heart failure and ischemic heart disease is still unclear. Therefore, caution should be taken despite growing evidence of the beneficial effects of urocortins. For example, exogenous urocortin I could cause undesired effects when treating ischemia–reperfusion injury, since it could increase the plasma ACTH level via CRF type1 receptor activation [28]. Since most reports were performed in experimental animal models, with only a few clinical studies, large prospective clinical studies with long-term follow up are needed to warrant the clinical significance of urocortins.

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Basic nutritional investigation

Effects of garlic on the induction of ventricular fibrillation

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Abstract

Objective: Previous studies have shown that oral and intravenous administrations of garlic provide a significant antiarrhythmic effect and improve defibrillation efficacy. We tested the hypothesis that garlic could decrease the inducibility of ventricular arrhythmia.

Methods: Twenty-one pigs (25–30 kg) were divided into three groups. In each group, the ventricular fibrillation threshold (VFT) and the upper limit of vulnerability (ULV) were determined. After the control VFT and ULV values were obtained, solutions containing 20 mg/kg (group 1, $n = 7$) and 40 mg/kg (group 2, $n = 7$) of garlic (1.3% allicin) were administered intravenously. The VFT and ULV were determined again at the end of garlic infusion. In group 3 ($n = 7$), 100 mL of normal saline was administered instead of garlic.

Results: The VFT values in groups 1 and 2 were not different from the control VFT. The ULV in group 1 was not different from the control ULV. However, the ULV in group 2 (328 ± 58 V, 8 ± 3 J) was significantly lower than the control ULV (415 ± 24 V, 13 ± 2 J), thus accounting for the reduction of ~21% by peak voltage and ~38% by energy. The effective refractory period and diastolic pacing threshold were not altered after garlic infusion. Saline did not alter VFT or ULV.

Conclusion: Garlic cannot alter the VFT, but it significantly decreases the ULV in a dose-dependent pattern, indicating that it can reduce the range of the stimulation strength between the VFT and ULV (vulnerability window) during the vulnerable period of a cardiac cycle.

Keywords: Garlic; Ventricular fibrillation; Arrhythmia; Sudden death; Heart

Introduction

The high mortality rate from sudden cardiac death, mainly caused by ventricular fibrillation (VF), is a major problem in national health worldwide [1–4]. Electrical defibrillation is the only successful clinical therapy available for the resuscitation of patients with VF [1,2]. Understanding the basic mechanisms of defibrillation and the high defibrillation efficacy are greatly needed for successful therapies.

Herbs have been used for medical treatment since the beginning of human civilization. With the high prevalence of herbal use in Eastern traditional medicines and Western medicinal drugs, garlic was found to have many significant cardiovascular effects. In the past few decades, garlic has been found to act as a significant antiarrhythmic agent, partly due to its free radical scavenging activity [5,6]. Garlic powder has been shown to reduce ischemia reperfusion–induced VF in isolated perfused rat heart [5,7]. Furthermore, garlic dialysate has been shown to suppress premature ventricular contraction and ventricular tachycardia in ouabain-intoxicated dogs and ectopic rhythms induced by isoprenaline and aconitine on electrically driven left rat atria, with a significant prolongation of the effective refractory period (ERP) [8]. These findings are possibly caused by garlic's inhibitory effect in relation to calcium availability in cardiac myocytes [8,9]. Recently, garlic powder has been shown to significantly improve the defibrillation efficacy in a dose-dependent pattern [10].

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It is known that VF can be induced with a sufficiently strong electrical stimulus given during the vulnerable period of normal rhythm [11]. The minimum current strength necessary to induce sustained VF is known as the VF threshold (VFT). There is a higher stimulus threshold above which VF is no longer induced and this is known as the upper limit of vulnerability (ULV) [12]. Because the effects of garlic on the inducibility of VF have not been investigated, this study was designed to determine whether garlic could reduce the efficacy of VF induction during a vulnerable period of a cardiac cycle. In this study, we tested the hypothesis that intravenous administration of garlic could increase the VFT and decrease the ULV.

Materials and methods

Animal preparation and electrode placement

This study was approved by the institutional animal care and use committees of the Faculty of Medicine, Chiang Mai University. All animals were cared for according to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996).

Twenty-one healthy juvenile pigs (25–30 kg) of either sex were anesthetized, monitored, and maintained under physiologic conditions. Anesthesia was induced with a combination of atropine (0.04 mg/kg), Zolitil 100 (tiletamine plus zolazepam; VIRBAC Laboratories, Carros, France; 5 mg/kg), and xylazine (2.2 mg/kg) injected intramuscularly and maintained by 0.5–2.0% halothane delivered in 100% oxygen. After cuffed-endotracheal intubation, mechanical ventilation (volume controlled, tidal volume 12 mL/kg, respiratory rate 10–15 cycles/min) was started with the pigs in a restrained dorsally recumbent position. Pavulon (pancuronium bromide; Organon, Inc., West Orange, NJ, USA; 1-mg/kg loading dose, 0.25-mg/kg maintenance dose every 45 min) was administered intravenously to minimize skeletal muscle contraction during shock testing.

The pigs were operated on to expose and isolate the right and left external jugular veins. A catheter with a 34-mm platinum-coated titanium coil electrode (Guidant Corp., St. Paul, MN, USA.) was inserted into the right ventricular apex. A 68-mm electrode catheter was positioned at the junction between the right atrium and the superior vena cava. The position of the catheters was verified by fluoroscopy. The catheters were secured at the venotomy sites to stabilize their positions. Blood pressure, electrocardiogram, heart rate, blood gas, plasma O_2 saturation, respiratory rate, and $EtCO_2$ were monitored continuously throughout the entire study.

Diastolic pacing threshold and ERP protocol

Diastolic pacing threshold (DPT) testing was performed by delivering trains of 10 S_1 stimuli (5-ms monophasic pulse, 500-ms interval) from 0.1 mA incremented in 0.1-mA steps until all drive trains elicited a ventricular response (capture). The DPT was defined as the minimum current strength necessary to capture the ventricle. The ERP was determined by delivering an S_2 stimulus on the last S_1 activation. The basic S_1–S_2 coupling interval was decreased decrementally in 10-ms steps until S_2 failed to elicit a capture. The ERP was defined as the longest S_1–S_2 interval at which an S_2 stimulus failed to elicit a ventricular response.

VFT determination

The VFT testing was performed by using the single-stimulus technique. An S_2 was introduced in the mid electrical diastole (mid T wave) of the last S_1 activation during ventricular pacing at an initial intensity of 100 V. If VF was induced, the S_2 strength was decremented in 20-V steps until VF was not induced. When VF was induced, a rescue shock (20–30 J) was delivered within 10 s to restore sinus rhythm. A minimum of 4 min was allowed to elapse between VF episodes [13]. The minimum S_2 strength necessary to induce sustained VF was defined as the VFT.

ULV determination and the vulnerability window

Ten S_1 stimuli at two-time DPT were delivered at an interval of 500 ms. The S_2 peak voltage was set initially at 400 V and delivered at the peak of the T wave. ULV shock strength was determined by the use of a three-reversal up/down protocol with 80-, 40-, and 20-V steps [14]. In the event of a 400-V shock failing to induce VF, the peak voltage was decreased in 80-V steps per VF induction attempt until the first reversal from VF induction failure to success was achieved. If the initial shock successfully induced VF, the voltage was increased in 80-V steps per VF induction attempt until a reversal from VF induction success to failure was achieved. At each reversal point, the algorithm was iterated in the opposite direction, but after the first reversal, the voltage step size was decreased to 40 V and 20 V after the second and third reversals, respectively. The lowest shock strength, which did not induce VF, was defined as the ULV [14].

After the VFT and ULV were obtained, the vulnerability window in each pig, defined as the difference of stimulation strength between the VFT and ULV, was calculated by subtracting the VFT from the ULV, and its mean value indicated the inducibility window of ventricular arrhythmia in each group.

The pigs were divided into three groups. The control VFT and ULV were determined at the commencement of the study in each group. In group 1 ($n = 7$), a 100-mL stock

Table 1
Mean DPT, ERP, heart rate, and systolic blood pressure before and after garlic or saline administration

	Group 1 (20 mg/kg)		Group 2 (40 mg/kg)		Group 3 (saline)	
	Before	After	Before	After	Before	After
DPT (mA)	0.4 ± 0.2	0.4 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
ERP (ms)	241 ± 28	250 ± 18	244 ± 28	240 ± 12	229 ± 28	247 ± 24
Heart rate (beats/min)	102 ± 15	100 ± 11	98 ± 19	102 ± 18	98 ± 15	97 ± 19
Systolic pressure (mmHg)	114 ± 13	114 ± 10	136 ± 13	135 ± 17	127 ± 11	136 ± 18

DPT, diastolic pacing threshold; ERP, effective refractory period

solution containing garlic powder (Immunytop; 1.3% allicin; Khao-La-Or Laboratories Ltd., Part., Samutprakarn, Thailand) at a concentration of 20 mg/kg was administered intravenously at the rate of 1.5 mL/min after the control VFT and ULV were obtained. This concentration was chosen after showing that it had antiarrhythmic effects [5,8]. Then, the VFT and ULV were determined again after garlic infusion. In group 2 ($n = 7$), garlic at 40 mg/kg (in a 100-mL stock solution) was administered, and the VFT and ULV were determined by a protocol similar to that of group 1. This concentration had been previously demonstrated to improve defibrillation efficacy [10]. In group 3 ($n = 7$), normal saline was administered intravenously at the same amount and rate as in group 1, and the VFT and ULV before and after normal saline infusion were determined.

Statistical analysis

Values are expressed as mean ± SD. Comparisons of variables in each group were performed using the Wilcoxon signed rank test. $P < 0.05$ was considered statistically significant.

Results

In group 1 (20-mg/kg garlic infusion), the DPT and ERP before and after garlic administration were not significantly different (Table 1). The VFT before and after garlic administration was not significantly different (Fig. 1A). The control ULV in this group was 363 ± 22 V for the peak voltage and 10 ± 1 J for total energy. After garlic administration, the ULV was not different from the control (370 ± 21 V, 10 ± 1 J) (Fig. 1B). The vulnerability window after garlic administration was also not different ($P = 0.53$; Table 2) from the control. There was no significant change in heart rate and systolic blood pressure after garlic infusion (Table 1).

In group 2 (40-mg/kg garlic infusion), the DPT and ERP before and after garlic administration were not significantly different (Table 1). The VFT after garlic administration was increased, but not to any statistical significance (Fig. 2A). The control ULV in this group was 415 ± 24 V for the peak voltage and 13 ± 2 J for total energy. After garlic administration, the ULV was 328 ± 58 V ($P = 0.03$ versus control) for the peak voltage and 8 ± 3 J ($P = 0.03$ versus control) for total energy (Fig. 2B), which accounted for a reduction of ~21% by peak voltage and ~38% by total energy. The vulnerability window after garlic administration was significantly lower than in the control ($P = 0.03$; Table 2). There was no significant change in heart rate and systolic blood pressure after garlic infusion in this group (Table 1).

In group 3 (saline infusion), the DPT and ERP before and after saline infusion were not significantly different (Table 1). The VFT and ULV before and after saline infusion were not different (Fig. 3A,B), resulting in no change in the vulnerability window (Table 2). Neither heart rate nor systolic blood pressure was changed after saline infusion (Table 1).

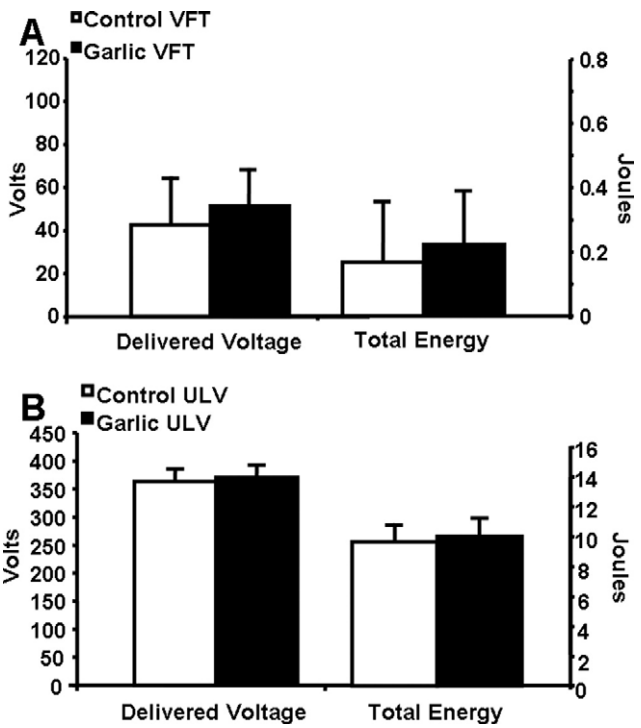

Fig. 1. The VFT and ULV in group 1. The peak voltage and total energy for the VFT (A) and ULV (B) were not significantly different from the control. ULV, upper limit of vulnerability; VFT, ventricular fibrillation threshold.

Table 2
Mean vulnerability window before and after garlic or saline administration

	Group 1 (20 mg/kg)		Group 2 (40 mg/kg)		Group 3 (saline)	
	Before	After	Before	After	Before	After
Vulnerability window (V)	319 ± 30	317 ± 25	362 ± 32	268 ± 63*	359 ± 68	362 ± 35

* $P < 0.05$ versus control.

Discussion

The major findings in this study are as follows: 1) intravenous administration of garlic powder at 20 mg/kg did not alter the VFT or ULV; 2) 40-mg/kg garlic infusion did not alter the VFT, but significantly decreased the ULV; and 3) there were no changes in DPT, ERP, heart rate, or systolic blood pressure after administration of garlic in both concentrations.

Garlic and inducibility of VF

Although the mechanisms by which VF is induced by a strong stimulus delivered during the vulnerable period is unclear, oscillation of intracellular calcium has been proposed as a possible mechanism [15]. Martin et al. [8,16] reported that garlic depressed a Ca^{2+} influx which related to a prevention of delayed afterdepolarizations (DADs). It is known that the mechanism underlying the generation of DADs involves an overload of intracellular Ca^{2+}, with oscillations of the transmembrane potential, giving rise to new activations [17,18]. Thereafter, arrhythmia can be induced from these activations (triggered activity) if they reach a critical threshold. In a recent study, it was suggested that normalization of intracellular calcium contributed to the reduction of VF by suppressing DADs in the isolated rat heart model [15]. Moreover, a defibrillation study in pigs has shown that DADs may be responsible for the earliest postshock activation arising focally after complete repolarization and consequently resulting in failed shocks [19]. The reduced inducibility of ventricular arrhythmia, demonstrated in the present study, could be due to the electrophysiologic effects of garlic on the heart. It is possible that the concentration of 40 mg/kg of garlic powder is sufficiently high to normalize intracellular Ca^{2+} to a higher degree than the 20-mg/kg garlic infusion. This could lead to a decrease in the occurrence of DADs in the ventricles and

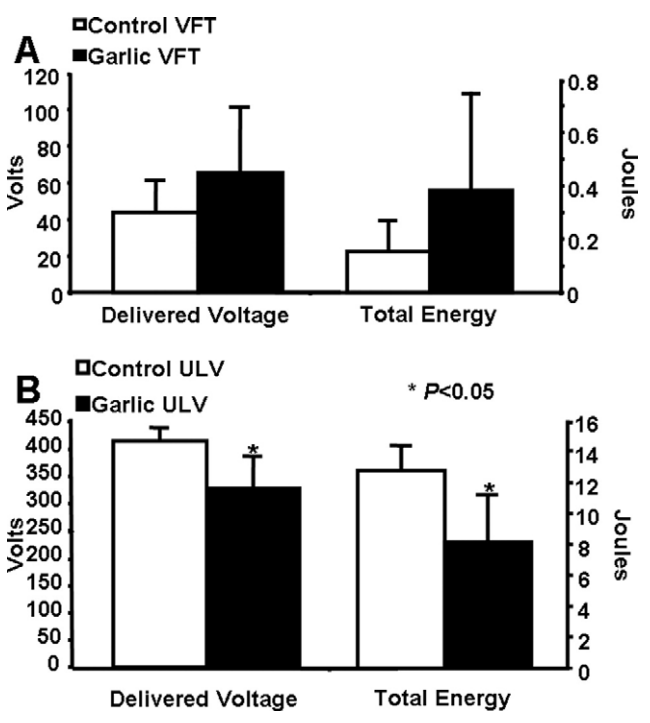

Fig. 2. The VFT and ULV in group 2. (A) The peak voltage and total energy for the VFT were not significantly different from the control, (B) but the peak voltage and total energy for the ULV were significantly lower than the control. *$P < 0.05$ versus control. ULV, upper limit of vulnerability; VFT, ventricular fibrillation threshold.

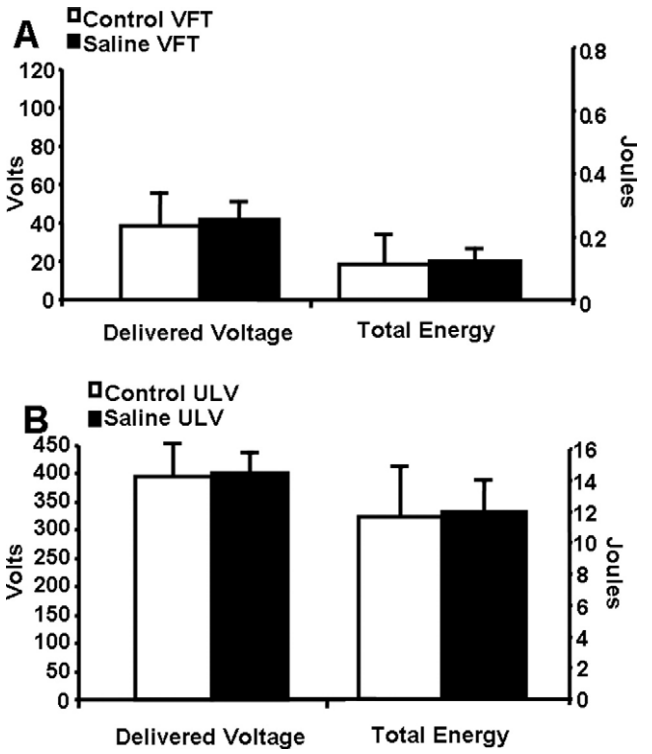

Fig. 3. The VFT and ULV in group 3. The peak voltage and total energy for the VFT (A) and ULV (B) were not significantly different from the control. ULV, upper limit of vulnerability; VFT, ventricular fibrillation threshold.

in the prevention of triggered activity and ventricular arrhythmias including VF. According to this mechanism, a 40-mg/kg garlic infusion should result in an increased VFT and a decreased ULV (consequently, a decrease in the vulnerability window). Nevertheless, from this study, we noticed only the latter, a decrease in the ULV but no change in the VFT, which may be due to the difference in the stimulation strength. The DAD suppression by garlic could obviously occur in relation to the high electrical strength of the stimulus, leading to intracellular Ca^{2+} oscillation [20,21]. Therefore, higher shock strength at the ULV level may augment the action of garlic, so that the ULV was decreased, with a similar effect to the previous demonstration on defibrillation efficacy [10]. In contrast, lower shock strength at the VFT level may not affect, or may attenuate, the action of garlic, thus causing unaltered VFT. Electrophysiologic studies at the cellular level are needed to validate this hypothesis. Nevertheless, our study has demonstrated for the first time that garlic powder significantly decreased the ULV and the vulnerability window during the vulnerable period of a cardiac cycle. Although Martin et al. [8] demonstrated that garlic dialysate prolonged the ERP in isolated rat atria in a dose-dependent manner, the ERP was not altered after garlic infusion in this study when using a large animal model that resembled humans. This may be due to the differences in the experimental model and the concentration of the garlic preparation. However, no alteration of ERP by garlic was found in this study, which suggests that ERP may not be involved in the improvement of VF inducibility by a strong stimulus delivered during the vulnerable period.

Study limitations

This study demonstrated a decrease in the inducibility of ventricular arrhythmia after intravenous administration of a garlic powder solution when using a large animal model that resembled humans. The use of normal pig hearts was the major limitation of this study. Results might have differed if the study were performed in diseased hearts or in humans. Also, the definite mechanistic effect of garlic on the electrophysiology of the heart during VF induction was not elucidated. Future studies at the cellular level are needed to investigate the electrophysiologic effects of garlic during VF induction, in particular the role of garlic on intracellular calcium in this model. The unaffected VFT after garlic infusion could be due to the coarse step-down VFT measurement protocol used in this study. It is possible that a 20-V step could be too large to detect fine VFT alteration. Despite these limitations, we believe that the findings in this study will initiate further investigations regarding the effect of garlic on VF induction. Studies at the cellular level in addition to further animal and human studies are the next step necessary to provide direct evidence to elucidate the mechanism by which garlic decreases the inducibility of ventricular arrhythmia and its clinical significance.

Conclusion

Garlic has been shown to improve defibrillation efficacy. In the present study, garlic significantly decreased the inducibility of ventricular arrhythmia in a dose-dependent manner. These electrophysiologic benefits of garlic on arrhythmia prevention could have a significant clinical impact in addition to its other benefits on the cardiovascular system.

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Letter to the Editor

Effects of garlic on defibrillation efficacy ☆

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Ventricular fibrillation (VF) has been shown to be responsible for most cases of sudden cardiac death in the industrialized world including Thailand [1–4]. Currently, electrical defibrillation is the only successful clinical therapy for patients with this lethal arrhythmia [1,2]. Despite its success, the shock strength required to successfully defibrillate during VF is still high. In the past few decades, the use of drugs as well as food supplement which can prevent arrhythmias has been investigated on their efficacy to decrease the defibrillation threshold (DFT) [5,6].

In traditional medicine, garlic (*Allium sativum*) and its preparations have been widely recognized as agents for the prevention and treatment of cardiovascular and other metabolic diseases, such as atherosclerosis, hyperlipidemia, thrombosis, hypertension and diabetes [7]. Previous studies have demonstrated that garlic has a significant anti-arrhythmic effect in both ventricular and supraventricular arrhythmias [8,9]. These beneficial effects have been proposed to be due to its alteration on cardiac electrophysiology including effective refractory period (ERP) prolongation, Ca^{2+} influx suppression, as well as its free radical scavenging activity [8,10]. Despite these anti-arrhythmic effects of garlic, its effect on defibrillation efficacy has never been verified.

In the present study, we tested the hypothesis that intravenous garlic administration can increase defibrillation efficacy by the reduction of the DFT. The study was approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University. All animals were cared for according to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). Eighteen pigs (20–25 kg) were used in this study. In each pig, shocking electrodes were placed at the right ventricular apex and the junction between superior vena cava and right atrium. VF was induced by 60-Hz alternating current delivered from an electrode at the tip of the RV catheter. After 10 s of VF, defibrillation was attempted with a biphasic truncated exponential shocks. DFT was determined by a three-reversal up/down protocol [5]. A minimum of 4 min was allowed to elapse between VF episodes. Pigs were divided into 3 groups ($n=6$/group). The control DFT was determined at the beginning of the study in each pig. Then, solution containing 20 mg/kg (group 1) and 40 mg/kg (group 2) garlic (1.3% allicin) were administered intravenously at a rate of 1.5 ml/min. The DFT was determined again after garlic infusion. In group 3, 100-ml normal saline was administered instead of garlic. Comparisons of data (mean±SD) were performed using Wilcoxon's test. $P<0.05$ was considered statistically significant.

In group 1, the DFT after 20 mg/kg garlic infusion was not different from the control DFT (see Table 1). In group 2, the DFT after 40 mg/kg garlic infusion was significantly lower than the control DFT, accounted for the reduction of ~13% by peak voltage and ~25% by total energy. In group 3, saline did not alter the DFT. There were no significant changes for mean systolic blood pressure and heart rate after garlic administration compared to those before garlic administration.

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Table 1
The DFT, heart rate, and systolic blood pressure before and after garlic or saline administration

	Group 1		Group 2		Group 3	
	Before	After	Before	After	Before	After
DFT						
Delivered voltage (Volts)	423±77	408±71	397±21	344±19*	404±28	406±21
Total energy (Joules)	13±4	12±4	12±1	9±1*	13±2	13±1
Heart rate (beats/min)	89±19	93±21	114±4	110±5	114±4	117±5
Systolic blood pressure (mmHg)	88±11	98±12	92±7	97±18	81±5	91±8

*$P<0.05$.

Garlic is a perennial plant that is cultivated worldwide. Its bulb has been used as a spice or medicinal herb for over centuries. One of the most biologically active compounds is allicin (diallylthiosulfinate or diallyl disulfide), which does not exist in garlic until it is crushed or cut. This is due to the fact that injury to the garlic bulb activates the enzyme allinase, which metabolizes alliin to allicin [11]. Although there are many preparations of garlic such as garlic oil, garlic powder, and aged garlic, only some of them that have physiologic effects. It has been shown that garlic oil, aged garlic and steam-distilled garlic do not contain significant amounts of alliin or allicin. Instead, they contain various products of allicin transformation in which none appears to have as much physiologic activity as fresh garlic or garlic powder [11,12]. Therefore, garlic powder was chosen in this study to investigate its effect on defibrillation efficacy.

Most anti-arrhythmic agents share the ability to prolong refractoriness relative to their effects on action potential duration (APD). Since reentry is believed to maintain fibrillation, if a drug prolongs refractoriness of fibers in the reentrant pathway, the pathway may not recover excitability in time to be depolarized by the reentering impulse, resulting in the cessation of reentrant propagation [13]. Following this assumption, lengthening of APD and ERP was accepted as important factors that determine anti-arrhythmic defibrillating ability. Previous defibrillation studies have shown that ERP prolongation is one of the underlying mechanisms for successful defibrillation, thus supporting this hypothesis [14,15]. Since garlic is known to prolong the ERP, the improved defibrillation efficacy demonstrated in the present study could be due to this electrophysiological effect of garlic on the heart. It is possible that the concentration of 40 mg/kg garlic powder is sufficiently high to extend the refractory period in a greater degree than 20 mg/kg garlic infusion. This could lead to a decrease in the degree of dispersion of refractoriness in the ventricles as well as the prevention of reentrant propagation, resulting in an improved defibrillation efficacy. However, the major limitation of this study is that the ERP was not measured. Future studies are needed to investigate whether ERP prolongation caused by garlic is dose-dependent.

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Review Article

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Ethnobotany & ethnopharmacology of *Tabernaemontana divaricata*

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Tabernaemontana divaricata **a common garden plant in tropical countries has been used as a traditional medicine. However, no recent review articles of** ***T. divaricata*****, particularly discussing its pharmacological properties, are available. This review presents the ethnobotany and ethnopharmacology of** ***T. divaricata*** **as well as its potential therapeutic benefits especially of the alkaloidal and non-alkaloidal constituents. Included, are the characteristics of 66 alkaloids isolated and identified from** ***T. divaricata*****. Non-alkaloids including the enzymes, pyrolytic oil, hydrocarbons, terpenoid and phenolic acids are also documented. Chemotaxonomic aspects of each alkaloid as well as information regarding the pharmacology of crude extracts and individual alkaloids from** ***T. divaricata*** **have been assembled and appraised. The beneficial properties of** ***T. divaricata*** **are antioxidant, anti-infection, anti-tumour action, analgesia and the enhancement of cholinergic activity in both peripheral and central nervous systems. The augmentation of cholinergic function may be of therapeutic benefit for many neurodegenerative diseases, particularly myasthenia gravis and Alzheimer's disease.**

Key words Alkaloids - non-alkaloids - pharmacological properties - *Tabernaemontana divaricata*

Introduction

Plants are well known as a major source of modern medicines. From ancient times, humans have utilized plants for the treatment or prevention of diseases, leading to the dawn of traditional medicine. *Tabernaemontana* is one of the genera that is used in Chinese, Ayurvedic and Thai traditional medicine for the treatment of fever, pain and dysentery[1,2]. *Tabernaemontana* plants are widely distributed in Thailand. Species found in Thailand are *T. bufalina, T. crispa, T. divaricata, T. pandacaqui, T. pauciflora* and *T. rostrata*[3-5]. One of the most interesting species is *Tabernaemontana divaricata* (L.) R. Br. Ex Roem. & Schult. (synonym: *Ervatamia coronaria, Ervatamia microphylla, Ervatamia divaricata, T. coronaria*). Growing evidence suggests that this plant has medicinal benefits and its extracts could possibly be used as pharmacological interventions in various diseases. In this review, information regarding ethnobotany, ethnopharmacology and therapeutic benefits of *T. divaricata* is discussed.

Description and taxonomy of *T. divaricata*

T. divaricata belongs to the *Apocynaceae* family,

Plumeroidae subfamily, *Tabermontanae* tribe and *Tabernaemontana* genus. The genus was named after the birthplace of its discoverer, J. Th. Mueller, Bergzabern, and Bergzabern was latinized into *Tabernaemontana*. The basionym of *T. divaricata* is *T. siamensis*[6] . Its homotypic synonym is *Ervatamia siamensis*[7]. The holotypes of *T. divaricata* are L, M and W[7]. The generic synonym, *Ervatamia*, is widely distributed in tropical countries as a garden plant, which usually has sweet-scented double flowers[2]. Approximately 100 species of this genus are widely distributed in tropical parts of the world, including Brazil, Egypt, India, Sri Lanka, Vietnam, Malaysia and Thailand. *T. divaricata* was first described by Linnaeus in 1753[2]. The complete taxonomy of *T. divaricata* is shown in Fig.1 according to Leeuwenberg[3]. *T. divaricata* has four typical characteristics including: *(i)* evergreen shrub forms shaped like symmetrical mounds 6-feet high, *(ii)* horizontal branches having the appearance of an attractive, almost horizontal shrub (the species name, *divaricata*, means an obtuse angle), *(iii)* large, shiny, deep green leaves, 6 or more inches in length and 2 inches wide, and *(iv)* waxy blossoms with white, five-petal pinwheels, gathered in small clusters on the stem tips.

Phytochemistry of *T. divaricata*

T. divaricata has been used in traditional medicine and for other purposes. The phytochemistry and a number of chemical constituents from the leaves, stems, and roots have been reported previously. Constituents studied include alkaloids[8-36], and non-alkaloid constituents such as terpenoids[29,37-40], steroids[37,41], flavonoids[42], phenyl propanoids[41,42], phenolic acids[16] and enzymes[43,44]. Since 1974, 66 different alkaloids of *T. divaricata* have been identified. The phytochemical data for each alkaloid provide information about its biosynthesis. Such information can assist in the search for new, medically interesting compounds that may be useful against diseases.

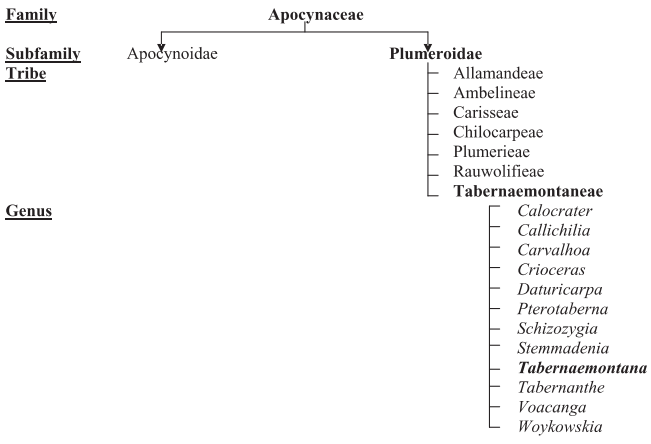

Fig. 1. The botanical classification of *Tabernaemontana* plants.

Alkaloids of T. divaricata: According to van Beek *et al*[2] alkaloids of *T. divaricata* are arranged in 11 main classes: Vincosan, Corynanthean, Vallesiachotaman, Strychnan, Aspidospermatan, Plumeran, Eburan, Ibogan, Tacaman, Bis-indole and Miscellaneous. The details of structure in each class and subdivision are shown in Tables I and II.

At least 66 alkaloids were extracted from *T. divaricata* by several methods such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography-mass spectrophotometry (GC-MS). Table III summarizes the currently known alkaloids isolated from *T. divaricata* in alphabetical order, together with their alkaloid subdivision, molecular weight, formula, plant part in which they occur and country of origin. Fig. 2 illustrates all chemical structures of alkaloids from *T. divaricata*.

Non-alkaloids of T. divaricata: Although most of the phytochemical work on *T. divaricata* has been concerned with the alkaloidal constituents, and most of the ethnomedical uses are probably related to the pharmacological activity of these substances, some non-alkaloidal constituents such as terpenoids, steroids, enzymes, and hydrocarbons have also been isolated from *T. divaricata*. Terpenoid-indole alkaloids are formally derived from a unit of tryptamine, obtained by decarboxylation of tryptophan catalyzed by the enzyme tryptophan decarboxylase (TDC), and a C_{10} unit of terpenoid origin (secologanin). The stereospecific condensation of these two units is catalyzed by the enzyme strictosidine synthase (SSS) as shown in Fig. 3[2] . The biosynthesis and metabolism of terpenoid is involved with many enzymes. Several studies demonstrated about the role of those enzymes that regulate biosynthesis and metabolism of terpenoids in *T. divaricata*[2]. For example, Pennings & Verpoorte[50] detected the enzyme anthranilate synthase from *T. divaricata* cell cultures by HPLC assay. Fulton and colleagues[51] also demonstrated five known enzymes that were detected for the first time in *T. divaricata* cell-suspension culture: isopentenyl diphosphate isomerase, prenyl transferase, squalene synthetase, qualene 2,3-oxide cycloartenol cyclase and squalene 2,3-oxide cyclase. These enzymes act as key regulatory agents in

Table I. Classification of the indole alkaloids occurring in *T.divaricata* (modified from van Beek *et al*)[2]*

Class	Abbreviation	Structure characteristics
Vincosan	(D)	C(2)-C(3)-C(14) unit, no N(4)-C(17) or N(4)-C(21) bond
Corynanthean	(C)	C(2)-C(3)-C(14) unit, N(4)-C(21) bond
Vallesiachotaman	(V)	C(2)-C(3)-C(14) unit, N(4)-C(17) bond
Strychnan	(S)	C(2)-C(16)-C(15) unit, C(3)-C(7) bond
Aspidospermatan	(A)	C(2)-C(16)-C(15) unit, no C(3)-C(7) bond
Plumeran	(P)	C(2)-C(16)-C(17)-C(20) unit
Eburnan	(E)	N(1)-C(16)-C(17)-C(20) unit
Ibogan	(I)	C(2)-C(16)-C(17)-C(14) unit
Tacaman	(T)	N(1)-C(16)-C(17)-C(14) unit
Miscellaneous	(M)	ND
Bis-indole	(B)	Two indole alkaloids attached to each other

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ND, not determined; C, carbon; N, nitrogen

Table II. Subdivision of the main class of indole alkaloids occurring in *T.divaricata* (modified from van Beek *et al)*[2]

Subdivision	References
Vincosan (D)	
C5 : Vobasine group	Cordell, 1974[45]
Aspidospermatan (A)	
A2 : Apparicine group	Kutney *et al* 1976[46]
A3 : Condylocarpine group	Cordell, 1974[45]
Plumeran (P)	
P1 : Voaphylline group	Cordell, 1974[45]
P2 : Tabersonine group	Cordell, 1974[45]; Kutney *et al* 1976[46]
Ibogan (I)	
I1 : Coronaridine group	Cordell, 1974[45]; Kutney, *et al* 1976[46]
Bis-indole (B)	
B2 : C-I group	van Beek, 1984[2]

controlling the flux of carbon into the cytosolic-microsomal pathway of terpenoid synthesis. Dagnino and colleagues[52] found five enzymes from *T. divaricata* cell lines including tryptophan decarboxylase, strictosidine synthase, strictosidine glucosidase, isopentenyl pyrophosphate isomerase and geraniol 10-hydroxylase. It has also been suggested that these five enzymes relate to the biosynthesis of terpenoid indole alkaloids of *T. divaricata*. In addition, the enzyme strictosidine α-D-glucosidase was partially purified from cell suspension cultures of *T. divaricata*[53]. Another non-alkaloidal enzyme, squalene synthase, was also partially purified from a membrane-rich fraction obtained from cell suspension cultures of *T. divaricata*[54]. Ramas-Valdivia and colleagues[55] discovered the enzyme farnesyl diphosphate synthase from *T. divaricata* cultured cells by chromatography and Western blotting assay.

Many plant species produce a wide range of chemical products that are not involved in primary metabolism and called secondary metabolites[56]. Secondary metabolites are metabolic intermediates or products found as differential products in restricted taxonomic groups and are not essential to the growth and life of the producing organism. They are biosynthesized from one or more primary metabolites by a wider variety of pathways than those available in primary metabolism[57]. Alkaloid and terpenoids are main secondary metabolites that have many physiological and pharmacological properties to living cells[56]. However, their biosynthesis are normally restricted to certain developmental stages of the organism[58]. Some of those biosynthesis are the phase-dependent formation for some enzymes[58]. Therefore, the expression of secondary metabolites is based on the process of plants' differentiation. Thus, it is not surprising that the synthesis of secondary metabolites does not occur in the meristematic cells of intact plants[59]. Moreover, some studies suggested that cell cultures of plants could produce secondary metabolites when they stopped being meristematic and rather acquired a certain degree of biochemical modification and maturation[56]. Therefore, several studies used cell cultures techniques to investigate the biosynthesis and metabolism of these secondary metabolites[13,14,60,61].

Other non alkaloidal constituents, investigated by Mandal and Mukherji[62], saw discovery of free radical-scavenging enzymes such as superoxide dismutase,

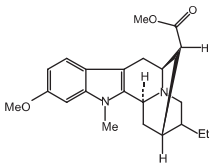
Table III. Summary of the alkaloids found in *T.divaricata*

Alkaloids	Class	Molecular weight	Formula	Plant part	Country of origin	References
11-Methoxy-N-methyldihydropericyclivine (1)	C	368.47	$C_{22}H_{28}N_2O_3$	leaves, flowers, roots	Sri Lanka	Arambewela & Ranatunge, 1991[8]
12-Hydroxyakuammicine (2)	S	338.40	$C_{20}H_{22}N_2O_3$	cell suspension culture	ND	Pawelka & Stoeckigt,1983[27]
19,20 Dihydrotabernamine (3)	B	618.86	$C_{40}H_{50}N_4O_4$	roots	Thailand	Ingkaninan *et al*, 200647
19,20-Dihydroervahanine A (4)	B	676.89	$C_{42}H_{52}N_4O_3$	stems	Brazil	Henriques *et al*, 1996[16]
19-Epivoacangine (5)	I	368.47	$C_{22}H_{28}N_2O3$	leaves, flowers, roots	Sri Lanka	Arambewela & Ranatunge, 1991[8]
19-Epivoacristine (6)	I	384.47	$C_{22}H_{28}N_2O_4$	leaves	Malaysia	Kam *et al*, 1992[18]
19-Heyneanine hydroxyindolenine (7)	I	370.44	$C_{21}H_{26}N_2O_4$	whole plant	Thailand	Sharma & Cordell, 1988[37]
19-Hydroxycoronaridine (8)	I	354.44	$C_{21}H_{26}N_2O_3$	root bark	India	Rastogi *et al*, 1980[29]
3-Oxocoronaridine (9)	I	352.43	$C_{21}H_{24}N_2O_3$	root bark	India	Rastogi *et al*, 1980[29]
3-Oxovoacangine(10)	I	382.45	$C_{22}H_{26}N_2O_4$	whole plant	Thailand	Sharma & Cordell, 1988[37]
3S-Cyanocoronaridine(11)	I	363.45	$C_{22}H_{25}N_3O_2$	stems, barks	Malaysia	Kam *et al*, 2004[24]
3S-Cyanoisovoacangine(12)	I	393.48	$C_{23}H_{27}N_3O_3$	stems, barks	Malaysia	Kam *et al*, 2004[24]
5-Hydroxy-6-oxocoronaridine (13)	I	368.43	$C_{21}H_{24}N_2O_4$	root bark	India	Rastogi *et al*, 1980[29]
5-Hydroxyvoaphylline (14)	P	312.18	$C_{19}H_{24}N_2O_2$	leaves	Pakistan	Atta-ur Rahman *et al*, 1986[11]
5-oxo-11-hydroxy voaphylline (15)	P	326.16	$C_{19}H_{22}N_2O_3$	leaves	Pakistan	Atta-ur Rahman & Muzaffar, 1985[10]
5-Oxocoronaridine (16)	I	352.43	$C_{21}H_{24}N_2O_3$	root bark	India	Rastogi *et al*, 1980[29]
6-Oxocoronaridine (17)	I	352.43	$C_{21}H_{24}N_2O_3$	root bark	India	Rastogi *et al*, 1980[29]
Appericine (18)	A	264.36	$C_{18}H_{20}N$	cell suspension culture	ND	Pawelka & Stoeckigt,1983[27]
Catharanthine (19)	I	336.43	$C_{21}H_{24}N_2O_2$	cell suspension culture	ND	Pawelka & Stoeckigt,1983[27]
Conodurine (20)	I	704.90	$C_{43}H_{52}N_4O_5$	roots	Thailand	Ingkaninan *et al*, 2006[47]
Conodusarine (21)	B	718.88	$C_{43}H_{50}N_4O_6$	stems, barks	Malaysia	Kam & Anuradha, 2004[48]
Conofoline (22)	B	736.90	$C_{43}H_{52}N_4O_7$	leaves	Malaysia	Kam & Anuradha, 1995[20]
Conolidine (23)	A	266.34	$C_{17}H_{18}N_2O$	stems, barks	Malaysia	Kam *et al*, 2004[24]
Conolobine A (24)	A	282.34	$C_{17}H_{18}N_2O_2$	stems, barks	Malaysia	Kam *et al*, 2004[24]
Conolobine B (25)	A	282.34	$C_{17}H_{18}N_2O_2$	stems, barks	Malaysia	Kam *et al*, 2004[24]
Conophyllidine (26)	B	778.89	$C_{44}H_{50}N_4O_9$	leaves	Malaysia	Kam *et al*, 1993[19]
Conophylline (27)	B	794.89	$C_{44}H_{50}N_4O_{10}$	leaves	Malaysia	Kam *et al*, 1992[18]
Conophyllinine (28)	B	812.90	$C_{44}H_{52}N_4O_{11}$	leaves	Malaysia	Kam *et al*, 2003[23]
Coronaridine (29)	I	338.44	$C_{21}H_{26}N_2O_2$	leaves	India	Raj *et al*, 1974[28]
Coronaridine hydroxyindolenine (30)	I	354.44	$C_{21}H_{26}N_2O_3$	root bark	India	Rastogi *et al*, 1980[29]
Dregamine (31)	C	354.44	$C_{21}H_{26}N_2O_3$	leaves, stems, barks, roots		EgyptKarawya & Aboutabl, 1979[26]
Ervaticine (32)	A	266.34	$C_{21}H_{26}N_2O_3$	leaves	Pakistan	Atta-ur Rahman *et al*, 1986[11]
Ervatinine (33)	P	326.39	$C_{19}H_{22}N_2O_3$	leaves	Pakistan	Atta-ur Rahman *et al*, 1985[12]
Heyneanine (34)	I	354.44	$C_{22}H_{28}N_2O_3$	whole plant	Thailand	Sharma & Cordell, 1988[37]

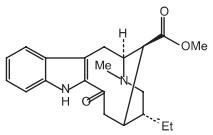
Hyderabadine (35)	P	340.46	$C_{21}H_{28}N_2O_2$	leaves	Pakistan	Atta-ur Rahman *et al*, 1983[9]
Ibogamine (36)	I	280.41	$C_{19}H_{24}N_2$	root bark	India	Rastogi *et al*, 1980[29]
Isovoacangine (37)	I	368.47	$C_{22}H_{28}N_2O_3$	leaves, flowers, roots	Sri Lanka	Arambewela & Ranatunge, 1991[8]
Isovoacristine (38)	I	384.47	$C_{22}H_{28}N_2O_4$	leaves, flowers, roots	Sri Lanka	Arambewela & Ranatunge, 1991[8]
Lahoricine (39)	C	194.39	$C_{19}H_{22}N_2O$	leaves	Pakistan	Atta-ur Rahman *et al*, 1985[49]
Lochnericine (40)	P	352.43	$C_{21}H_{24}N_2O_3$	leaves	India	Raj *et al*, 1974[28]
Mehranine (41)	P	310.43	$C_{20}H_{26}N_2O$	leaves	Pakistan	Atta-ur Rahman *et al*, 1983[9]
N1-Methylvoaphylline(42)	P	310.43	$C_{20}H_{26}N_2O$	leaves	Malaysia	Kam & Anuradha, 1995[20]
N-methylvoafinine (43)	A	326.43	$C_{20}H_{26}N_2O_2$	leaves	Malaysia	Kam & Anuradha, 1995[21]
O-Acetylvallesamine (44)	A	382.45	$C_{22}H_{26}N_2O_4$	cell suspension culture	ND	Van der Heijden *et al*, 1990[36]
Pachysiphine (45)	P	352.43	$C_{21}H_{24}N_2O_3$	leaves	Malaysia	Kam & Anuradha, 1995[20]
Pericyclivine (46)	C	322.40	$C_{20}H_{22}N_2O_3$	cell suspension culture	ND	Van der Heijden *et al*, 1988[35]
Perivine (47)	C	338.40	$C_{20}H_{22}N_2O_3$	cell suspension culture	ND	Van der Heijden *et al*, 1988[35]
Pseudovobparicine (48)	B	600.79	$C_{36}H_{44}N_4O_2$	root, bark	Vietnam	Van Beek *et al*, 1985[34]
Stemmadenine (49)	A	354.44	$C_{21}H_{26}N_2O_3$	cell suspension culture	ND	Van der Heijden et al, 1990[36]
Taberhanine (50)	A	428.48	$C_{23}H_{28}N_2O_6$	leaves	Malaysia	Kam *et al*, 2003[23]
Tabernaelegantine A (51)	I	706.91	$C_{43}H_{54}N_4O_5$	roots	Thailand	Ingkaninan *et al*, 2006[47]
Tabernaemontanine (52)	C	354.44	$C_{12}H_{26}N_2O_3$	leaves	India	Raj *et al*, 1974[28]
Tubotaiwine (53)	A	324.42	$C_{20}H_{24}N_2O_2$	cell suspension culture	ND	Pawelka & Stoeckigt,1983[27]
Vallesamine (54)	A	340.42	$C_{20}H_{24}N_2O_3$	cell suspension culture	ND	Van der Heijden *et al*, 1988[35]
Voacamine (55)	B	704.90	$C_{43}H_{52}N_4O_5$	leaves, stems, barks, roots		EgyptKarawya & Aboutabl, 1979[26]
Voacangine (56)	I	368.47	$C_{22}H_{28}N_2O_3$	leaves	India	Raj *et al*, 1974[28]
Voacangine hydroxyindolenine (57)	I	384.47	$C_{22}H_{28}N_2O_4$	whole plant	Thailand	Sharma & Cordell, 1988[37]
Voacristine (58)	I	384.47	$C_{22}H_{28}N_2O_4$	whole plant	Thailand	Sharma & Cordell, 1988[37]
Voacristine hydroxyindolenine (59)	I	400.47	$C_{22}H_{28}N_2O_5$	whole plant	Thailand	Sharma & Cordell, 1988[37]
Voafinidine (60)	A	328.45	$C_{20}H_{28}N_2O_2$	leaves	Malaysia	Kam *et al*, 1996[22]
Voafinine (61)	A	312.41	$C_{19}H_{24}N_2O_2$	leaves	Malaysia	Kam & Anuradha, 1995[21]
Voaharine (62)	P	326.39	$C_{19}H_{22}N_2O_3$	leaves	Malaysia	Kam *et al*, 1992[18]
Voalenine (63)	A	326.39	$C_{19}H_{22}N_2O_3$	leaves	Malaysia	Kam *et al*, 1996[22]
Voaphylline (64)	P	296.41	$C_{19}H_{24}N_2O$	leaves	India	Raj *et al*, 1974[28]
Voaphylline hydroxyindolenine (65)	P	312.41	$C_{19}H_{24}N_2O_2$	cell suspension culture	ND	Van der Heijden *et al*, 1988[36]
Vobasine (66)	C	352.43	$C_{21}H_{24}N_2O_3$	leaves, stems, barks, roots		EgyptKarawya & Aboutabl, 1979[26]

ND, Not determined; C, corynanthean; S, strychnan; I, ibogan; B, bis-indole; P, plumeran; A, aspidospermatan. Figures in the parentheses represent alkaloids from *T.divaricata* in alphabetic order. The chemical structures shown in Fig. 3.

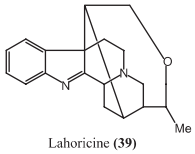
Corynanthean class



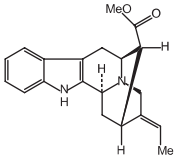
11-Methoxy-N-methyldihydropericyclivine **(1)**



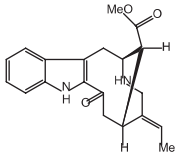
Dregamine **(31)**



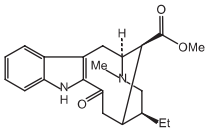
Lahoricine **(39)**



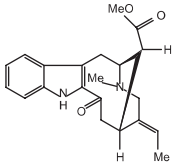
Pericyclivine **(46)**



Perivine **(47)**

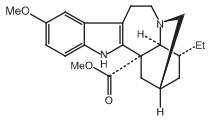


Tabernaemontanine **(52)**

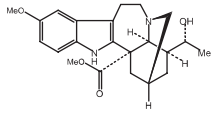


Vobasine **(66)**

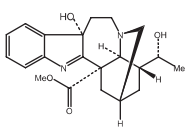
Ibogan class



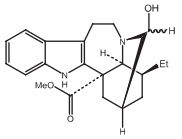
19-Epivoacangine**(5)**



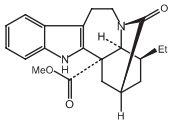
19-Epivoacristine**(6)**



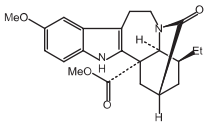
19-Heyneanine hydroxyindolenine **(7)**



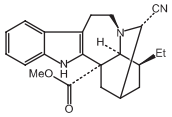
19-Hydroxycoronaridine **(8)**



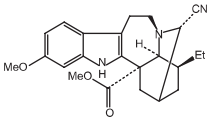
3-Oxocoronaridine **(9)**



3-Oxovoacangine**(10)**

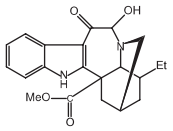


3S-Cyanocoronaridine**(11)**

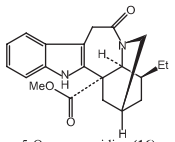


3S-Cyanoisovoacangine**(12)**

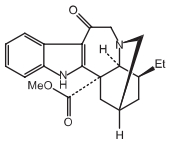
Ibogan class



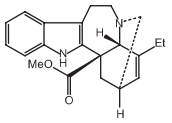
5-Hydroxy-6-oxocoronaridine **(13)**



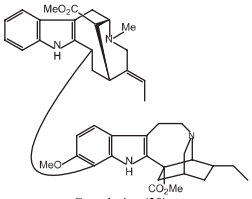
5-Oxocoronaridine **(16)**



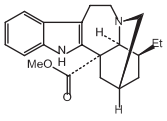
6-Oxocoronaridine **(17)**



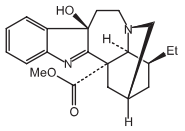
Catharanthine **(19)**



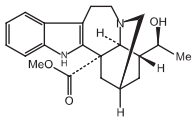
Conodurine **(20)**



Coronaridine **(29)**

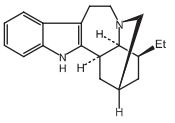


Coronaridine hydroxyindolenine **(30)**

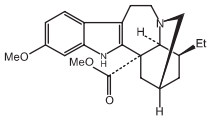


Heyneanine **(34)**

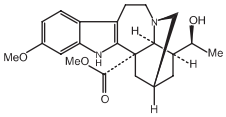
Ibogan class



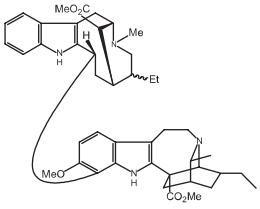
Ibogamine **(36)**



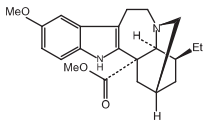
Isovoacangine **(37)**



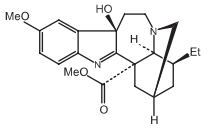
Isovoacristine **(38)**



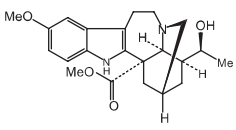
Tabernaelegantine A **(51)**



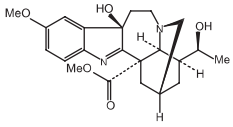
Voacangine **(56)**



Voacangine hydroxyindolenine **(57)**

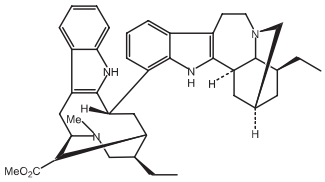


Voacristine **(58)**

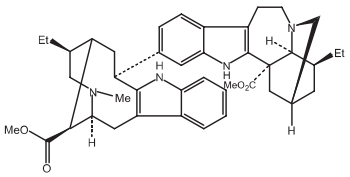


Voacristine hydroxyindolenine **(59)**

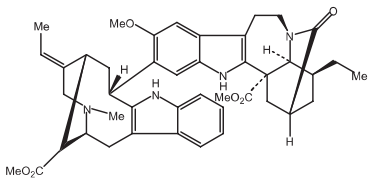
Bis-indole class



19,20 Dihydrotabernamine**(3)**

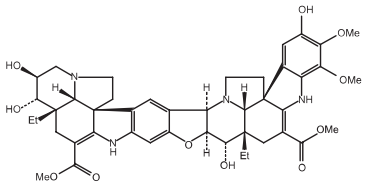


19,20-Dihydroervahanine A **(4)**

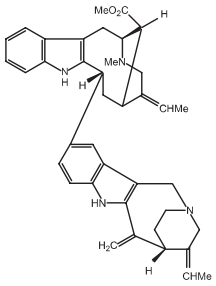


Conodusarine **(21)**

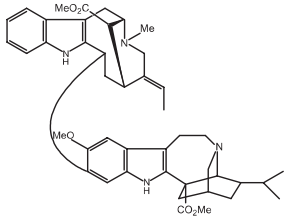
Bis-indole class



Conophyllinine **(28)**

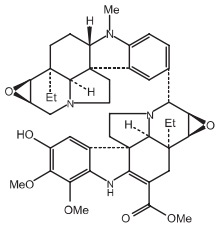


Pseudovobparicine **(48)**

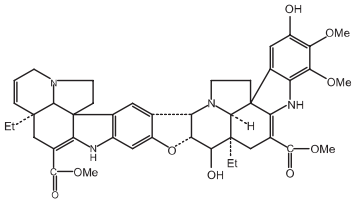


Voacamine **(55)**

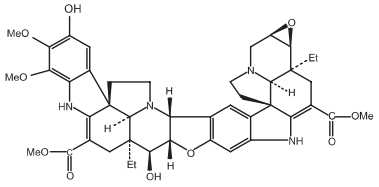
Bis-indole class



Conofoline **(22)**

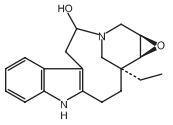


Conophyllidine **(26)**

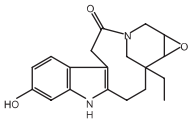


Conophylline **(27)**

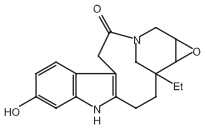
Plumeran class



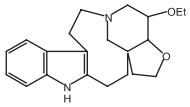
5-Hydroxyvoaphylline **(14)**



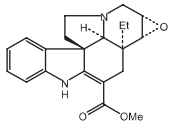
5-oxo-11-hydroxy voaphylline **(15)**



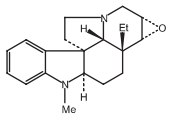
Ervatinine **(33)**



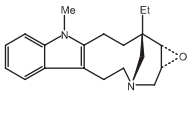
Hyderabadine **(35)**



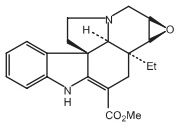
Lochnericine **(40)**



Mehranine **(41)**

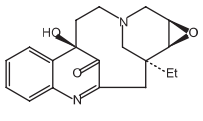


N1-Methylvoaphylline**(42)**

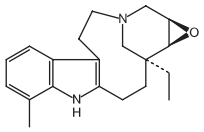


Pachysiphine **(45)**

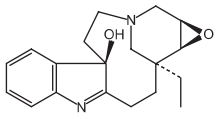
Plumeran class



Voaharine **(62)**

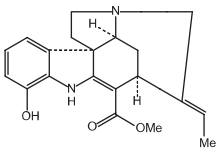


Vophylline **(64)**



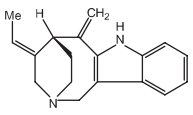
Voaphylline hydroxyindolenine**(65)**

Strychnan class

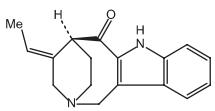


12**-**Hydroxyakuammicine **(2)**

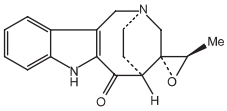
Aspidospermatan class



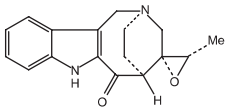
Apparicine **(18)**



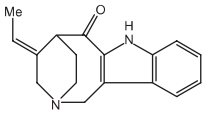
Conolidine **(23)**



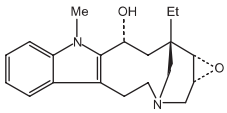
Conolobine A **(24)**



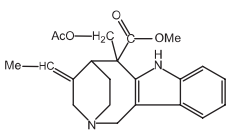
Conolobine B **(25)**



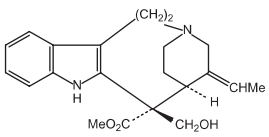
Ervaticine **(32)**



N**-**methylvoafinine **(43)**

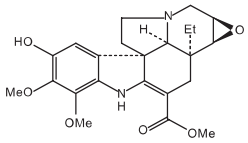


O**-**Acetylvallesamine **(44)**

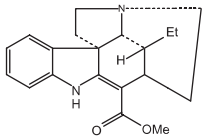


Stemmadenine **(49)**

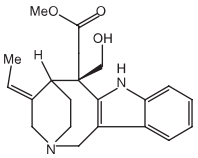
Aspidospermatan class



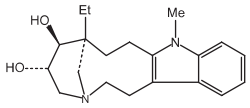
Taberhanine **(50)**



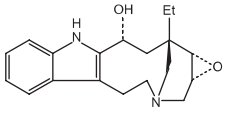
Tubotaiwine **(53)**



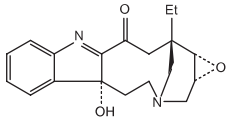
Vallesamine **(54)**



Voafinidine **(60)**



Voafinine **(61)**



Voalenine **(63)**

Fig. 2. All chemical structures of alkaloids from *T. divaricata*. Numbers in the parentheses represent alkaloids from *T. divaricata* in alphabetic order.

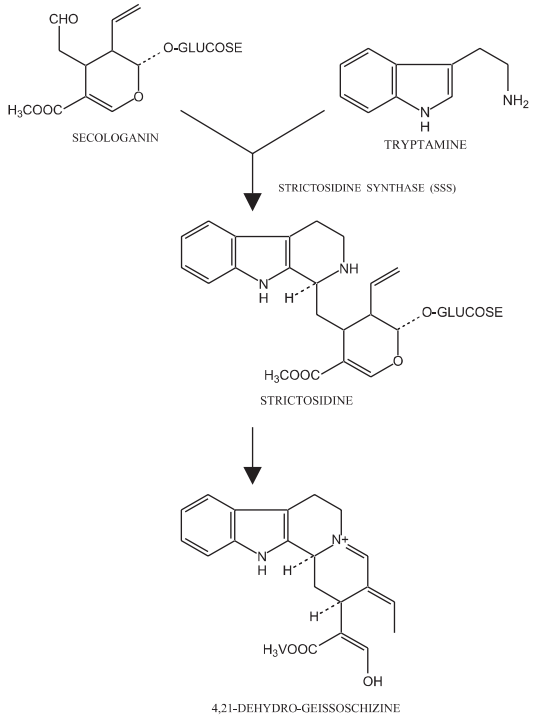


Fig. 3. The first step in the biosynthesis pathway of terpenoid-indole alkaloids.Terpenoid-iodole alkaloids are formally derived from secologanin and tryptamine. These two units are catalyzed by the enzyme strictosidine synthase (SSS).

Table IV. Summary of the non-alkaloids found in *T. divaricata*

Non-alkaloids	Plant part	Specimen from	References
Enzyme:			
Anthranilate synthase	cell culture	Netherlands	Poulsen *et al*, 1991[50]
Isopentenyl diphosphate isomerase Prenyl transferase Squalene synthetase Qualene 2,3–oxide: cycloartenol cyclase, Squalene 2,3-oxide: cyclase	cell culture	United Kingdom	Fulton *et al*,1994[51]
Tryptophan decarboxylase Strictosidine synthase Strictosidine glucosidase Isopentenyl pyrophosphate isomerase Geratinol 10-hydroxylase	cell culture	Netherlands	Dagnino *et al*, 1995[52]
Strictosidine β–D-glucosidase	cell culture	Netherlands	Luijendijk *et al*, 1996[53]
Squalene synthase	cell culture	United Kingdom	Kroon and Threlfall, 1997[54]
Isopentenyl diphosphate isomerase Farnesyl diphosphate synthase	cell culture	Netherlands	Ramas-Valdivia *et al*, 1998[55]
Superoxide dismutase Catalase, Ascorbate peroxidase Glutathione reductase Phenolic peroxidase		India	Mandal and Mukherji, 2001[62]
Pyrolytic oil and solid char	stems, leaves	India	Sharma and Prasad, 1986[63]
Hydrocarbon	leaves, roots, flowers, stems	India	Behera *et al*, 1995[64]
Other (terpenoid & phenolic acid):			
α–amyrin acetate Lupeol acetate α-amyrin lupeol Cycloartenol β-sitosterol Campesterol Benzoic acid Aurantiamide acetate	root bark	India	Rastogi *et al*, 1980[29] Dagnino *et al*, 1994[15] Van der Heijden *et al*, 1989[40]

catalase, ascorbate peroxidase, glutathione reductase and phenolic peroxidase in *T. divaricata* from roadside plants in India. Their discoveries indicated that *T. divaricata* was a very good scavenging system to combat the effects of air pollution. Other non alkaloidal compounds in *T. divaricata* such as pyrolytic oil, solid char, amino acid and hydrocarbon were also found to have some beneficial effects. Sharma and Prasad[63] showed that the stems and leaves of Indian *T. divaricata* have pyrolytic oil and solid char that can be converted into petroleum and ethanol, which can be exploited to produce gasohol fuel. Behera and colleagues[64] also demonstrated that the hexane extract from old leaves, roots, flowers and stems of *T. divaricata* was rich in hydrocarbons. Rastogi and colleagues[29] isolated eight non alkaloid compounds from the root bark of *T. divaricata* such as α -amyrin acetate, lupeol acetate, α-amyrin lupeol, cycloartenol, β-sitosterol,

campesterol, benzoic acid and aurantiamide acetate. Their compounds are terpenoids and phenolic acid, and plant metabolites, which exhibit pharmacological properties such as anti-inflammatory and anti-oxidant activity *in vitro*[65]. A summary of the non alkaloids extracted from *T. divaricata* is shown in Table IV.

Pharmacological properties of *T. divaricata*

Both *in vivo* and *in vitro* pharmacological properties of *T. divaricata* have previously been investigated. The first direction was to investigate the pharmacological properties of *T. divaricata* by using crude ethanol extracts or crude alkaloid fractions. The second direction was to identify pharmacological effects of pure alkaloid compounds isolated from *T. divaricata* or biologically active alkaloids of *T. divaricata*. The details of pharmacological properties of *T. divaricata* are summarized in the following paragraphs.

Pharmacological properties of T. divaricata crude extracts or crude alkaloid fractions

Role of *T. divaricata* in anti-infection and anti-inflammation - The most common medicinal use of crude *T. divaricata* extract involves its antimicrobial action against infectious diseases such as syphilis, leprosy, and gonorrhoea, as well as its antiparasitic action against worms, dysentery, diarrhoea, and malaria[2].

The anti-inflammatory effect of *T. divaricata* was studied in carageenin-induced paw oedema in rats[66-69]. In this model, male rats were injected with 0.1 ml of carageenin into one of the hind paws. The *T. divaricata* extracts (150-200 mg/kg) were administrated either orally or intra-peritoneally 1 h prior to the sub-plantar injection of carageenin. Oedema measurements were made using a modified plethymograph 1, 2 and 4 h after carageenin injection. This study demonstrated that *T. divaricata* extracts had significant anti-inflammatory effect on carageenin-induced paw oedema compared to animals without *T. divaricata* administration and that this anti-inflammatory effect of *T. divaricata* was dose dependent[16]. The anti-inflammatory mechanism of *T. divaricata* is thought to be due to the presence of phenolic acid, a chemical agent that has a potential anti-inflammatory benefit[42].

Role of T. divaricata in anti-tumour effects: Immunoglobulin A nephropathy (IgA-N) is the most common pattern of glomerulonephritis (GN). Fifteen to twenty five per cent of GN patients develop end-stage renal disease, typically through a slow progression of renal insufficiency over 10 yr or more[70]. The characteristic of histological renal lesion of IgA-N is a tumour of mesangial cell proliferative GN. Causes of IgA-N may involve the activation of mesangial cells in the kidney by the deposition of IgA immune complexes. The activation of those cells stimulates the secretion of immunomodulatory factors from inflammatory cells in the circulation, such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α)[71,72]. These factors have been shown to increase the volume of extracellular matrix and activate mesangial cell proliferation in the kidney. Both changes are characteristics of nephritis. Therefore, the suppression of mesangial cell proliferation may have a therapeutic benefit in the treatment of nephropathy. Kuo and colleagues[73] investigated the effect of crude methanol extract from 15 Chinese herbs on human mesangial cell proliferation in an *in vitro* study. Their results indicated that *T. divaricata*, one of the 15 herbs, could suppress mesangial cell proliferation via the reduction of IL-1, IL-6 and TNF-α expression. These findings suggest that *T. divaricata* could have benefits as an anti-tumour drug in IgA-N.

Anti-oxidative effects of T. divaricata: The anti-oxidative effects of *T. divaricata* have been studied by various investigators using the carbon tetrachloride (CCl_4)-induced hepatotoxicity model[62,74]. The hepatotoxicity is due to the metabolite of CCl_4, a free radical that causes the peroxidation of lipids in the endoplasmic reticulum that leads to cell death[75]. Gupta *et al*[74] found, in an *in vivo* study, that methanol extract from leaves of *T. divaricata* produced a significant hepatoprotective effect by decreasing lipid peroxidation and significantly increasing the level of anti-oxidant agents such as glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) in a dose dependent manner.

Furthermore, Mandal and Mukherji[62] demonstrated that *T. divaricata* is a very good scavenging system to combat the effects of air pollution. Their results demonstrated that *T. divaricata* plants in nature have high levels of activity of anti-oxidant agents such as SOD, CAT, GSH, ascorbate peroxidase and phenolic peroxidase.

Analgesic effects of T. divaricata: Roots of *T. divaricata* have reportedly been used in folk medicine for their analgesic properties. The study of Henriques and colleagues[16] confirmed the anti-nociceptive effect of *T. divaricata*. They demonstrated that mice treated with

150 mg/kg of *T. divaricata* extract either orally or intraperitoneally 30 min before being placed on a heated plate (50-55°C), had significantly greater response times to the heat stimulus than the control group without *T. divaricata* treatment. However, the mechanisms underlying this analgesic effect have not been verified.

Neuropharmacological activities of T. divaricata: Hippocratic or behaviour screening in an *in vivo* study of ethanol extract from *T. divaricata* was studied by Taesotikul and colleague[76]. The hippocratic screening test is commonly used in the preliminary screening test of medicinal plants to detect interesting pharmacological activities. According to their screening studies, the ethanol extract of *T. divaricata* was found to cause dose-related decreased motor activity, ataxia, loss of righting reflex, decreased respiratory rate and loss of screen grip. These effects indicated that *T. divaricata* has depressive effects on the central nervous system (CNS). In addition, the loss of screen grip and decreased muscle tone in the rat model following *T. divaricata* administration suggest that *T. divaricata* may act as a skeletal muscle relaxant. Their findings in the animals suggest that *T. divaricata* has depressive effects on both peripheral and central nervous systems.

In contrast, Ingkaninan *et al*[77] reported that the roots and stems of *T. divaricata* have been used in Thai traditional medicine as components of rejuvenating and neurotonic remedies. It is believed that these remedies can prevent forgetfulness and improve memory as well as being a CNS stimulant. However, there was no scientific evidence to support this belief, until a *in vitro* study demonstrated that ethanol extracts from *T. divaricata* root at a concentration of 0.1 mg/ml inhibit more than 90 per cent of acetylcholinesterase (AChE) activity[72]. Our recent study also demonstrated that *T. divaricata* administration in various doses can significantly decrease neuronal AChE activity in the cerebral cortex[78]. Our data showed that the percentages of AChE inhibition in the cortex at 2 h after *T. divaricata* administration at 250, 500 and 1,000 mg/kg was 11, 18 and 12 per cent, respectively. These results suggest that *T. divaricata* has inhibitory effects on neuronal AChE activity in animal models. Therefore, *T. divaricata* may be a new therapeutic target for Alzheimer's disease. Moreover, *T. australis* has also shown AChE inhibiting activity *in vitro*[77,79].

Pharmacological properties of T. divaricata alkaloids

12-hydroxy akuammicine (2): In an *in vivo* study, a concentration of 1 mg/kg of 12-hydroxy akuammicine(2), administered intravenously, caused an increase in frequency or tone of the rabbit uterus[75]. In mice and rats, the intraperitoneal administration of 12-hydroxy akuammicine(2), at a concentration of 5-15 mg/kg/day for 9-20 days, inhibited the growth of ascites and alveolar lymphoma[2]. In addition, 12-hydroxy akuammicine(2) had gonadotropic activity via follicular stimulation[2]. These findings suggest that this alkaloid of *T. divaricata* may play roles in both gonadotropic and anti-tumour effects.

19, 20 dihydrotabernamine (3) and 19,20 dihydroervahanine A (4): The 19, 20 dihydrotabernamine(3) and 19,20-dihydroervahanine A (4) are alkaloids found in the roots of *T. divaricat*a. They can inhibit acetylcholinesterase (AChE) activity *in vitro*[47]. The inhibitory effect of both alkaloids was proved to be specific, reversible and competitive. In addition, the compounds showed greater inhibitory activity on AChE than galanthamine, a well known acetylcholinesterase inhibitor[47].

Apparicine (18): An *in vitro* study demonstrated that apparicine(18) at the concentration of 250 µg/ml can inhibit the activity of *Polio III virus*[79,80]. This alkaloid at a concentration of 1.2 per cent also exhibited antimicrobial activity against *Shigella*, *Salmonella*, *Pseudomonas*, *Escherichia*, *Proteus*, *Staphylococcus* and *Corynebacterium* in an *in vitro* study[81]. Moreover, apparicine(18), as shown in an *in vitro* study, acts as an opioid agonist to opioid receptors[82].

Catharanthine (19): In an *in vitro* study by Ehrlich of ascite tumour cells, a convenient biological model for the investigation of tumour cells[83], catharanthine(19) inhibited the effect of α –aminoisobutyric acid, an amino acid transporter in tumour cells. This finding suggested that catharanthine(19) could have anti-tumour properties via inhibition of tumour cell proliferation[84]. In addition, catharanthine(19) has been shown to inhibit the calcium-calmodulin-stimulated activity of brain cyclic adenosine monophosphate (cAMP) phosphodiesterase in an *in vitro* study, which resulted in an increased intracellular level of cAMP[85] . The increased intracellular level of cAMP in neurons may lead to improved neuronal activity[86].

Conophylline (27): Conophylline (27) is a vinca alkaloid from *T. divaricata*. It has been shown to induce differentiation of pancreatic precursor cells[86]. In the rat pancreatic rudiment of organ culture, conophylline (27) inhibited the formation of cystic structure and increased the number of insulin-positive cells[86]. In addition,

conophylline (27) has also been shown to induce insulin production in rat pancreatic acinar carcinoma cells[87]. Conophylline (27) is effective in reversing the condition of hyperglycaemia in neonatal streptozotocin-treated rats[87]. Both the insulin content and the beta cell mass are increased by the administration of conophylline (27) in these animal models. The histological study demonstrated that conophylline (27) increased the numbers of ductal cells positive for pancreatic-duodenal-homeobox protein-1 and islet-like cell clusters[88]. Therefore, conophylline (27) induced pancreatic beta cells differentiated both *in vivo* and *in vitro*. Conophylline (27) has recently been employed as a health food for preventing and ameliorating diabetes and obesity. It is used for lowering blood glucose level[88] and, also, it is a new anti-tumour alkaloid[89-92]. A study showed that conophylline (27) inhibited both TNF-alpha-induced activations and phosphorylation as well as degrading of I-kB-α in human T-cell leukaemia cells[88]. In addition, conophylline (27) inhibited TGF-β-induced apoptosis in rat hepatoma cells. The conophylline (27) inhibition of TGF-β-induced promoter activity could be attributed to its potency in modulating the interaction of downstream transcriptional factors via up-regulation of *c-Jun* expression[89]. Conophylline(27) also inhibited expression on tumour cell adhesion and infiltration in the human endometrial cancer cell line[91]. The effect of conophylline (27) on the growth properties of K-ras-NRK cells was investigated both *in vitro* and *in viv*o. Conophylline (27) induced reversible flattening and almost complete growth inhibition of K-ras-NRK and K-ras-NIH3T3 cell lines, and lowered the increased uptake of 2-deoxyglucose. It inhibited the growth of K-ras-NRK and K-ras-NIH3T3 tumours transplanted in nude mice at a concentration of 0.01-0.5 and 0.001-0.1 mg/mouse, respectively[92].

Coronaridine (29): Coronaridine (29) is an alkaloid found in the leaves, stems, barks and roots of *T. divaricata*. It has been demonstrated as having an effect on autonomic and central nervous system activity[2]. The administration of coronaridine (29) produced an analgesic effect on noxious stimulation and was effective in suppressing rage caused by foot shock in an animal model[93]. Taesotikul and colleagues[94] also demonstrated that coronaridine (29) has both analgesic and anti-inflammatory activities in rats in the writhing and pain response to tail immersion in hot water as well as in the carageenin-induced paw edema test in mice. An intravenous injection of coronaridine (29) has been shown to cause dose-related hypotensive and bradycardial responses in a normal rat model[95,96]. Moreover, it has been shown, in an *in vitro* study, to decrease estrogenic activity and could lead to the anti-fertility action of *T. divaricata*[95]. An *in vivo* study reported by Mehrotra and Kamboj[97] demonstrated that this alkaloid did not have any effect on reproductive activity, except for partial inhibition of oxytocin-induced uterine response. Recently, coronaridine (29) has also been shown to have a significant AChE inhibitory activity, at the same concentration, of the physostigmine and galantamine (AChE inhibitors) in an *in vitro* study[79].

Dregamine (31): Dregamine (31) can be found in the leaves, stems, bark and roots of *T. divaricat*a. Dregamine (31) has been shown to have convulsive and respiratory stimulating effects[97]. It inhibited muscular fatigue in both *in vitro* and *in vivo* studies, similar to the activity of ibogaine[97]. Dregamine (31) has been used for the treatment of muscular and nervous asthenia and respiratory depression[97].

Ibogamine (36): Ibogamine (36) is an indole alkaloid found in the roots of *T. divaricata*. It has been shown to reduce the monosynaptic knee-jerk reflex in the cat when a 2 mg/kg concentration of ibogamine (36) was administered intraperitonealy. The mechanism of this action affected neither postsynaptic reflex arcs nor neuromuscular transmission[30], but possibly had effect via blockade of nicotinic receptors at the neuromuscular junction[98]. In addition, ibogamine (36) could act as a weak anticonvulsant agent, as demonstrated in a mouse model[99]. More recently, the indole alkaloid ibogamine (36) was explored as an agent that combats the symptoms of drug withdrawal[100-102]. Additionally, preclinical studies of ibogamine (36) in rodent models of cocaine and opiate self-administration support the notion that it is an anti-addictive agent[103-109]. Ibogamine (36) has been reported to effectively reduce drug cravings, withdrawal symptoms[110], and their tremorigenic, hallucinogenic, neurotoxic and cardiovascular side effects in addicts[111]. The predominant action mechanism of ibogamine (36) as an anti-addictive agent possibly occurs via blocking of the kappa opioid receptors, N-methyl-D-asparate (NMDA) receptors, the serotonin uptake sites and nicotinic receptors[107].

Isovoacangine (37): Isovoacangine (37) has been shown to have a high affinity for pacemaker tissue

in the regulation of the heart[112]. Isovoacangine (37) has been demonstrated to cause a negative chronotropic activity on the spontaneously beating isolated guinea pig atrium and a negative inotropic activity on the electrically driven isolated guinea pig left atrium.

Isovoacristine (38): When this alkaloid was tested on the isolated guinea pig ileum, both anti-cholinergic and antihistaminic activities were observed[113]. In addition, it has been shown that rabbit skeletal muscle was relaxed when this alkaloid was applied. This was due to the anti-cholinergic mechanism. Isovoacristine (38) hydrochloride also caused a negative chronotropic effect in both frog and rabbit models[113]. This effect on the heart has been thought to occur via the anti-cholinergic activity of this alkaloid.

Tabernaemontanine (52): Tabernaemontanine (52) is an alkaloid found in the leaves, stems, bark and roots of *T. divaricat*a. Tabernaemontanine (52) has been shown to have a vasodilatory effect in dogs[2]. It is used to dilate the blood vessels in humans following cases of arteriosclerosis, cerebral trauma and circulatory irregularities[2].

Voacamine (55): Voacamine (55) is an alkaloid found in the leaves, stems, bark and roots of *T. divaricata*. Voacamine (55) has been shown to have cardiotonic

Table V. Summary of the general pharmacological activity of alkaloids found in *T. divaricata*

Alkaloids	Pharmacological activity
12-hydroxy akuammicine (2)	Uterine contraction (*in vivo*)[2]
	Inhibition of ascites and alveolar lymphoma (*in vivo*)[2]
19, 20 dihydrotabernamine (3)	AChE inhibition (*in vitro*)[47]
19,20 dihydroervahanine A. (4)	AChE inhibition (*in vitro*)[47]
Apparicine (18)	Anti-Polio III virus (*in vitro*)[79,80]
	Anti-microbial activity (*in vitro*)[81]
	Opioid against opiated receptors (*in vitro*)[82]
Catharanthine (19)	Anti-tumour effect *(in vitro)*[83,84]
	Inhibition of cAMP phosphodiesterase activity (*in vitro*)[85]
Conophylline(27)	Anti-tumour effect *(in vitro)*[89-91]
	Ameliorate blood glucose level (*in vitro* and *in vivo*)[87,88]
Coronaridine (29)	Analgesic effect (*in vivo*)[2,93]
	Anti-inflammation (*in vivo*)[94]
	Hypotension and Bradycardia (*in vivo*)[95,96]
	Antifertility with estrogenic and uterine relaxant effect (*in vitro*)[2]
	AChE inhibition (*in vitro*)[79]
Dregamine (31)	CNS stimulation (*in vivo*)[2]
	Analeptic effect (*in vitro* & *in vivo*)[2]
Ibogamine (36)	CNS stimulation (*in vivo*)[30, 98]
	Anticonvulsant effect (*in vivo*)[99]
	Anti-addictive effect (*in vivo*)[103-109]
Isovoacangine (37)	Negative chronotropic activity (*in vitro*)[112]
	Negative inotropic activity (*in vitro*)[112]
Isovoacristine (38)	Bradycardia (*in vivo*)[84]
	Anticholinergic and antihistaminic (on guinea pig ileum) effect (*in vitro*)[113]
	Skeletal muscle relaxation (due to cholinergic mechanism) (*in vivo*)[113]
Tabernaemontanine (52)	Vasodilation (*in vivo*)[2]
Voacamine (55)	Cardiotonic effect (comparable with cardiac glycoside) (*in vivo*)[2,114]
	Mild analgesic effect (*in vivo*)[2]
	Anti-microbial activity (*in vitro*)[2]
Voacangine (56)	Slight CNS stimulation (potentiated hypnotic effect of barbiturates) (*in vivo*)[115]
	Negative chronotropic activity (*in vitro*)[112]
	AChE inhibition (*in vitro*)[79]
Voacristine (58)	Weak CNS stimulation (*in vivo*)[2]
	Negative chronotropic activity (*in vitro*)[112]
Vobasine (66)	Weak CNS depression (*in vivo*)[117]

Table VI. Possible alkaloids in *T.divaricata* that could play roles in traditional use

Traditional use of *T.divaricata*	Possible alkaloids in *T.divaricata* that could play roles in traditional use
Chemotherapeutic activity such as antimicrobial activities and antiinflammatory activities	Apparicine, Voacamine, Coronaridine, Isovoacristine
Anti-tumour activities	12-hydroxy akuammicine, Catharanthine, Conophylline
Central and peripheral nervous systems activity	Dregamine, Vobasine, Ibogaine, Voacangine, Voacristine, Isovoacristine, 19, 20 dihydrotabernamine, 19, 20 dihydroervahanine A
Analgesic activities	Coronaridine, Voacamine, Apparicine
Smooth muscles and endocrine activities	Coronaridine, 12-hydroxy akuammicine, Conophylline
Cardiovascular system activities	Tabernaemontamine, Coronaridine, Voacangine, Isovoacangine, Voacristine, Coronaridine, Isovoacristine, Voacamine

effects and use as a treatment of heart conditions[2,114]. This alkaloid has a positive inotropic effect without chronotropic effect on the heart. In addition, voacamine (55) demonstrated a strong antimicrobial activity against Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococus aureus*, and moderate activity against Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*[2].

Voacangine (56): Voacangine (56) is an alkaloid found in the leaves, stems, bark and roots of *T. divaricata*. Voacangine (56) potentiated the hypnotic effects of barbiturates and had an analgesic as well as a local anesthetic activity in a mouse model[115]. Voacangine (56) had negative chronotropic and inotropic activities on the spontaneously beating isolated guinea pig atrium and the electrically driven isolated guinea pig left atrium[112]. In another study with isolated guinea pig atria, it antagonized the positive chronotropic and inotropic effects of noradrenaline on the heart[116]. In contrast, one study showed that voacangine (56) had no effect on the heart rate[114]. The discrepancy of results obtained from these studies suggests that further investigation is required to determine the effect of voacangine (56) on the heart, as well as determine its definite pharmacological properties. Voacangine (56) was also shown to have an AChE inhibitory effect *in vitro*[79], which may explain the variation in heart beat in previous studies.

Voacristine (58): In general pharmacological screening tests, voacristine (58) exhibited a weak stimulating effect on the central nervous system. For example, head-shaking behaviour in mice occurred when this compound was administrated[2]. Moreover, this alkaloid has been shown to cause a negative chronotropic effect in rats[112].

Vobasine (66): Vobasine (66) is an alkaloid found in the leaves, stems, bark and roots of *T. divaricat*a. In the hippocratic screening test, the administration of vobasine (66) at 300 mg/kg into mice caused lacrimation, mydriasis, respiratory depression and a depression of central nervous system activity[117].

Since at least 66 alkaloids have been isolated from this species and the yield of alkaloid fraction obtained from dry stems of *T. divaricata* was at least 0.98 per cent[16], it is these alkaloidal activities that possibly justify its use in traditional medicines. The alkaloidal components of *T. divaricata* could play important roles in these pharmacological activities of *T. divaricata* extracts as summarized in Table V and VI. The most interesting is its effect on cholinergic activity. This effect may be the basis for the traditional use of *T. divaricata* in cardiovascular and nervous systems. According to previous studies, several alkaloids in *T. divaricata* enhance cholinergic activity[94,118]. However, the alkaloid, isovoacristine, acts as an anti-cholinergic agent. Therefore, further investigations into each alkaloid from *T. divaricata* should be greatly beneficial for human medicine.

Other *Tabernaemontana* species in Thailand: *Tabernaemontana pandacaqui (T. pandacaqui)* and its pharmacological activities

In addition to *T. divaricata, T. pandacaqui* is another species commonly grown in the northern part of Thailand and used as traditional medicine for treatment of fever and pain[4]. Its synonyms are *Ervatamia pandacaqui* and *Ervatamia angustisepala*. *T. pandacaqui* is known in Thailand as 'Phut'. The characteristics of *T. pandacaqui* are similar to those of *T. divaricata* in that their flowers are numerous, white, with a slender tube and five spreading lobes. However, the flowers of *T. pandacaqui* are smaller than those of *T. divaricata*[4]. From the crude alkaloid fraction of the *T. pandacaqui* stem, at least 24 indole alkaloids can be

isolated. Among these alkaloids, coronaridine is the main alkaloid found in *T. pandacaqui*. Since these alkaloids are similar to those isolated from *T. divaricata*, their pharmacological activity might be the same. It has been demonstrated that the extract of *T. pandacaqui* could cause dose-related decreased motor activity, ataxia, and loss of righting reflex, decreased respiratory rate, analgesia and hyperemia of the ear[76]. These effects were generally similar to those obtained from the extract of *T. divaricata*. However, at the same dose, the intensity of the pharmacological activity caused by *T. pandacaqui* was stronger than that observed with *T. divaricata*. Other interesting properties of *T. pandacaqui* extracts include hypotensive and negative chronotropic and inotropic effects observed in a rat model[95]. They further indicated that hypotensive and bradycardia responses to *T. pandacaqui* administration might involve cholinergic and central mechanisms[118]. In Thai folk medicine, the root of *T. pandacaqui* is boiled in water and used for the treatment of pain and inflammation. Taesotikul and colleagues[94,118] studied the effect of *T. pandacaqui* administration on carrageenin-induced rat paw odema, yeast-induced hyperthermia in rats and writhing response induced by acetic acid in mice. Their results demonstrated that the alcoholic extract of *T. pandacaqui* stems has significant anti-inflammatory, anti-pyretic and anti-nociceptive activities. The opioid active compounds isolated from the leaves of *T. pandacaqui* support their analgesic effect[17]. Furthermore, it is possible that the analgesic activities of *T. pandacaqui* in traditional use are due to the activity of its other alkaloids such as voacangine and coronaridine[115], both of which are also found in *T. divaricata*.

Toxicity of *T. divaricata*

Since *T. divaricata* has a number of pharmacological activities, further toxicological studies were necessary. Henriques and colleagues[16] investigated the toxicity of *T. divaricata* using the behaviour screening test in mice treated with alcoholic or aqueous extracts of *T. divaricata* at doses of 150-200 mg/kg. They reported that the results were indistinguishable from control animals, indicating that no toxicity was found at these concentrations. On the other hand, Melo and colleagues[119] demonstrated that voacristine, one of the major alkaloids of *T. divaricata*, presented dose-dependent cytostatic and cytotoxic effects on cultures of yeast. Since only a few studies have reported the toxicity of *T. divaricata*, further investigations on its toxicity will be needed to understand its adverse effects.

Conclusions

Tabernaemontana plants have been used in folk medicine for the treatment of high blood pressure, pain and inflammation, as well topical application for healing wounds. *T. divaricata* exhibit different roles in CNS, cardiovascular, gonadotropic, anti-tumour, anti-infectious and anti-oxidative activity and most recently enhancement of cholinergic activity in the nervous system. Evidence suggests that *T. divaricata* could possibly be a useful therapeutic agent for several neurodegenerative diseases such as Alzheimer's disease, vascular dementia and delirium, since the possible cause of these disorders is cholinergic deficiency. The possible cholinergic candidate alkaloids in *T. divaricata*, are coronaridine, voacangine, isovoacristine, 19, 20 dihydrotabernamine, and 19, 20 dihydroervahanine A . However, further detailed studies of *T. divaricata* and its alkaloids *in vivo* are needed to investigate this possibility. There are still many *T. divaricata* alkaloids and their derivatives, whose pharmacological activities have not yet been investigated. It is possible that they may contain beneficial pharmacological properties. Therefore, *in vivo* investigations regarding their effects could provide insights into the benefits of *T. divaricata* for future clinical management of many human diseases.

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Review

Heart rate variability in myocardial infarction and heart failure ☆

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Abstract

The need to refine the identification of patients who might benefit from implantation of an implantable cardioverter defibrillator has been risen by the results of many clinical trials on ICD therapy. Traditional parameters such as left ventricular ejection fraction and the presence of non-sustained ventricular tachycardia were not strong enough to achieve this goal with reasonable cost-effectiveness. Heart rate variability (HRV) is one of the most popular parameters used to assess the autonomic tone. HRV has been reported as a strong predictor of cardiovascular mortality. Currently, three different categories of methods in HRV analysis are being used; the time domain, frequency domain, and non-linear dynamic analysis. Both time domain and frequency domain analyses of HRV have been investigated extensively regarding their use as a prognostic marker for cardiovascular mortality. The non-linear dynamic analysis is the latest tool that has shown to have an even higher predictive value than any of the traditional parameters. However, standardized and supporting evidence on this new technique is still lacking. In this article, the current role of HRV in the prediction of cardiovascular mortality in myocardial infarction and heart failure patients has been reviewed.

Keywords: Heart rate variability; Myocardial infarction; Heart failure; Arrhythmia; Implantable cardioverter defibrillator

1. Introduction

Despite new therapeutic advances in the management of ischemic heart disease, patients who are admitted with acute coronary syndrome still show a high incidence of mortality during admission and in the first few months after discharge [1–10]. Sudden cardiac death due to arrhythmic events is the major cause of death among these patients, and antiarrhythmic drugs have not as yet been shown to reduce mortality after myocardial infarction (MI) [1,4,9]. Implantable cardioverter defibrillators (ICD) is the only effective treatment for both primary and secondary prevention of this mortality [2,5,7,11]. Early identification of patients who might benefit from ICD remains difficult, due partly to an unclear understanding in the pathophysiology of acute coronary syndrome. Although traditional risk stratifiers such as reduced left ventricular ejection fraction (LVEF) and frequent ventricular premature complexes double the risk of cardiac mortality, the positive predictive value of these predictors is still not sufficiently high for use in clinical practice with reasonable cost-effectiveness.

RR interval variability has been used for many years as a tool to evaluate an autonomic nervous system modulation of the heart. The difference between RR intervals in successive beats was first noticed in 1965 by Hon and Lee. They found that fetal distress was preceded by alterations in interbeat intervals before any appreciable change occurred in the heart

Abbreviations: BRS, Baroreflex sensitivity; CABG, Coronary artery bypass graft; CI, Confident interval; ECG, Electrocardiogram; HF, High frequency; HRT, Heart rate turbulence; HRV, Heart rate variability; ICD, Implantable cardioverter defibrillator; LF, Low frequency; LVEF, Left ventricular ejection fraction; MI, Myocardial infarction; NSVT, Non-sustain ventricular tachycardia; NYHA, New York heart association; SDNN, Standard deviation of NN intervals; ULF, Ultralow frequency; VF, Ventricular fibrillation; VLF, Very low frequency.

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rate itself [12]. In 1970, Ewing et al. introduced a simple bedside method to detect diabetic neuropathy in diabetic patients with RR interval variability [13]. The prognostic significance of RR interval variability in post-MI patients was first published by Wolf et al. in 1978 [14]. Although possible benefits of RR interval variability had been observed, understanding of the mechanism underlying it was still vague and only improved rapidly when the power spectral analysis was first introduced by Akselrod et al. in 1981 [15]. This power spectral analysis has led to an understanding in the autonomic background of RR interval variability [15,16]. Since autonomic imbalance has been strongly implicated in the pathophysiology of arrhythmogenesis [17–20], this finding from the spectral analysis of the variability of RR intervals has provided an explanation of the mechanism underlying its relationship with mortality in post-MI patients. However, the mechanisms underlying some frequency ranges remain controversial [16,21–23].

In different aspects of approach, nonlinear dynamic analysis has been the technique recently proposed because it can be used to analyze the complexity of the variability of RR intervals. This technique has not only shown how it can be an independent prognostic factor from traditional RR interval variability parameters, but also a provision of an even higher predictive value than traditional parameters [24–27].

Classically, there was a difference between using the term RR interval variability and heart rate variability (HRV). As its name suggests, RR interval variability represents the parameters describing the variability of RR interval, whereas HRV represents the parameters describing the variability of heart rate. Although the parameters derived from the variability of RR interval were more popular in usage, the term HRV was generally accepted as representative of the parameters calculated from both RR interval and heart rate.

Until now, HRV is one of the most promising markers and has been extensively studied in the last two decades. The noninvasiveness and easy derivation of HRV measurement make it more practical and widely studied. Since technical aspects regarding the measurement and analysis of HRV have been excellently described elsewhere [28–33], only the role of HRV as a risk stratifier for the prediction of cardiovascular mortality in MI and heart failure are mainly presented and discussed in this review article. The time and frequency domain of HRV parameters are summarized in Table 1.

2. HRV in myocardial infarction

A number of studies on HRV in acute MI patients have been reported since Wolf et al. [14] first proposed that there was a relationship between the two [9,27,34–44]. In the acute phase of MI, a depressed HRV has been observed for two weeks, followed by recovery [38,45]. In a retrospective analysis of an ATRAMI (Autonomic Tone and Reflex After Myocardial Infarction) study, which enrolled 1248 patients with recent MI [41,42], the two most popular autonomic markers, HRV (SDNN) and baroreflex sensitivity (BRS), were investigated to test their prediction on cardiac mortality during a two-year follow up. Patients with recent MI (<28 days) have been recorded for BRS, LVEF, and 24-h ECG. SDNN<70 ms has been shown as an independent and potent predictor of cardiovascular mortality (relative risk ratio 3.2, 95% CI 1.6–6.3). The combination of SDNN, BRS and NSVT has an even higher relative risk ratio of 22 for cardiac mortality, which is greater than any other combination of LVEF. The combinations of SDNN and NSVT, which are derived from only an ECG, also have a strong predictive value (relative risk ratio 17, 95% CI 7.2–40.5). However, the combination of LVEF and depressed SDNN was not a strong predictor of cardiac mortality (relative risk ratio 4.1, 95% CI 1.3–12.2) in the ATRAMI study.

Although there were no prospective studies in post-MI patients directly designed to test the predictive value of HRV, there were prospective studies in this group of patients that also measured HRV parameters. In a substudy from an EMIAT (European Myocardial Infarct Amiodarone Trial) population, SDNN<50 ms and HRV index<20 U were shown as independent predictors of total mortality in 1216 post-MI patients with LVEF<40% [37]. That study also demonstrated that patients with depressed HRV benefited from prophylactic antiarrhythmic treatment with amiodarone. More recent data came from the ALIVE (Azimilide Post-Infarct Survival Evaluation) trial, which was a prospective randomized placebo-controlled trial of Azimilide that enrolled 3717 post-MI patients [9]. That study demonstrated that the HRV index<20 U was an independent predictor of a 1-year total mortality (15% versus 9.5%, $p<0.0005$). However, in the DINAMIT study, which was a prospective open-label randomized controlled trial with 674 enrolled patients designed to test the effective of prophylactic ICD therapy, the reduced SDNN (combined with poor LVEF) failed to show its usefulness in the qualification of post-MI patients for ICD [8]. In a prospective study of 70 patients with idiopathic dilated cardiomyopathy who required ICD interventions, Grimm et al. also demonstrated that SDNN on 24-h ECG is not a good marker to select idiopathic dilated cardiomyopathy patients for prophylactic

Table 1
Commonly used parameters of time and frequency domains of HRV [30]

Parameters	Description	Unit
Time domain		
SDNN	Standard deviation of all NN intervals	ms
SDANN	Standard deviation of 5-min averaged NN intervals	ms
ASDNN	Average of 5-min standard deviation of NN intervals	ms
RMSSD	Root mean square of successive differences	ms
Frequency domain		
Total power		ms^2
Low frequency (LF) power	Power between 0.15–0.4 Hz	ms^2
High frequency (HF) power	Power between 0.04–0.15 Hz	ms^2
LF/HF ratio	Ratio between LF and HF power	–

ICD therapy [46]. A summary of these large predictive studies ($N>500$) of HRV in post-MI patients can be found in Table 2.

Although the origins of some spectral components are still unclear, the frequency domain technique has been widely investigated in post-MI patients. The depression of total power, ULF, HF, an increase of LF in the normalize unit and an increase of LF/HF ratio, which is believed to reflect sympathovagal imbalance, have been observed and shown as strong predictors of cardiovascular mortality in post-MI patients [38,47,48]. Although decreased ULF has been reported as a predictor of cardiovascular mortality, replacement of this parameter by SDNN has been suggested, since long term spectral analysis has encountered problems dealing with ectopic beats [49].

The timing recommended for performing HRV analysis in MI patients is still not clearly defined. Due to the nature of autonomic tone recovery, which usually begins at two weeks after the onset of MI, recommendation has been made to perform HRV analysis at 1 week post-MI [30,38,45]. The study on predischarge HRV analysis in post-MI patients was reported to have prognostic significance. The studies evaluating HRV during the first 48 h also demonstrated its significant predictive value [50–52]. A CAST (Cardiac Arrhythmia Suppression Trial) population data retrospective analysis demonstrated that an HRV analysis on 1-year post-MI follow up patients also had prognostic significance [27].

Non-linear dynamic parameters have been reported as the predictors of cardiac mortality in post-MI patients [27,35]. In a subgroup analysis of 446 patients in a prospective DIAMOND (Danish Investigations of Arrhythmia and Mortality On Dofetilide in survivors of myocardial infarction) study [35], it was found that DFA1 was a stronger predictor of total mortality, arrhythmic mortality and cardiac mortality than traditional time and frequency domain parameters in patients with LVEF < 36% (relative risk ratio 3.0, 95% CI 2.5–4.2) during a 2-year follow up. In a CAST population analysis, three parameters; power law slope, DFA1 and SD12 showed stronger predictive values than the traditional time and frequency domain parameters [27,53].

Although the use of HRV as a predictor of long term mortality has been proposed with increased supporting evidence, the predictive values of these parameters are still too low for use as a standalone routine screening test. A combination of HRV and other traditional parameters should help to minimize this limitation, particularly in parameters derived from an ECG, which should have no additional cost and be more practical with high cost-effectiveness.

3. HRV in heart failure

The evidence of HRV in heart failure patients has not been extensively investigated when compared to that in post-MI patients. After the first report of HRV in heart failure patients by Saul et al. in 1988 [54], many studies have investigated the change of HRV in heart failure patients in order to seek the correlation between HRV and current clinical status [37,55–58]. Only a few studies have focused on HRV as a

Table 2
Summary of predictive studies of HRV (time and frequency domain parameters) in post-MI ($N>500$) and heart failure patients (see text for details)

Authors	Population/size	Type of study	Follow-up period	Predictive results
Bigger et al. [49].	Post-MI/715 patients	Prospective	4 years	Total, ULF, and VLF power have strong association with mortality
La Rovere et al. [41].	Post-MI/1284 patients	Prospective	21 ± 8 months	Depressed HRV (SDNN < 70) was a strong predictor of cardiac mortality independently of LVEF and of ventricular arrhythmias
Stein et al. [36].	Post-MI/769 patients	Retrospective	–	Decreased HRV did not predict mortality for the entire study group. In CABG subgroup, decreased HRV was not associated with increased mortality. In patients without CABG or diabetes, decreased SDANN predicted mortality.
Malik et al. [37].	Post-MI/1216 patients	Retrospective	–	Patients with LVEF ≤ 40% and depressed HRV (SDNN < 50, HRV index < 20) benefit from prophylactic antiarrhythmic treatment with amiodarone.
La Rovere et al. [42].	Post-MI/1071 patients	Prospective	21 ± 8 months	Depressed HRV (SDNN < 70) was associated with increased mortality.
Hohnloser et al. [8].	Post-MI/674 patients	Prospective	30 ± 13 months	Decreased SDNN (with poor LVEF) did not demonstrate its usefulness in identifying post-MI patients at risk of increased mortality for ICD.
Camm et al. [9]	Post-MI/3717 patients	Prospective	1 year	Low HRV had a higher 1-year mortality than high HRV patients.
Nolan et al. [59].	CHF/433 patients	Prospective	482 ± 161 days	Reduced SDNN was a strong predictor of mortality due to progressive heart failure.
La Rovere et al. [60].	CHF/202 patients	Retrospective	–	Reduced short-term LF power during controlled breathing was a strong predictor of sudden death in CHF patients.
Aronson et al. [62].	Decompensated CHF/199 patients	Prospective	312 ± 150 days	SDNN, SDANN, total power, and ULF power were useful as predictors of survival after hospital discharge.
Anastasiou-Nana et al. [61].	CHF secondary to ischemic or idiopathic dilated cardiomyopathy/52 patients	Prospective	2 years	HRV parameters were not associated with all-cause mortality.

predictor of long term mortality [59–62]. A summary of these predictive studies of HRV in heart failure patients can be found in Table 2.

Strong evidence came from the prospective analysis data of a UK-Heart study, which assessed the reduction of SDNN (<50 ms), creatinine, serum sodium, NSVT, cardiothoracic ratio and LV end-diastolic diameter in 433 out-patients with congestive heart failure (NYHA functional class I to III; mean ejection fraction 0.41±0.17) [59]. In that analysis, the reduction in SDNN was a more powerful predictor of death risk, due to progressive heart failure, than the other conventional clinical measurements. A short term HRV analysis of moderate to severe congestive heart failure (age 52±9 years, LVEF 24±7%, NYHA class 2.3±0.7) patients was reported by La Rovere et al [60]. Their study used 202 patients (1991–1995) as derivative samples to develop the predictive model. The model was then validated with another 242 patients (1996–2001) for its predictive value. The results of that study demonstrated that the reduction of absolute LF power ($\leq$13 ms^2 in derivative samples and $\leq$11 ms^2 in validation samples) was a strong and independent predictor of sudden death in heart failure patients (derivative samples: relative risk ratio 3.7, 95% CI 1.5 to 9.3, validation samples: relative risk ratio 3.0, 95% CI 1.2 to 7.6). In a more severe group of patients, HRV analysis was done in 199 patients (131 men, aged 60±14 years), who were admitted to hospital for decompensate congestive heart failure after previously being diagnosed as NYHA class III or IV congestive heart failure [62]. HRV analysis in both time and frequency domain from a 24-h holter ECG recording on the day of admission demonstrated that the index of overall HRV (SDNN: relative risk ratio 2.2, 95%CI 1.05 to 4.3, p=0.036, SDANN: relative risk ratio 2.1, 95% CI 1.05 to 4.2, p=0.04, total power: relative risk ratio 2.2, 95% CI 1.08 to 4.2, p=0.03, ULF power: relative risk ratio 2.6, 95% CI 1.3 to 5.3, p=0.007) provided useful prognostic information in correlation with a 1-year total mortality.

Recently, a prospective investigation of HRV and iodine-123-metaiodobenzylguanidine myocardial uptake in 52 chronic congestive heart failure, secondary to ischemic or idiopathic dilated cardiomyopathy patients, with a mean LVEF of 31±12%, has been reported [61]. In that study, HRV in both time and frequency parameters was similar between survivors and nonsurvivors, except that decreased HF power was associated with an increased risk of sudden death (relative risk ratio 0.310, 95% CI 0.101 to 0.954, p=0.041), but not all-cause mortality. The cause of this disparity in the predictive power among these heart failure cases is still being investigated. The apparent difference is the clinical status of patients included in each study. The clinical status of the population in the study of Anastasiou-Nana et al. was more severe than that in the UK-Heart study. LVEF in the Anastasiou-Nana et al. study was also higher than that reported by La Rovere et al [60]. Since heart failure is a continually progressive process of autonomic imbalance, whereas MI is an impact of events followed by the recovery of this imbalance, it is difficult to find the reference point to define the timing of HRV measurement in heart failure patients. Comparisons of HRV determined in heart failure patients with similar clinical conditions in all populations are also difficult. It is possible that these limitations may be the cause of variation and disparity in HRV predictive values.

4. Modification of HRV by interventions and drugs

Efforts to modify HRV in order to improve prognostic values in post-MI patients have come from its physiological background, which is believed to be an autonomic modulation of heart rate. Although there is much evidence supporting the benefits of an increased vagal activity, it is not known how much vagal activity has to be increased to provide the best improvement in outcome. Another point that needs to be considered in trying to modify HRV is the change of autonomic balance measured by HRV being the physiologic response to the need of higher cardiac performance. An attempt to improve HRV by reducing the need of this higher performance may improve the prognosis, while an improved HRV by reducing the ability of the heart to respond to these physiologic needs should worsen the prognosis. Furthermore, since the origins of many frequency components are still unclear, especially LF, these interventions or drugs that try to modify HRV may still lack strong supporting evidence.

The beta blocker, which is believed to increase cardiac vagal efferent activity and reduce sympathetic efferent activity, has been noted for preventing the rise of the LF component observed in the morning hours [63]. This benefit has been demonstrated in coronary occluded pigs, in which the incidence of VF was reduced [64]. Some antiarrhythmic drugs such as flecainide, encainide and moricizine were reported to increase HRV parameters in post-MI patients [65,66]. Aprindine and procainamide also demonstrated a similar effect in an animal model [67]. However, no correlation between HRV changes and mortality was found [65]. Cardiac glycosides have been reported to modify HRV [68,69]. In contrast to the consequence of mortality, digoxin has been shown to increase cardiac vagal control assessed by HRV.

Alteration of the renin–angiotensin system, which has been proposed as a mechanism underlying ULF spectral components by angiotensin converting enzyme inhibitors, shows the ability to increase cardiac vagal control, as demonstrated by a significant increase in total power, ULF, VLF, and LF power [70].

Endurance exercise training is commonly believed to enhance cardiac parasympathetic tone. Although there are several reports that used power spectral density analysis of HRV in athletes, or followed endurance training, their findings still demonstrated a greater, lesser, or no different HF or vagal spectral component between athletes and controls [71–76]. Since a clear respiratory or vagal power component was not isolated, this may contribute to the discrepancy in findings. A recent study, which attempted to correlate the frequency band to respiration instead of the classical HF band range, suggested

that training-induced adaptations in the intrinsic heart rate may contribute to training-induced bradycardia [77]. However, more research to investigate the mechanisms that account for possible adaptive changes or basal differences are needed in order to explain resting bradycardia found in endurance-trained individuals.

Many interventions in post-MI patients also change HRV parameters. Coronary artery bypass grafting (CABG) strongly depressed HRV in the time and frequency domain and nonlinear dynamic analysis in many studies [78–82]. While the mechanism underlying depressed HRV after CABG is still unclear, some studies demonstrated a complete recovery of HRV in 2–3 months after CABG [79,81], whereas some failed to demonstrate a recovery even after 6 months of follow up [80]. The prognostic significance of this depressed HRV has also been investigated, but no correlation between depressed HRV and prognosis was found [82]. The effect of early reperfusion by thrombolytic agents or interventions after acute MI on HRV parameters has also been investigated [83–87]. Successful reperfusion increased HRV in some studies [83–85], however, Chakko et al. reported that immediate, transient, and seemingly paradoxical depressed HRV was observed in post-MI after reperfusion [86]. Nevertheless, increased HRV after successful reperfusion still has prognostic significance in this group of patients [87].

5. Clinical use of HRV

Although traditional time and frequency domain analyses have been integrated in many ECG monitoring systems, and can be automatically operated, these analyses have yet to be entered into routine clinical use for risk stratification [88–90]. The most discussed benefit of HRV is the ability to identify high risk sudden cardiac death patients, who would benefit from an ICD. This benefit has been investigated. However, the major limitation for HRV use as a routine screening test is its low predictive value. This limitation needs to be improved by combining HRV with other ECG derived parameters or even with traditional ones. Other uses of HRV have been reported such as a screening test for diabetic neuropathy and tetraplegia or as a marker in many research studies to assess autonomic tone and prognostic factor [34,91–93].

6. Future development for clinical use

Although HRV has been demonstrated in many studies as a strong predictor of cardiovascular mortality with a high value of specificity, its sensitivity is too low to be used alone as a routine screening test [88–90]. Nevertheless, HRV has succeeded in its primary goal to refine the identification of patients who might benefit from the implantation of an ICD, when combined with other traditional markers such as NSVT, BRS, and LVEF. Non-linear dynamic is a new tool that can be used to analyze other aspects of the data in the variability of heart rate. However, this technique still requires more evidence from large systematic studies and needs to be standardized before it can be used as a standard marker, similar to those in traditional parameters.

While investigations on its predictive value is ongoing, it is essential that clarification of physiological evidence must be provided, especially the origin of each frequency domain and the link between the complexity of HRV and mortality [16,94]. These explanations will not only answer the question about HRV mechanisms, but also open a new path to the selection of interventions or drugs that modify HRV parameters. Furthermore, many new methods have been introduced to analyze the ECG, with different supporting mechanisms [95–104]. Heart rate deceleration capacity [107], which measures vagal activity affected heart-rate variability, and heart rate turbulence [106], which measures baroreflex responded to ventricular ectopic beats, were shown to be even more a powerful predictor of mortality after myocardial infarction and is more accurate than LVEF and the conventional measures of heart rate variability. T-wave alternans, ventricular late potential and prevalent of low frequency were also proposed as prognostic markers in this group of patients but with lower predictive values than the conventional HRV parameters. These measurements and HRV are easy to perform and highly reproducible at a low cost, since no additional costs besides ECG recordings are needed. Any combination of traditional HRV, HRT, NSVT, non-linear dynamic parameters of HRV, and some of these new methods with excellent sensitivity and specificity in the cardiovascular mortality prediction could be of great benefit, and they will enable easier and more practical identification of patients at risk. Furthermore, due to the wide availability of ECG monitoring systems together with their simple recording technique, most physicians can perform these investigations in general circumstances. Future investigations are needed to confirm the clinical application of HRV as a specific prognostic marker, particularly in MI and heart failure patients

7. Conclusion

HRV, as both a traditional and non-linear dynamic parameter, has shown to be a strong predictor of cardiovascular mortality, which may help to refine the identification of patients who might benefit from the implantation of an ICD. Further studies are needed to understand the mechanisms of HRV components and improve their sensitivity and specificity as a prognostic marker. The combination between HRV and other parameters that can be obtained from ECG recordings may be an effective solution for this limitation.

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Preliminary Report

Effects of Combined Sildenafil-Nitric Oxide Donor On Defibrillation Efficacy

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Background: *A previous study demonstrated that supra-therapeutic concentration of sildenafil citrate attenuates defibrillation efficacy. However, the effect of combined sildenafil and NTG administration on defibrillation efficacy is not known.*
Objective: *The present study investigated whether sildenafil administration at the therapeutic level increases the defibrillation threshold (DFT) when combined with NTG.*
Material and Method: *Twenty-four pigs (20-25 kg) were randomized into four groups. After the control DFT was obtained, a stock solution of 50-mg (group 1, therapeutic concentration) and 100-mg (group 2, supra-therapeutic concentration) of sildenafil, and 100-mL of saline (groups 3 and 4) were infused at 2 mL/min. Then, NTG was administered in groups 1-3 at 5 □g/min, with an increment of 5 □g/min every 5 min. The DFT was determined again after NTG was infused for 20 minutes.*
Results: *In group 1, the DFT (402 ± 33V, 11 ± 2J) was not different from the control (404 ± 28V, 11 ± 2J). In group 2, the DFT (521 ± 18V, 19 ± 1J) was higher ($p < 0.004$) than that in the control group (444 ± 31V, 14 ± 2J). Saline did not alter the DFT, either individually or in combination with NTG.*
Conclusion: *Supratherapeutic dose of sildenafil-NTG combination significantly increased the DFT (17% of peak voltage, 37% of total energy). This effect on DFT appears to be driven by sildenafil and not NTG.*

Keywords: *Sildenafil citrate, Nitroglycerin, Fibrillation, Defibrillation*

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Sildenafil is a highly selective inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE-5), which is used for the treatment of Erectile Dysfunction (ED)[1-5]. Growing evidence has also demonstrated that sildenafil is an effective agent for treating pulmonary arterial hypertension, leading to an increased use of this drug[6-9]. PDE-5 is found not only in the vasculature of the corpus cavernosum, but also in the systemic arteries and veins throughout the body[10]. Accordingly, PDE-5 inhibitors are effective as mild vasodilators by increasing intracellular cGMP levels in vascular smooth muscle[11].

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Although sildenafil has been used clinically for many years, its cardiac effects are still unclear and need to be investigated further[12-14]. In clinical use since 1998, PDE-5 inhibitors have an excellent safety profile, and it is now clear that sildenafil is not arrhythmogenic at the therapeutic level[15-17]. However, it has been reported that some of the patients who died suddenly, presumably by fatal ventricular arrhythmia, were on sildenafil in combination with nitrate therapy[18]. Furthermore, administration of sildenafil together with nitric oxide donor at the supra-therapeutic level has demonstrated the promotion of fatal arrhythmias, whereas sildenafil alone at the therapeutic level is not arrhythmogenic[19]. Recently, we demonstrated that defibrillation efficacy was significantly attenuated when sildenafil was administered intravenously at the supra-therapeutic level, but not at a therapeutic dose[20]. Since nitroglycerine (NTG) is known to have a synergistic

effect on blood pressure when given with sildenafil[21], and sildenafil administered in combination with nitric oxide donor promotes fatal arrhythmias[19], the authors sought to determine whether combined sildenafil-NTG administration significantly affected the defibrillation threshold (DFT) when sildenafil is administered at the therapeutic level. In the present study, the authors tested the hypothesis that intravenous administration of sildenafil at a therapeutic dose, together with NTG, attenuates defibrillation efficacy.

Material and Method

Animal preparation and electrode placement

Experiments were performed on the swine model; twenty-four healthy pigs (20-30 kg) of either sex that were anesthetized and maintained under physiologic conditions as previously described[20,22]. The swine model was chosen since it has been widely used to study defibrillation due to its anatomy and arrhythmia characteristics[20,22-26]. Experiments were performed in accordance with Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University. All animals were housed and maintained in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). A catheter, with a 34-mm platinum coated titanium coil electrode (Guidant Inc., USA), was inserted into the right ventricular apex. A 68-mm electrode catheter was positioned at the junction between the right atrium and superior vena cava. The position of the catheters was verified with fluoroscopy. The blood pressure, ECG, heart rate, core body temperature, arterial blood gas, plasma O_2 saturation, respiratory rate, and $EtCO_2$ were monitored continuously throughout the entire study.

Defibrillation protocol

Ventricular fibrillation (VF) was induced by 50-Hz alternating current delivered via an electrode at the tip of the right ventricular catheter. After 10 seconds of VF, defibrillation was attempted with biphasic shocks (Ventak, Guidant Inc., USA), with electrodes at the right ventricular apex as a cathode, and at the superior vena cava as an anode for the first phase. A minimum of 4 minutes was allowed to elapse between VF episodes. If the shock failed to defibrillate, a rescue shock (20-30 J) was delivered within 10 sec.

The DFT in each group was determined using a three-reversal up/down protocol[20,27]. Briefly, the initial shock strength was started at 400 V. For a successful defibrillation episode, the leading edge voltage was decreased in 80 V steps per defibrillation attempt until a first reversal from the successful defibrillation to a failed defibrillation episode was achieved. If the initial shock was unsuccessful, the leading edge voltage was increased in 80 V steps per defibrillation attempt until a first reversal from the failed defibrillation to a successful defibrillation episode was achieved. At each reversal point, the algorithm was iterated in the opposite direction. After the first reversal point was obtained, the strength of the voltage step was diminished to 40 V, and 20 V after the second reversal point. The DFT was defined as the lowest energy requirement for successful defibrillation after the three reversal points were obtained, when the next lower setting failed to defibrillate the heart.

Twenty-four pigs were randomly assigned into four groups (Fig. 1). In group 1, the DFT (control-DFT) was determined at the beginning of the study. After the control DFT was obtained, a 50-mg stock solution (0.5 mg/mL) of sildenafil was administered intravenously at the rate of 2 mL/minute over 50 minutes[11]. In group 2, a stock solution of 100-mg of sildenafil (1 mg/mL) was administered at the same rate as that in group 1.To confirm that an increase in plasma volume did not affect the DFT, a similar protocol to the one in group 1 was performed in groups 3 and 4, except for saline (100 mL), which was administered at the same rate as that in group 1. At the end of sildenafil and saline infusion, NTG was administered intravenously in groups 1, 2 and 3 at a rate of 5 □g/min, with an increment of 5 □g/min every 5 minutes. The DFT was determined again after NTG was infused for 20 minutes. The animal was euthanized by VF induction at the end of the study.

Drug administration

Sildenafil (Pfizer) in tablet form (50 and 100-mg) was dissolved in 100 mL of normal saline just before the experiment to form a stock solution. The solution was then filtered, and a clear solution was obtained. Two different concentrations of sildenafil were injected intravenously at the rate of 2 mL/min over 50 minutes. Intravenous administration of 50-mg of sildenafil has been shown to represent ~100-mg of sildenafil citrate taken orally, whereas 100-mg of sildenafil infusion has been shown to be a supra-therapeutic dosage (~ > 200-mg taken orally)[1,11]. In the present study, NTG was used as a nitric oxide donor. NTG (Schwarz/Berli Jucker) at 5 mg (5 mL) was added into 0.9% NaCl 45 mL, forming a stock solution of NTG at 100 □g/mL. NTG was

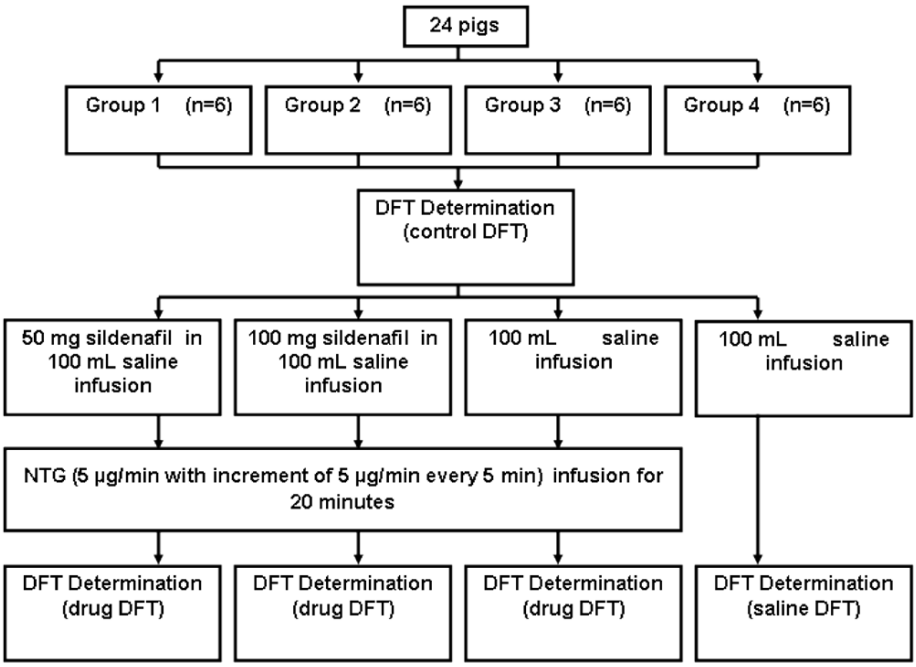

Fig. 1 Illustrated diagram of the study protocol for DFT determination,. pigs were randomly assigned into four groups equally, in each group, the DFT was determined at the beginning of the study and after interventions

administered intravenously at a rate of 5 □g/min, with an increment of 5 □g/min every 5 minutes. The increment was terminated if the systolic blood pressure fell to 20 mmHg below the baseline.

Statistical analysis

Values are expressed as mean ± SD. Comparisons of data between control and drug-DFT within each animal were performed using the student's t-test. A $p < 0.05$ was considered statistically significant.

Results

The average weight of the pigs used in the present study was 26 ± 1 kg. The total number of shocks delivered to each animal was 13 ± 1. The average number of shocks delivered to the animal before obtaining the DFT was 6 ± 1 in the control group and 6 ± 1 in the drug (sildenafil-NTG) or saline (either individually or in combination with NTG) injected groups.

In group 1, the peak voltage and total energy for the control DFT was 404 ± 28 V and 11 ± 2 J, respectively. After 50-mg of sildenafil-NTG infusion, the DFT (402 ± 33 V, 11 ± 2 J, for the peak voltage and total energy respectively) was not different from the control DFT ($p = 0.7$ and $p = 0.9$, respectively, Table 1). Both the impedance and pulse width were not changed after drug administration. The systolic blood pressure after 50-mg of sildenafil-NTG infusion was significantly lower than that in the control ($p < 0.05$, Table 1). However, the heart rate before and after drug administration was not significantly different (Table 1).

In group 2, the peak voltage and total energy for the control DFT was 444 ± 31 V and 14 ± 2 J, respectively. After 100-mg of sildenafil-NTG infusion, the DFT (521 ± 18 V, 19 ± 1 J, for the peak voltage and total energy respectively) was significantly higher than that in the control group (Table 1). The 100-mg of sildenafil-NTG infusion significantly increased the DFT by 17% of the leading-edge voltage and 37% of total energy. No changes in the impedance and pulse width were found in this group. The systolic blood pressure after 100-mg of sildenafil-NTG infusion was significantly lower than that in the control ($p < 0.05$, Table 1). The heart rate before and after drug administration was not different ($p = 0.3$).

In group 3, the peak voltage and total energy for the control DFT was not different from that in the control group (Table 1). The systolic blood pressure after saline-NTG infusion was significantly lower than that in the control group ($p < 0.05$, Table 1). The im-

Table 1. DFT and hemodynamic parameters measured before and after drug (sildenafil-NTG) or saline (either individually or in combination with NTG) administration

Parameters	Group I (n = 6)		Group II (n = 6)		Group III (n = 6)		Group IV (n = 6)	
	Control	50-mg Sildenafil + NTG	Control	100-mg Sildenafil + NTG	Control	100-mL Saline + NTG	Control	100-mL Saline only
Delivered voltage (Volts)	404 ± 28	402 ± 33	444 ± 31	521 ± 18*	424 ± 26	427 ± 34	439 ± 14	417 ± 17
Total energy (Joules)	11 ± 2	11 ± 2	14 ± 2	19 ± 1□	12 ± 2	13 ± 2	14 ± 1	13 ± 1
Impedance (Ohm)	61 ± 2	61 ± 2	60 ± 2	59 ± 3	62 ± 2	62 ± 1	61 ± 3	58 ± 3
Pulse width (msec)	15 ± 1	15 ± 1	15 ± 1	15 ± 1	15 ± 1	15 ± 1	16 ± 1	15 ± 1
Systolic BP (mmHg)	97 ± 3	84 ± 3□	89 ± 4	76 ± 4□	106 ± 3	95 ± 5□	90 ± 3	102 ± 3□
Heart rate (bpm)	88 ± 4	94 ± 5	106 ± 4	95 ± 7	99 ± 4	100 ± 7	106 ± 2	116 ± 6

* $p < 0.004$ compared to control; □ $p < 0.002$ compared to control; □ $p < 0.05$ compared to control

pedance, pulse width, and heart rate before and after drug administration were not different ($p = 0.7$).

In group 4, the peak voltage and total energy for the control DFT was not different from that in the control group (Table 1). The impedance and pulse width were not altered after drug infusion. The systolic blood pressure after saline infusion was significantly higher than that in the control group ($p < 0.05$, Table 1). However, the heart rate before and after drug administration was not different ($p = 0.1$).

Discussion

The findings of the present study could be divided into 2 areas of concern: effects on the DFT and those on blood pressure and heart rate. The major findings were as follows: (1) Intravenous administration of 100-mg of sildenafil-NTG combination (group 2) significantly increased the DFT; (2) Infusion of 50-mg of sildenafil-NTG (group 1) as well as saline either individually (group 4) or in combination with NTG (group 3) did not change the DFT; (3) The administration of 50-mg of sildenafil-NTG (group 1), 100-mg of sildenafil-NTG (group 2), and saline-NTG (group 3) significantly decreased the systolic blood pressure (SBP); and (4) The heart rate was not altered after drug (sildenafil-NTG) and saline (either individually or in combination with NTG) administration.

Sildenafil-NTG combination and defibrillation efficacy

It was shown previously that sildenafil at the supra-therapeutic level in combination with nitric oxide donor promotes fatal arrhythmias[19]. In that study, sildenafil alone was not arrhythmogenic even at a supra-therapeutic concentration, suggesting that nitric oxide may play a role in the arrhythmogenicity of sildenafil. Recently, sildenafil demonstrated an increase in the DFT when a supra-therapeutic dose was given intravenously, but not when the therapeutic dose was administered[20]. However, the effect of the sildenafil-nitric oxide donor combination on defibrillation efficacy has never been investigated. To the best of the authors' knowledge, the present study is the first to investigate the effect of sildenafil-nitric oxide donor on defibrillation efficacy. The present results demonstrated that intravenous administration of 50-mg of sildenafil (i.e. representing the therapeutic plasma level[11]) in combination with NTG does not affect the DFT.

Since the authors' previous study demonstrated that 50-mg of sildenafil alone does not affect the DFT when administered intravenously, the results of the present study indicate no detrimental effect of added NTG on defibrillation efficacy. Furthermore, the present study demonstrated that the combination of 100-mg of sildenafil (i.e. a supra-therapeutic plasma level[11]) and NTG significantly increased the DFT when administered intravenously. It was shown previously that the intravenous administration of 100-mg of sildenafil alone significantly increased the DFT by 19% of voltage and 38% of total energy[20]. In the present study, a combined 100-mg of sildenafil-NTG raised the DFT by 17% of voltage and 37% of total energy. Since the results from using 100-mg of sildenafil alone or 100-mg of sildenafil-NTG combination demonstrated a similar percentage of DFT increment by both voltage and total energy, these findings indicate no beneficial or worsening effect of added NTG on defibrillation efficacy over sildenafil administered in a supra-therapeutic dose.

Many studies have been carried out to validate the cardiac electrophysiological effects of sildenafil[13,14,16,17,28,29]. By using human ether-a-go-go-related gene (HERG)-transfected HEK293 cells, Geelen et al [13] demonstrated that sildenafil at the supra-therapeutic level prolongs cardiac repolarization by blocking the rapid component of the delayed rectifier potassium current (I_{Kr}). Accordingly, sildenafil should be expected to improve defibrillation efficacy by lowering the DFT, as do most other I_{Kr} blockers, such as sotalol and dofetilide[30,31]. However, a recent study performed by Chiang et al [12] showed different findings. They demonstrated that sildenafil at a therapeutic concentration neither blocked I_{Kr} and I_{Ks} in guinea pig ventricular myocytes, nor prolonged cardiac repolarization in guinea pig papillary muscles and canine purkinje fibers. Moreover, they also found that sildenafil at supra-therapeutic concentrations caused shortening of cardiac repolarization, presumably through its blocking effect on $I_{Ca,L}$ [12]. In an in vivo study using a canine model, Sugiyama et al demonstrated that intravenous administration of sildenafil at therapeutic to moderate supratherapeutic concentrations did not affect the action potential duration[14]. Therefore, the definite effect of sildenafil on cardiac ion channels must be investigated further and the discrepancy of results verified. Nevertheless, the authors' previous study[20], and this present study, demonstrated the undesired effect on defibrillation efficacy of the supra-therapeutic plasma level of sildenafil.

In the present study, the increase in the DFT after the combination of 100-mg of sildenafil and NTG administration could be due to its effects on cellular electrophysiological alterations in cardiomyocytes. Sildenafil is known to inhibit PDE-5, causing a net increase in intracellular cGMP concentrations[1,32]. Musialek et al[33] found that an increase in cellular cGMP could stimulate the hyperpolarization-activated inward current (I_f), which may promote an "automatic" tachycardia[33]. However, the constant heart rate after sildenafil administration at both therapeutic and supra-therapeutic concentrations reported in the present study suggests that this mechanism should not be the cause of a high DFT. At supra-therapeutic concentrations, sildenafil blocks $I_{Ca,L}$ and accelerates cardiac repolarization[12], which may possibly lead to a shortening of the action potential duration (APD) and effective refractory period (ERP). These electrophysiological changes could be responsible for an attenuation of defibrillation efficacy, resulting in the increase in the DFT found in the present study. As for the sildenafil-NTG combination, Yoo et al recently reported that sildenafil did not augment NTG to increase the plasma cGMP concentration in dogs[34]. This might explain no augmentative effect of NTG on defibrillation efficacy when given at either 50-mg or 100-mg of sildenafil, as reported in the present study. Further defibrillation studies such as cardiac mapping are necessary to elucidate the mechanism of increased DFT by supra-therapeutic doses of sildenafil.

Conclusion

The intravenous administration of combined 100-mg of sildenafil (i.e. a supra-therapeutic concentration) and NTG significantly increased the DFT (37% total energy). No synergistic effect of NTG was found on the DFT, when administered together with sildenafil. Sildenafil is used increasingly in both ED and pulmonary arterial hypertension, and if this adverse effect is also consistent in humans, the drug should be used with caution in patients with relatively high DFT. Care should be taken when prescribing sildenafil, individually or in combination with nitric oxide donors, to patients with a high DFT at baseline, pulmonary hypertension that requires prolonged use of these drugs, and impaired drug elimination such as hepatic or renal insufficiency.

Study limitations

Although the present study demonstrated an increase in the DFT after intravenous administration of a supra-therapeutic plasma concentration of sildenafil combined with NTG using a standardized swine model of approximate human heart size, the present study was performed in normal pig hearts and results may differ in diseased hearts. Moreover, the pharmacokinetics and metabolism of these drugs vary substantially between species, therefore the results could be similar or different in humans. Although the effective refractory period was not measured in the present study, it was shown in the introduction of the present study that intravenous administration of sildenafil at the sub- to supra-therapeutic level did not affect either monophasic action potential duration or effective refractory period. Defibrillation studies at cellular and molecular levels are needed to verify the mechanistic insight by which sildenafil increases the DFT.

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ผลของการให้ยา sildenafil ร่วมกับ nitric oxide donor ต่อประสิทธิภาพในการทำ defibrillation

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การศึกษาก่อนหน้านี้พบว่าการให้ยา sildenafil ในระดับสูงว่าระดับที่ใช้ในการรักษา ทำให้ประสิทธิภาพในการทำ defilbrillation ลดลง อย่างไรก็ตามผลของการให้ยา sildenafil ร่วมกับ nitroglycerine (NTG) ต่อประสิทธิภาพการทำ defibrillation ยังไม่มีการศึกษา งานวิจัยนี้ศึกษาว่าผลของยา sildenafil ในระดับปกติที่ใช้ในการรักษา เมื่อให้รวมกับ nitroglycerine จะมีผลต่อประสิทธิภาพในการทำ defibrillation หรือไม่ การศึกษานี้ใช้สุกร 24 ตัว (20 -25 กิโลกรัม) โดยทำการวัด defibrillation threshold (DFT) หลังจากนั้นแบ่งสุกรเป็น 4 กลุ่ม โดยที่กลุ่มที่ 1 ได้รับสารละลายที่มียา sildenafil อยู่ 50 มิลลิกรัม (ระดับ therapeutic), กลุ่มที่ 2 ได้รับสารละลายที่มียา sildenafil 100 มิลลิกรัม (ระดับสูงกว่า therapeutic), กลุ่มที่ 3 และ 4 ได้รับน้ำเกลือ 100 มิลลิลิตรเข้าทางหลอดเลือดด้วยอัตราเร็ว 2 มิลลิลิตรต่อนาที จากนั้น NTG จะถูกให้ทางเส้นเลือดดำในกลุ่มที่ 1-3 ด้วยอัตรา 5 ไมโครกรัมต่อนาที โดยเพิ่มขนาดของ NTG 5 ไมโครกรัมต่อนาที ทุก ๆ 5 นาที จากนั้น DFT จะถูกวัดอีกครั้งหนึ่งหลังได้ NTG ไปแล้ว 20 นาที ผลการศึกษาพบว่าในกลุ่มที่ 1 นั้นค่า DFT หลังได้รับยาไม่แตกต่างจากค่า DFT ตอนเริ่มต้น ในกลุ่มที่ 2 พบว่าค่า DFT หลังได้รับยามีค่าสูงขึ้นกว่าค่า DFT ตั้งต้น ในกลุ่มที่ 3 และ 4 นั้น ค่า DFT ทั้ง 2 ไม่มีความแตกต่างกัน ดังนั้นจึงสรุปได้ว่า ยา sildenafil ที่ระดับ supratherapeutic ร่วมกับ NTG เพิ่มค่า DFT อย่างมีนัยสำคัญ ซึ่งผลต่อค่า DFT นี้นั้น น่าจะเกิดมาจากผลของ sildenafil ไม่ใช่มาจาก NTG

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Letter to the Editor

Fish oil does not improve defibrillation efficacy ☆

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1. Introduction

Sudden cardiac death, caused mainly by ventricular fibrillation (VF), is the major cause of death in many nations around the world [1]. Despite the use of an implantable cardioverter-defibrillator (ICD), the therapeutic goal is still far from ideal. Investigators have been investigating the means that could lower the shock strength required to successfully defibrillate, thus minimizing cellular damage in VF patients [2]. In the past decades, fish oil has been shown to be antiarrhythmic and decreases cardiovascular mortality [3,4]. However, its effect on the defibrillation efficacy has never been investigated.

The n-3 class of polyunsaturated fatty acids such as docosahexaenoic acid (DHA) has been shown to be related to a prevention of cardiac arrhythmias as well as a reduction in sudden cardiac death [3]. Previous studies have demonstrated that the occurrence of VF during ischemia and reperfusion can be prevented by the acute intravenous administration of DHA [5,6]. Ventricular arrhythmia threshold has also been raised by the application of fish oil [7]. Recently, we demonstrated that acute intravenous infusion of DHA could significantly decrease the upper limit of vulnerability shock, therefore reducing the VF induction window during the vulnerable period of a cardiac cycle [2]. Despite the increasing evidence of its antiarrhythmic effect, the role of fish oil on defibrillation efficacy has never been verified. In the present study, we sought to determine whether DHA can improve the defibrillation efficacy. The study was approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University. All animals were cared for according to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No.85–23, revised 1996).

Ten healthy pigs (20–30 kg) of either sex were anesthetized and maintained under physiologic conditions [8]. Catheters with a 34-mm and 68-mm platinum coated titanium coil electrodes (Guidant Corp.) were inserted into the RV apex and at the junction between right atrium and superior vena cava, respectively, and were used as defibrillation electrodes. The blood pressure, ECG, heart rate, plasma O_2 saturation, respiratory rate and $EtCO_2$ were monitored continuously throughout the entire study. In the study protocol, VF was induced by 50-Hz AC delivered via an electrode at the tip of the RV catheter. After 10 s of VF, defibrillation was attempted with biphasic truncated exponential shocks (Ventak ECD, Guidant Inc.). A minimum of 4 min was allowed to elapse between VF episodes or until the blood pressure and heart rate returned to normal values. If the shock failed to defibrillate, a rescue shock (20–30 J) was delivered within 10 s. The animals were euthanized by VF at the end of the study. The DFT was determined using a 3-reversal up/down protocol as described elsewhere [8]. Pigs were divided into 2 groups equally. In each pig, the control DFT was determined at the beginning of the study. Then, a solution contained 1.0 g of DHA (Sigma Chemical Co.) in 100 mL normal saline with 10 g purified delipidated bovine serum albumin (BSA) was infused intravenously within

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90 min in group 1 [6]. The DFT was determined again immediately after the end of DHA administration (DHA-DFT). To confirm that the BSA, which was used as a vehicle for the DHA, did not alter the DFT, a BSA of the same volume as used in the DHA administration in group 1 was infused intravenously within 90 min in group 2. Then, the DFT (BSA-DFT) was determined again after the end of BSA infusion. Comparisons of data (mean±standard deviation) were performed using a Student's paired *t*-test. Differences were considered significant when $P<0.05$.

In group 1, the control DFT delivered voltage was 421±94 V and the total energy was 15±7 J. The DHA-DFT was not different (416±92 V and 15±7 J), compared to the control DFT ($P=0.5$). The impedance (47±6 vs. 46±7 Ω) measured before and after DHA infusion was not different ($P=0.3$), indicating that it did not attribute to the unaltered DFT in this group. The mean systolic blood pressure (93±13 vs. 98±17 mmHg) and the heart rate (91±13 vs. 97±9 beats/min) recorded before and after DHA administration were not different ($P=0.7$). In group 2, the vehicle did not alter the DFT delivered voltage (341±37 vs. 345±42 V, $P=0.7$) and total energy (9±2 vs. 9±2 J, for control-DFT and BSA-DFT, respectively, $P=0.5$). The impedance (54±6 vs. 54±7 Ω, $P=0.8$), systolic blood pressure (95±10 vs. 92±14 mmHg, $P=0.5$) and heart rate (102±10 vs. 96±16 beats/min, $P=0.4$) measured before and after BSA infusion was not different.

Although a number of studies have demonstrated the potential antiarrhythmic benefits of fish oil, our present study indicates that acute intravenous administration of 1.0 g DHA cannot improve defibrillation efficacy. Previous studies have shown that administration of fish oil, either orally or intravenously, can raise the arrhythmia induction threshold [7], prevent the occurrence of VF [6], as well as decrease the upper limit of vulnerability shocks [2]. Our results suggest that despite its antiarrhythmic effects, fish oil could not help to improve the defibrillation efficacy by decreasing the DFT. To the best of our knowledge, this is the first study designed to specifically test the effect of fish oil on defibrillation. Our study has several limitations. First, the study was performed in normal hearts; therefore the results could differ if the study was performed in diseased hearts as well as in human. However, recent clinical study has also reported no beneficial effects of fish oil on the DFT [9]. Also, a single dose of 1.0-g DHA was tested in the present study. It is possible that higher concentration of DHA infusion could give a different result. Future studies with different DHA concentrations as well as defibrillation studies at the cellular level in normal and diseased hearts are needed to determine the effect of DHA on the DFT.

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EDITORIAL COMMENTARY

Preshock phase singularity and defibrillation outcome: Another piece to solve the jigsaw puzzle?

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Sudden cardiac death is a major cause of death in most industrialized nations around the world, and ventricular fibrillation (VF) is known to be mainly responsible for this fatality.[1] Despite the fact that VF has been known for over a century as a lethal culprit often perpetrating sudden cardiac death, electrical defibrillation is still the only known effective clinical therapy for this fatal arrhythmia.[2] In the past few decades, the use of an implantable cardioverter-defibrillator has been shown to decrease the mortality rate significantly in patients with prior myocardial infarction and depressed ejection fraction.[1] Together with the mortality reduction, our understanding on how the shock terminates VF has been much improved.[3] This is partly due to the development of investigating tools such as multichannel electrical and optical cardiac mapping as well as cardiac computer simulations that allow investigators to study electrical activity in the heart in two or three dimensions, which mainly contributes to the advancement in the field of defibrillation.[4–8] Despite the fact that many of the "defibrillation puzzle" pieces are in place, a few still remain elusive.[3] Why some shocks fail and others of the same strength succeed in effecting defibrillation is not well understood.

Reentry is known to maintain VF and give rise to reinitiation of VF after a failed defibrillation attempt.[6,9] When a shock fails to terminate VF, reentry has been seen to follow and be responsible for failed defibrillation.[6] Since a phase singularity (PS) represents the existence of reentry, the number of PSs has been thought to be associated with defibrillation outcome.[10,11]

Influence of shock strength on postshock PS and defibrillation outcome

Influence of shock strength on defibrillation has been extensively investigated, and its important role in postshock activation patterns was suggested.[3,10,11] Previous electrical mapping studies in dogs demonstrated that the earliest postshock activation appeared early (i.e., short isoelectric window) after weak shocks (well below the defibrillation threshold) and were associated with failed defibrillation, whereas strong defibrillation shocks had a long isoelectric window after the shock and were generally associated with successful defibrillation.[4,5] When shocks at a strength near the defibrillation threshold were used and failed to defibrillate, repetitive responses were observed before degenerating into VF.[7,12–15] In contrast, most optical mapping studies in isolated, perfused rabbit hearts reported that there was no isoelectric window after defibrillation shocks and that reentry was responsible for failed defibrillation.[6,16,17] The discrepancy among these findings has been proposed as largely due to different shock strengths used among studies, since the shock strength used in those optical mapping studies was frequently much weaker than the defibrillation threshold.[3,15]

The influence of shock strengths on activation patterns after defibrillation shocks has been strongly emphasized and supported by recent optical mapping reports in isolated pig hearts using shocks at various strengths from 100 to 900 V.[10,11] In that report,[10] it was demonstrated that (1) postshock interval or an isoelectric window did not exist at low strength shocks but existed and monotonically increased as shock strength increased; (2) the number of postshock PSs progressively decreased (and reached zero) as defibrillation shock strength increased, whereas the number of preshock PSs during VF did not differ among those shocks; and (3) low-strength shocks were not able to defibrillate as the number of postshock PSs was still high and reentry was observed as a postshock activation pattern for failed defibrillation. In that study, however, defibrillation only reached a 100% success rate at 900 V, even though postshock PSs were no longer observed from 600 to 900 V and only a focal pattern of epicardial activation was observed in failed defibrillation.[10] These findings indicate that shock strengths play a major role in determining the number of postshock PSs and postshock activation patterns; however, the number of postshock PSs did not critically determine defibrillation outcome.

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Postshock activation patterns and defibrillation outcome for near-DFT_{50} shocks

Although a number of studies have been carried out to investigate defibrillation mechanisms over the past decades, there are only a few that used a shock strength of around 50% defibrillation success (DFT_{50}) to investigate the relationship between myocardial responses to the shock and defibrillation outcome.[7,12–14] Since the nature of defibrillation is probabilistic and shock-strength dependent, investigators have been trying to find the determining factors by which DFT_{50} shocks of the same strength sometimes succeed while at other times they fail to defibrillate. Electrical and optical cardiac mapping studies in pigs have demonstrated that when the strength of the shock was kept constant at DFT_{50}, three types of defibrillation outcome, that is, successful defibrillation type A or type 1 (immediate resumption of sinus rhythm); successful type B or type 2 (a few repetitive responses before sinus rhythm); and failed defibrillation, could be observed.[7,12] At this DFT_{50}, repetitive responses similar to type B successful shocks were often observed after failed defibrillation.

In electrical mapping studies, investigated parameters immediately after the shock indicated no differences between type B success and failed defibrillation and that the first postshock activation between the two was almost indistinguishable, whereas successive cycles demonstrated divergent patterns.[7,13] In failed defibrillation episodes, the intercycle interval (i.e., an interval between onsets of successive cycles) was found to be shorter, whereas the wave front conduction time (i.e., time that each cycle takes to traverse the heart) was longer for the first five successive cycles after the shock, when compared with those in type B success. All of these findings suggest that electrophysiologic variables immediately after the shock might not be a crucial factor in determining the outcome of defibrillation. Instead, the number and rapidity of postshock cycles could be determining factors of defibrillation outcome for near-DFT_{50} shocks. However, electrical state at shock onset was not investigated in those studies.

In an optical mapping study using near-DFT_{50} shocks, similar findings to previous electrical mapping results were demonstrated.[12] However, the electrical state of the heart at the time of the shock was different between type B success and failed defibrillation. These findings indicated that the phase of VF action potentials at shock onset is crucial in determining defibrillation outcome. However, immediate myocardial responses to the shock (i.e., depolarization pattern of the first postshock cycle) were no different between type B success and failed defibrillation. These findings, again, could be interpreted to mean that the number and rapidity of postshock activations rather than cardiac state at the time of the shock may be important factors in determining defibrillation outcome at DFT_{50} shocks. In those studies, however, type A success episodes were often excluded from data analysis and the preshock PS was never investigated for its role in determining defibrillation outcome.

Preshock PS and defibrillation outcome for near-DFT_{50} shocks

In this issue of *Heart Rhythm*, Hayashi et al[18] reported for the first time an important finding concerning the relationship between preshock PS and defibrillation outcome at near-DFT_{50} shocks. They studied the process of ventricular defibrillation in isolated, perfused rabbit hearts using an optical mapping system that provides an elegant method to dynamically monitor electrophysiological activation and recovery of a significant portion of the ventricular heart muscle. They found that when shocks near the DFT were applied either early (10 seconds) or late (1 minutes) after VF was induced, the number of PSs present just before shocks was related to postshock activation dynamics. Their major findings that a low number of preshock PSs were associated with type A success reemphasize the importance of electrical states of myocardium at the time of the shock when determining defibrillation outcome. However, because of the complex nature of defibrillation, Hayashi et al found that the number of preshock PSs was no different between failed and type B successful defibrillation. This suggested that there are possibly parameters other than cardiac state at shock onset that determined defibrillation outcome for type B success and failed defibrillation.

In summary, the article by Hayashi et al[18] has helped to explain the discrepancy in previous findings in terms of shock strength influence, activation patterns and electrical state of myocardium at the time of the shock, and defibrillation outcome. Do the findings of the Hayashi et al study add a piece to the defibrillation puzzle? In a word, yes. Their results solidify further the notion that electrophysiological activity just before the shock importantly influences the postshock activation dynamics and defibrillation outcome. Their findings suggest the possibility that if these dynamics could be actively and reliably controlled in specific regions of the heart,[19–21] significant and clinically important reductions in DFT might be achieved.

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Letter to the Editor

Heart rate variability in β-thalassemic mice ☆

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Keywords: Thalassemia; Heart rate variability; Knockout mice

Heart rate variability (HRV) has been accepted as a new risk stratifier for cardiac complications in post-myocardial infarction and heart failure patients. However, there are only a few reports regarding HRV in thalassemia. Since genetically engineered thalassemic mice have been used to investigate thalassemic complication and therapeutic interventions [1], the characteristics of HRV in the β-thalassemic mice were investigated in the present study.

Although chelating therapy has been shown to improve the prognosis in β-thalassemia, sudden cardiac death and cardiac failure are still the major cause of death in these patients [2]. Early identification of patients who are at risk of developing cardiac complications is essential since better therapeutic approach such as intensify chelating therapy may be required to prevent mortality. It is known that basic parameters such as hemoglobin level cannot be used for this purpose, while traditional predictors such as left ventricular ejection fraction and history of arrhythmia are still not useful because of their low positive predictive value and low cost-effectiveness [3]. HRV has been proposed as a new risk stratifier in post-myocardial infarction and heart failure patients [4,5]. However, only a few studies investigated the role of HRV in thalassemia [6–8]. Using a small number of patients, Franzoni et al. [6] demonstrated that β-thalassemic patients had depressed HRV without clinical signs of cardiac functional involvement.

In the last decade, 'humanized' transgenic mice containing β-thalassemia mutations in the context of the human β-globin locus have been generated, which display hematological abnormalities at the phenotypic and genotypic level similar to thalassemia patients [9,10]. More recently Jamsai et al. reported the creation of the HbE/β-thalassemia mouse model [11]. This mouse model represents ~40% of β-thalassemia mutations in southern China and Thailand and is as an ideal *in vivo* model system for pathophysiological studies [12].

Although the genetic expression of HbE/β-thalassemia mouse is similar to that found in the human counterpart, the alteration of autonomic modulation in these thalassemic mice has never been investigated. Therefore, we evaluated whether the HRV is changed during the early stage of thalassemia in this mouse model. Thirteen wild-type mice, 13 double heterozygous HbE/β-thalassemia mice and 13 heterozygous β-globin knockout mice ($^{mu}\beta^{+/-}$) (mean age = 4 ± 1 months) were studied. Short-term ECG recording was performed in all conscious mice with ADInstruments Powerlab. Time and frequency domain HRV were evaluated using MATLAB 7.0, as previously described by Just et al. [13]. Hemoglobin level, the production of reactive oxygen species (ROS), and serum iron (SI) were also measured [14,15]. When compared to the wild type mice, all HRV parameters were depressed in the $^{mu}\beta^{+/-}$ group (Table 1). In double heterozygous HbE/β-thalassemia mice, all HRV parameters were also depressed except SDNN which was reduced but not reach statistical significant. The hemoglobin

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Table 1
HRV and basic measured parameters in wild-type, double heterozygous β/E and heterozygous β-thalassemic mice

Parameter	Wild-type (n=13)	Double Heterozygous HbE/β (n=13)	Heterozygous β-globin KO (n=13)
SDNN	5.6±4.3	3.1±2.3	1.8±0.7*
rMSSD	4.7±4.1	1.8±1.7*	1.0±0.4*
HFnu	40±8	24±10*	23± 6*
LFnu	59±8	76±10*	77± 6*
LF/HF	1.57±0.5	3.8±1.8*	3.8±1.6*
HR	643.8±51	670.5±46.9	682.1±34.1
Hb	16.4±1.6	15.0±2.4	9.0±1.6*†
SI	20.3±6.1	22.1±4.6	43.3±24.3*†
ROS	106.4±25.7	116.0±16.8	215.6±69.1*†

Values are expressed as (mean±SD). *p<0.01 compared with wild-type, †p<0.01 compared with double heterozygous ; SDNN: standard deviation of all RR intervals (msec); rMSSD: root mean square of successive difference of RR (ms); HFnu: normalized high frequency power; LFnu: normalized low frequency power; HR: mean heart rate (beat per minute); Hb: hemoglobin (g/dl); SI: Serum Iron (μM); ROS: Production of Reactive Oxygen Species (Fl Unit).

level was decreased only in the $^{mu}\beta^{+/-}$ group with increased SI and ROS production.

The depressed HRV, i.e. an expression of cardiac autonomic modulation, in $^{mu}\beta^{+/-}$ and double heterozygous HbE/β-thalassemia mice could be due to the effect of chronic anemia associated with a certain degree of iron overload that leads to oxidative damage, similar to that in thalassemic patients [6]. However, a reduced hemoglobin with increased SI and ROS production was found only in the $^{mu}\beta^{+/-}$ group. This finding raises the question about the causes of impaired autonomic modulation in double heterozygous HbE/β-thalassemia group. It is possible that depressed HRV may reflect an early physiologic change in this mouse model. Further investigations are required to validate the cause of these depressed HRV parameters.

In conclusion, the present study demonstrates depressed HRV in the $^{mu}\beta^{+/-}$ and double heterozygous HbE/β-thalassemia mice. Although the prognostic value of this depressed HRV still needs to be further investigated, these findings indicate that autonomic modulation in these thalassemic mice could well represent that in β-thalassemic patients, suggesting that these could be used to investigate cardiac complications as well as other pharmacological interventions in thalassemia.

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Effects of Sildenafil Citrate on Defibrillation Efficacy

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Effects of Sildenafil Citrate on Defibrillation Efficacy. ***Introduction:*** **Although fatal arrhythmia and sudden death have been reported in patients taking sildenafil citrate, its effect on defibrillation efficacy has not been investigated. The aim of this study was to test the hypothesis that sildenafil citrate increases the shock strength required to successfully defibrillate during ventricular fibrillation (VF).**

Methods and Results: **A total of 26 pigs (20–25 kg) were randomly assigned into three groups. In each group, the defibrillation threshold (DFT) was determined at the beginning of the study using a three-reversal up/down protocol. Each shock (RV-SVC, biphasic) was delivered after 10 seconds of VF. Group 1 ($n = 10$) received 50 mg and group 2 ($n = 10$) received 100 mg of sildenafil citrate intravenously at a rate of 2 mL/minute for 50 minutes. Group 3 ($n = 6$) received 100 mL of saline intravenously at the same rate as in group 1. The DFT was determined again after the drug (drug-DFT) and saline (saline-DFT) administration. For 100-mg sildenafil citrate infusion, the DFT (483 ± 39 V, 18 ± 3 J) was significantly ($P < 0.003$ and $P < 0.01$, respectively) higher than the control-DFT (407 ± 123 V, 13 ± 7 J). This sildenafil citrate infusion increased the DFT ~19% by voltage, and ~38% by total energy. After 50-mg sildenafil citrate infusion, the DFT (454 ± 28 V, 15 ± 2 J) was not different than the control DFT (449 ± 28 V, 15 ± 2 J). Saline infusion (391 ± 18 V, 12 ± 1 J) did not alter the control DFT (399 ± 22 V, 12 ± 1 J).**

Conclusion: **The 100-mg sildenafil citrate infusion, representing a supra-therapeutic plasma level, significantly increased the DFT. This finding indicates that VF occurring during supra-therapeutic sildenafil citrate treatment would require a stronger shock to successfully defibrillate.** *(J Cardiovasc Electrophysiol, Vol. 17, pp. 292-295, March 2006)*

sildenafil citrate, defibrillation, ventricular fibrillation, sudden cardiac death

Introduction

Sildenafil citrate is a highly selective inhibitor of cGMP-specific phosphodiesterase type 5 (PDE-5) and has been widely used for the treatment of erectile dysfunction.[1-5] Reports by the U.S. Food and Drug Administration (FDA) have shown the incidence of significant cardiovascular events, including sudden cardiac death, associated with the use of sildenafil citrate.[6] As of February 1999, the number of spontaneous deaths reported to the FDA among men who had taken sildenafil citrate was 401.[7] Of these cases, a total of 219 were presumed to be cardiac in origin, including myocardial infarction and sudden cardiac death caused by ventricular tachycardia/ventricular fibrillation (VT/VF).[7]

In the past few years, a number of studies have investigated the effects of sildenafil citrate on cardiac electrophysiological alterations that could cause VT/VF and sudden cardiac death.[8-10] A recent report by Swissa et al. indicated that administration of sildenafil citrate at a supra-therapeutic level combined with a nitric oxide donor could cause arrhythmia in isolated swine right ventricles.[10] However, the effect of this drug on defibrillation efficacy has not been reported to date.

The group of men who use sildenafil citrate for erectile dysfunction may also be at risk for sudden cardiac death caused mainly by VF. It is essential to know how the drug affects defibrillation efficacy. Furthermore, growing evidence suggests that sildenafil citrate is an effective agent for treating pulmonary arterial hypertension, leading to an increased use of this drug.[11-14] Therefore, the aim of this study was to test the hypothesis that sildenafil citrate increases the shock strength required to successfully defibrillate during VF.

Materials and Methods

Animal Preparation and Electrode Placement

Twenty-six healthy pigs (20–25 kg) of either sex were anesthetized and maintained under physiologic conditions as described previously.[15] Swine were chosen as a study model since they have been widely used to study defibrillation.[15-19] The study was approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University. All animals were cared for according to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). A catheter with a 34-mm platinum-coated titanium coil electrode (Guidant Inc.) was inserted into the right ventricular apex. A 68-mm electrode catheter was positioned at the junction between the right atrium and the superior vena cava. The position of the catheters was verified with fluoroscopy. Blood pressure, ECG, heart rate, plasma

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O_2 saturation, respiratory rate, and $EtCO_2$ were monitored continuously throughout the entire study.

Defibrillation Protocol

VF was induced by 60-Hz alternating current delivered via an electrode at the tip of the right ventricular catheter. After 10 seconds of VF, defibrillation was attempted with biphasic shocks (Ventak, Guidant Inc., MN) with electrodes at the right ventricular apex as cathode and at the superior vena cava as anode for the first phase. A minimum of 4 minutes were allowed to elapse between VF episodes. If the shock failed to defibrillate, a rescue shock (20–30 J) was delivered within 10 seconds.

DFT in each group was determined using a three-reversal up/down protocol.[18] Pigs were randomly assigned into three groups. In group 1 (n = 10), the DFT (control-DFT) was determined at the beginning of the study. After the control DFT was obtained, a 50-mg stock solution (0.5 mg/mL) of sildenafil citrate was administered intravenously at the rate of 2 mL/minute over 50 minutes.[20] The DFT (Drug-DFT) was determined again at the end of sildenafil citrate infusion. In group 2 (n = 10), a similar protocol as in group 1 was performed, except that a stock solution of 100-mg sildenafil citrate (1mg/mL) was administered at the same rate as in group 1. To confirm that an increase in plasma volume did not affect the DFT, a similar protocol as in group 1 was performed in group 3 (n = 6), except that saline (100 mL) was administered at the same rate as in group 1. The animal was euthanized by VF induction at the end of the study.

Drug Administration

Sildenafil citrate (Pfizer) in tablet form (50 and 100 mg) was dissolved in 100 mL of normal saline just before the experiment to form a stock solution. Two different concentrations of sildenafil citrate were injected intravenously at the rate of 2 mL/minute over 50 minutes. Intravenous administration of 50-mg sildenafil citrate has been shown to represent ∼100-mg sildenafil citrate taken orally, whereas 100-mg sildenafil citrate infusion has been shown to be a supra-therapeutic dosage (∼>200 mg taken orally).[1,20]

Statistical Analysis

Values are expressed as mean ± SD. Comparisons of data between the two DFT groups were performed using a paired *t*-test. $P < 0.05$ was considered statistically significant.

Results

The weight of the pigs used in this study was 24.3 ± 1 kg. The total number of shocks delivered to each animal was 14 ± 1. The average number of shocks delivered to the animal before the DFT was obtained was 7 ± 1 in the control group and 7 ± 1 in the sildenafil citrate and saline injected group.

In group 1, the delivered leading-edge voltage and total energy for the control DFT was 449 ± 28 V and 15 ± 2 J, respectively. Drug-DFT (454 ± 28 V, 15 ± 2 J) was not different than the control DFT (Table 1). The systolic blood pressure after drug infusion was significantly lower than the control systolic blood pressure ($P < 0.05$, Table 1). However, the heart rate before (102 ± 7 bpm) and after (110 ± 7 bpm) drug administration was not different ($P = 0.2$).

In group 2, the delivered leading-edge voltage and total energy for the control DFT was 407 ± 123 V and 13 ± 7 J, respectively. Drug-DFT (483 ± 39 V, 18 ± 3 J) was significantly higher than the control DFT (Table 1). A 100-mg sildenafil citrate infusion significantly increased the DFT ∼19% by leading-edge voltage and ∼38% by total energy. The systolic blood pressure after drug infusion was significantly lower than the control systolic blood pressure ($P < 0.05$, Table 1). The heart rate before (93 ± 19 bpm) and after (100 ± 7 bpm) drug administration was not different ($P = 0.2$).

In group 3, the delivered leading-edge voltage and total energy for the control DFT was 399 ± 22 V and 12 ± 1 J, respectively. Saline DFT (391 ± 18 V, 12 ± 1 J) was not different than the control DFT (Table 1). The systolic blood pressure before and after saline infusion was not different ($P = 0.1$, Table 1). The heart rate before (107 ± 4 bpm) and after (115 ± 4 bpm) drug administration was not different ($P = 0.1$).

Discussion

The major findings of the present study are as follows: (1) intravenous administration of 100-mg sildenafil citrate significantly increased the DFT; (2) infusion of 50-mg sildenafil citrate and saline did not change the DFT; and (3) both 50-mg and 100-mg sildenafil citrate administration significantly decreased the systolic blood pressure.

Sildenafil Citrate and Defibrillation Efficacy

In the past few years, cardiac electrophysiological effects of sildenafil citrate have been investigated extensively.[8,21-26]

TABLE 1

Defibrillation Threshold (DFT) and Hemodynamic Parameters Measured Before and After Drug or Saline Administration

	Group I		Group II		Group III	
Parameters	**Control**	**50-mg Sildenafil**	**Control**	**100-mg Sildenafil**	**Control**	**100-mL Saline**
Delivered voltage (volts)	449 ± 28	454 ± 28	407 ± 123	483 ± 39*	399 ± 22	391 ± 18
Total energy (joules)	15 ± 2	15 ± 2	13 ± 7	18 ± 3†	12 ± 1	12 ± 1
Impedance (ohm)	58 ± 2	56 ± 2	59 ± 6	57 ± 2	62 ± 2	60 ± 2
Pulse width (msec)	15 ± 1	15 ± 1	15 ± 3	14 ± 1	16 ± 1	16 ± 1
Systolic BP (mmHg)	87 ± 5	73 ± 3‡	98 ± 16	85 ± 3‡	84 ± 4	94 ± 5
Heart rate (bpm)	102 ± 7	110 ± 7	93 ± 19	100 ± 7	107 ± 4	115 ± 4

*$P < 0.003$ compared to control.
†$P < 0.01$ compared to control.
‡$P < 0.05$ compared to control.

These effects include its ability to decrease the threshold of ventricular tachyarrhythmias and prolong cardiac repolarization.[10] However, its effect on the defibrillation efficacy has never been reported. To the best of our knowledge, the present study is the first to investigate the effect of sildenafil citrate on defibrillation efficacy. Our results demonstrate that intravenous administration of 50-mg sildenafil citrate (∼ 100 mg taken orally[20]) does not affect the DFT. However, 100-mg sildenafil citrate administered intravenously (∼>200 mg taken orally[20]) significantly increased the DFT. These findings suggest that a dose-dependent effect on DFT exists, in which the administration of the drug at a supra-therapeutic dosage, but not at a therapeutic dosage, increases DFT.

Supra-therapeutic concentrations of sildenafil citrate have been shown to have harmful effects in patients with conditions such as impaired drug elimination or drug overdose.[27] A recent study using isolated swine right ventricles demonstrated that the threshold for ventricular tachyarrhythmia was decreased with the administration of a high-dose sildenafil citrate combined with a nitric oxide donor.[10] Although Geelen et al.[8] have shown that sildenafil citrate prolongs cardiac repolarization by blocking the rapid component of the delayed rectifier potassium current in HERG-transfected HEK293 cells, a recent study performed by Chiang et al.[28] has shown differently. They demonstrated that sildenafil at a therapeutic concentration does not block I_{Kr} and I_{Ks} in guinea pig ventricular myocytes, nor does it prolong cardiac repolarization in guinea pig papillary muscles and canine Purkinje fibers. They also found that sildenafil citrate blocks $I_{Ca,L}$ in a dose-dependent manner.[28]

In the present study, the increase in DFT after supra-therapeutic sildenafil citrate administration could be due to its effects on cellular electrophysiological alterations in cardiomyocytes. Sildenafil citrate is known to inhibit PDE-5, causing a net increase in intracellular cGMP concentrations.[1,29] Musialek et al.[30] demonstrated that an increase in cellular cGMP could stimulate the pacemaker current, which may promote an "automatic" tachycardia.[30] Moreover, sildenafil citrate at supra-therapeutic concentration blocks $I_{Ca,L.}$[28] These electrophysiological changes could be responsible for an increase in the DFT found in this study.[28,30]

Effects of Sildenafil Citrate on Arterial Blood Pressure and Heart Rate

Sildenafil citrate belongs to a class of compounds called PDE inhibitors. PDEs comprise a diverse family of enzymes that hydrolyze cyclic nucleotides (cAMP and cGMP) and therefore play a critical role in the modulation of second messenger signaling pathways.[31] It has been shown that the blood pressure-lowering effects of sildenafil citrate are dependent on the degree of drug-activated nitric oxide-guanylate cyclase pathway.[27,32] In the present study, both 50-mg and 100-mg sildenafil citrate administration significantly decreased the systolic blood pressure (∼10 mmHg reduction). The heart rate after 50-mg and 100-mg sildenafil citrate administration was slightly increased but did not reach statistical significance ($P = 0.2$). These results of the hemodynamic effects of sildenafil citrate are consistent with previous reports.[33]

Conclusion and Clinical Implications

Sildenafil citrate significantly increased DFT (∼38% by energy) at a concentration above the therapeutic level. Since supra-therapeutic plasma level of the drug may be seen clinically after drug overdose or in the presence of impaired drug elimination,[9] this finding indicates that VF occurring during sildenafil citrate treatment in patients with impaired drug elimination such as hepatic or renal insufficiency, as well as in patients with pulmonary hypertension, may require a much stronger shock to successfully defibrillate. Therefore, care should be taken when prescribing sildenafil citrate to patients with cardiac disease, as well as in patients at risk for impaired drug elimination.[5,34] This finding also suggests that a high-energy implantable defibrillator could be useful in this group of patients.

Study Limitations

The present study demonstrated an increase in the DFT after intravenous administration of a supra-therapeutic plasma concentration of sildenafil citrate using a large animal model to approximate human heart size. However, this study was performed in normal pig hearts, and the results may differ in diseased hearts or in different species. Moreover, the pharmacokinetics and metabolism of this drug varies substantially between species. Therefore, the concentration of the drug used in this study may not represent the plasma level of the drug when used in humans. Also, the definite mechanistic effect of the drug on the electrophysiology of the heart during defibrillation was not elucidated. Future studies are needed to investigate the electrophysiologic effects of the drug during VF as well as after the defibrillation shock. Despite these limitations, we believe that the finding in the present study will initiate further investigations regarding the mechanistic effects of sildenafil citrate on defibrillation. Optical and electrical cardiac mapping studies could be the next step to better understanding how supra-therapeutic dosages of the drug increased the DFT. Defibrillation studies at the cellular level are also necessary to provide direct evidence elucidating the mechanism by which sildenafil citrate increases the DFT.

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Tabernaemontana divaricata extract inhibits neuronal acetylcholinesterase activity in rats

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Abstract

The current pharmacotherapy for Alzheimer's disease (AD) is the use of acetylcholinesterase inhibitors (AChE-Is). A previous *in vitro* study showed that *Tabernaemontana divaricata* extract (TDE) can inhibit AChE activity. However, neither the AChE inhibitory effects nor the effect on neuronal activity of TDE has been investigated *in vivo*. To determine those effects of TDE in animal models, the Ellman's colorimetric method was implemented to investigate the cortical and circulating cholinesterase (ChE) activity, and Fos expression was used to determine the neuronal activity in the cerebral cortex, following acute administration of TDE with various doses (250, 500 and 1000 mg/kg) and at different time points. All doses of TDE 2 h after a single administration significantly inhibited cortical AChE activity and enhanced neuronal activity in the cerebral cortex. The enhancement of Fos expression and AChE inhibitory effects in the cerebral cortex among the three TDE-treated groups was not significantly different. A 2 h interval following all doses of TDE administration had no effect on circulating ChE activity. However, TDE significantly inhibited circulating AChE 10, 30 and 60 min after administration. Our findings suggest that TDE is a reversible AChE-I and could be beneficial as a novel therapeutic agent for AD.

Keywords: Acetylcholinesterase inhibitor; Cerebral cortex; Fos

1. Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease, having many cognitive and neuropsychiatric manifestations that result in progressive disability. Neuropathologically, AD is characterized by the appearance of neuritic plaques and neurofibrillary tangles in the brain. The cholinergic system has been shown as the predominantly affected neurotransmitter system in this disease (Giacobini, 2003a). The degeneration of cholinergic afferents to the cerebral cortex from the medial septum is one hallmark of AD (Coyle et al., 1983). In fact, the severity of cognitive decline is correlated with cholinergic impairment (Perry et al., 1978, 1999). Currently, the most effective available pharmacotherapy for AD is the use of acetylcholinesterase inhibitors (AChE-Is), which indirectly elevate the attenuated acetylcholine concentrations in the AD-affected brain, thereby enhancing cholinergic function (Bickel et al., 1991; Barnes et al., 2000; Disterhoft and Matthew, 2003; Giacobini, 2003b; Liston et al., 2004). Although the use of AChE-Is (e.g. donezepil, rivastigmine and galantamine), a symptomatic pharmacological treatment of AD, has been shown as beneficial to cognitive, functional and behavioral symptoms of the disease, it also causes undesired side effects (Sweeney et al., 1989; Bickel et al., 1991; Woodruff-Pak et al., 2001; Zarotsky et al., 2003; Liston et al., 2004). The most common adverse effects, related to cholinergic stimulation in the brain and peripheral tissues, include gastrointestinal, cardiorespiratory, extrapyramidal, genitourinary, and musculoskeletal symptoms, as well as sleep disturbances (Thompson et al., 2004). Therefore, the search for

Abbreviations: AChE, acetylcholinesterase; ChE, cholinesterase; TDE, *Tabernaemontana divaricata* extract

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new AChE-Is, particularly from natural products, with higher efficacy and fewer side effects has been extensively investigated.

Tabernaemontana divaricata (L.) is a common garden plant in Southeast Asia and other tropical countries. It has been reported as a rich source of various alkaloids, with various pharmacological properties (van Beek et al., 1984). It has been used in traditional rejuvenation remedies in Thailand (Ingkaninan et al., 2003). In Thai herbal medicine, these remedies are believed to improve memory. In addition, native people in America, Africa and Continental Asia have used this plant as a central nervous system stimulant (Taesotikul et al., 1998). Despite its long-time use, there have been very few scientific studies to explain how *Tabernaemontana divaricata* can improve memory. Recently, Ingkaninan et al. (2003) demonstrated that methanolic extracts of *Tabernaemontana divaricata* (0.1 mg/ml) inhibited more than 90% of AChE activity in their *in vitro* study. However, neither its effects on AChE inhibition nor its neuronal activity enhancement in the cerebral cortex, a brain region critical for learning and memory, have ever been investigated in an *in vivo* model. Therefore, in this study, the hypotheses that acute administration of *Tabernaemontana divaricata* extract (TDE) can (1) inhibit the activity of circulating and cortical acetylcholinesterase (AChE) as well as butyrylcholinesterase (BuChE), and (2) enhance neuronal activity in the cerebral cortex, were tested. To the best of our knowledge, this is the first study investigating the above effects of TDE in rats.

2. Materials and methods

2.1. Plant materials

Tabernaemontana divaricata was collected from Phitsanulok, Thailand. The voucher specimen (collection no. Changwijit 0020) was deposited at the PBM herbarium, Faculty of Pharmaceutical Sciences, Mahidol University, Thailand.

2.2. Extract of Tabernaemontana divaricata

Roots of *Tabernaemontana divaricata* were separated from the whole plants and dried at 55 °C. The dried materials were ground, macerated with 95% ethanol twice (for 3 and 7 days) and dried by evaporating the ethanol extracts under a reduced pressure. The yield of the ethanolic extracts from roots of *Tabernaemontana divaricata* was 9.16% of dried materials. To confirm the quality of TDE in each experiment, each lot of TDE was microplate assayed for AChE and BuChE activity *in vitro* prior to its use in animals.

2.3. In vitro analysis for AChE and BuChE activity

The assay for measuring ChE activity was modified from the assay described by Ellman et al. (1961) and Ingkaninan et al. (2003). Briefly, 125 μl of 3 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 25 μl of 15 mM acetylthiocholine iodide (ATCI) for AChE activity or butyrylthiocholine idoide (BTCI) for BuChE activity, 50 μl of buffer, and 25 μl of sample dissolved in buffer containing not more than 10% methanol were added to microplate wells, followed by 25 μl of 0.28 U/ml AChE. The microplate was then read at 405 nm every 5 s for 2 min by a CERES UV 900C microplate reader (Bio-Tek instrument, USA). The velocities of the reactions were measured. Enzyme activity was calculated as a percentage of the velocities compared to that of the blank sample. Inhibitory activity was calculated by subtracting the percentage of enzyme activity from 100%. Every experiment was done in triplicate. Stock solutions of samples in Trisma–hydrochloride (Tris–HCl) buffer containing not more than 10% methanol were diluted serially with Tris–HCl buffer to obtain eight or nine different concentrations. The concentration of TDE and galantamine required to inhibit 50% enzymatic activity (IC_{50}) were determined in triplicated by using the software package Prism (Graph Pad Inc., San Diego, USA).

2.4. In vivo study protocol

A total of 136 male Wistar rats, weighing 100–150 g, were purchased from the National Animal Center, Salaya campus, Mahidol University, Bangkok, Thailand. All rats were housed, two or three per cage, with free access to food and water for at least 1 week prior to the study. The research protocol adhered to the "Guide for the Care and Use of Animals in compliance with the National Institute of Health guideline for the care and treatment of animals" and followed the appropriate Faculty of Medicine, Chiang Mai University, Standard Operating Procedure for animal identification (approved protocol number 07/2547), housing and diet before and during the experiments.

For the study of ChE activity, 112 rats were divided into 16 groups ($n = 7$ per group). Each group was injected intraperitoneally with one of the following doses of TDE dissolved in ethanol (EtOH): 0, 250, 500 or 1000 mg/kg. The animals administered with each TDE dose were divided into four sets. Each set was sacrified 10, 30, 60 or 120 min following TDE injection intraperitoneally. The minimum dose of TDE used in this study was 250 mg/kg, since this dose was half of the lowest dose (500 mg/kg) reported to affect parasympathetic activity, such as vasodilatation or mild respiratory depression in animal models (Taesotikul et al., 1989). The intraperitoneal lethal doses of the ethanolic extracts from roots was 2000 mg/kg, in which the signs and symptoms observed after this lethal dose were paralysis of skeletal muscles, irregular breathing, cyanosis, asphyxia, tremor, clonic convulsion, coma and death within 8–25 min after injection (Taesotikul et al., 1989). No animals in this study showed any signs and symptoms while receiving any doses of TDE. For the positive control of TDE on ChE activity, eight rats were divided into two groups ($n = 4$ per group). Each group was injected intraperitoneally with either 0 or 10 mg/kg of galantamine dissolved in saline. These eight animals were sacrificed 120 min following galantamine injection.

For the study of neuronal activity in the cerebral cortex, the Fos immunohistochemistry technique was used. In this study, rats were divided into four groups ($n = 6$ per group). Each group was injected intraperitoneally with one of the following doses of TDE dissolved in ethanol (EtOH); 250 mg/kg (group 1), 500 mg/kg (group 2), or 1000 mg/kg (group 3). Control animals (0 mg/kg TDE, group 4) were intraperitoneally injected

with EtOH alone. For the positive control, the other two sets of animals were used. Each group ($n = 2$ per group) was injected intraperitoneally with either 0 or 10 mg/kg galantamine dissolved in saline. Since Fos protein exhibits maximal expression after 2 h of stimulation, each rat was sacrified 2 h after intraperitoneal TDE injection for the Fos immunohistochemistry study.

2.5. ChE activity determinations in blood and cortical samples

Determination of ChE activity was based on the colorimetric method originally described by Ellman et al. (1961), and adapted for determining the enzyme activity in rat blood and supernatants of cortical homogenates.

All animals were euthanized with pentobarbital (50 mg/kg, i.p.) and then blood samples from the heart were collected before the animals were intracardially perfused with normal saline. Cortical tissues were separately dissected and rapidly removed.

The blood was centrifuged for 6 min at 17,000 rpm at room temperature to separate red blood cells (RBC) and serum. Both RBC and serum samples were immediately measured for ChE activity.

The fresh cortical tissues were weighed and then homogenized in 10 parts of 0.1 M phosphate buffer pH 7.4, which contained 1% Triton-X 100. Following centrifugation at 15,000 rpm for 15 min at 4 °C, the clear supernatants were removed and served as the enzyme source. AChE activity was determined in 50 μl aliquots of RBC or the cerebral supernatants (run as duplicates). The reaction was started by adding 0.25 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DNTB) and either (1) 0.5 mM acetylthiocholine-iodide (ATCI), the commonly used substrate for *in vitro* AChE determinations (Ellman et al., 1961) or (2) 0.5 mM butyrylthiocholine iodide (BTCI), the commonly used substrate for *in vitro* BuChE determinations, each combination dissolved in phosphate buffer pH 7.4 (0.1 M). The plate was then immediately placed into an automatic microplate reader and the yellow reaction product was quantified at 22 °C using a wavelength of 405 nm. The reaction was monitored over a period of 10 min with readouts taken every 10 s. The reaction was then processed by a program controlled by the plate reader. Data were stored in a computer. Quantification of the enzymatic activity was based on a change in optical density in the linear range over time, using the molar extinction coefficient of the reaction products. The spectrophotometric absorption was quantitatively measured and expressed as nmol acetylcholine hydrolysed/min/ml RBC or /mg cortical tissue and nanomole butyrylcholine hydrolysed/min/ml plasma or /mg cortical tissue. All chemicals used in this study were purchased from Sigma–Aldrich Co. (St. Louis, MO).

2.6. Fos immunohistochemistry

To address the possibility that TDE enhanced neuronal activity, the expression of Fos proteins in cortical neurons following acute TDE administration was determined. Fos is a cellular marker in which the pattern of its staining can provide clues about the involvement of specific neuronal populations as being involved in learning-associated signal processing or having been activated by external stimuli (Marcus et al., 1998; Aggleton and Pearce, 2001; Bozon et al., 2002). Therefore, if neurons are activated via TDE, Fos-positive neurons in brain tissues, particularly the cerebral cortex, following TDE administration should be observed.

In this study, each animal was deeply anesthetized 2 h after TDE and galantamine injections with pentobarbital (80 mg/kg, intraperitoneally) and perfused intracardially with phosphate buffer saline, followed by 4% paraformaldehyde. The whole brain was removed, post-fixed and placed in 30% sucrose overnight and then frozen and cut into 40 μm-thick, transverse frozen sections. Every section was processed for Fos, using a rabbit polyclonal antibody (Santa Cruz, 1:1,000 dilution). A black reaction product was produced with a standard ABC reaction (Vectastatin Elite kit; Vector Labs) with nickel intensification using Vectastain, and the sections were mounted on slides. Processing for Fos was similar to that described previously (Chattipakorn et al., 1999).

2.7. Data analysis

For Fos immunohistochemistry, changes in the number of Fos-positive cells in the cortical regions between different treatment groups were counted using a double-blinded technique as described in Chattipakorn et al. (1999). Data from Fos immunohistochemistry and ChE activity were expressed as mean ± S.E. Statistical analyses were carried out using parametric analysis (ANOVA) and Fisher's L.S.D. test for post hoc testing to calculate the significance for both TDE-treated and control rats. Significance was set at $P < 0.05$.

3. Results

The IC_{50} of TDE for AChE and BuChE activity was 2.56 ± 0.37 and 76.95 ± 0.11 mg/l, respectively. The AChE inhibitory effect of TDE from the microplate assay was less potent than that of galantamine (IC_{50} for AChE and BuChE: 0.22 ± 0.04 and 23.96 ± 9.03 mg/l) (Fig. 1). These findings confirm those of a previous report (Ingkaninan et al., 2003) and suggest that TDE is a ChE inhibitor, but it inhibited AChE activity more than BuChE activity in this *in vitro* study.

The percentage inhibition of AChE activity in the cerebral cortex compared to that in the control was $17.4 \pm 6.3\%$, $22.7 \pm 6.9\%$ and $16.6 \pm 5.0\%$ for 250, 500, and 1000 mg/kg TDE, respectively, 2 h after intraperitoneal administration. These levels of AChE activity in the TDE groups were significantly different from those in the controls ($P < 0.05$, Fig. 2). No difference was found among these TDE-treated groups ($P = 0.39$, Fig. 2). In contrast to AChE activity, the % BuChE inhibitory effects in the cerebral cortex of all three TDE-treated groups were $12.3 \pm 5.3\%$, $15.2 \pm 5.2\%$ and $8.6 \pm 4.4\%$ for 250, 500, and 1000 mg/kg TDE, respectively, 2 h after intraperitoneal administration (Fig. 2). BuChE activities in the cerebral cortex from all three TDE-treated groups were not significantly different from those in the controls ($P = 0.1$). In the positive control study, it was found that galantamine, 2 h after the acute

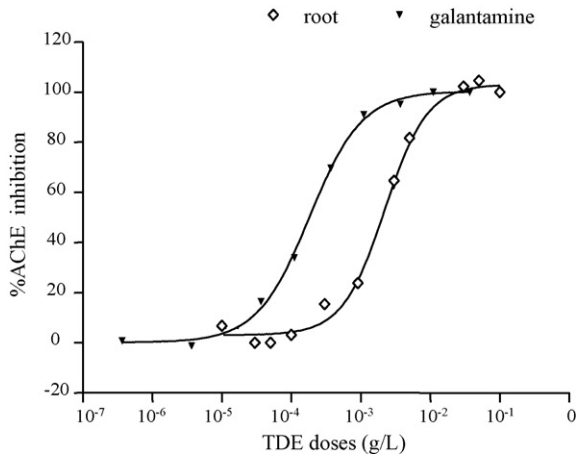

Fig. 1. The dose response curve of % AChE inhibition from roots of *Tabernaemontana divaricata* extract (◊) in comparison with that of galantamine (▼). Values are the mean of one typical experiment performed in triplicate.

single intraperitoneal administration of 10 mg/kg, could inhibit the activity of cortical AChE ($28 \pm 6\%$) and BuChE ($0.6 \pm 4\%$) when compared to the activity seen in the control (0 mg/kg galantamine). Our study used 10 mg/kg of galantamine as a positive control for TDE administration because this dose had been previously reported as having AChE inhibitory effects in mouse forebrain (Bores et al., 1996). The percentage of AChE and BuChE inhibitory activities in cortical tissues 2 h after galantamine injection was similar to that of the TDE administration. The different time points in cortical AChE and BuChE inhibitory activities following TDE administration were investigated further in this study. No significant differences in cortical AChE and BuChE activity were found between all doses of TDE-treated groups and the control group at 10, 30 and 60 min after TDE administration ($P > 0.1$, Figs. 3 and 4).

In contrast to the findings in cortical tissue, TDE had no effect on ChE activity in the circulation, either in erythrocyte AChE or plasma BuChE activities 2 h after TDE administration (Fig. 5). The percentage inhibition of circulating AChE and BuChE activities 2 h after 10 mg/kg-galantamine administration intraperitoneally was $2.6 \pm 1\%$ and $5 \pm 1\%$, respectively. The circulating ChE activity after galantamine administration was not significantly different from that in the control ($P = 0.063$). The results of this study are consistent with those of a previous report, which demonstrated that the AChE inhibitory effects of galantamine in the circulation were insignificant 2 h after administration (Bores et al., 1996). These findings suggest that TDE may be a short-acting and reversible agent in inhibiting ChE activity in the circulation, similar to galantamine. To investigate that possibility, this study also analyzed the erythrocyte AChE or plasma BuChE activities at 10, 30, and 60 min after TDE administration. We found that the circulating AChE activity significantly decreased at those time points after TDE administration when compared to the control ($P < 0.05$). The mean percentage of circulating AChE inhibition at all three time points

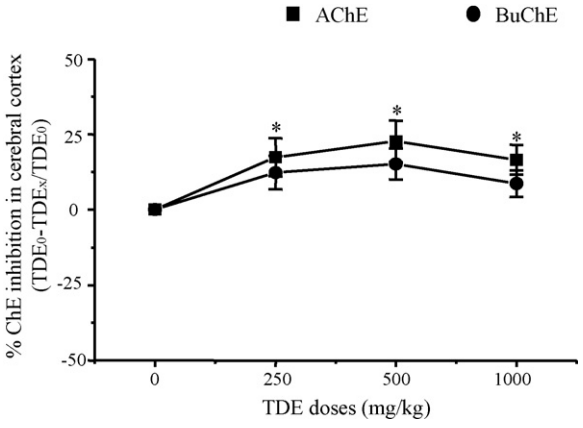

Fig. 2. Normalized % cortical ChE inhibition levels in rats (cortical ChE activity of $\{(TDE_0 - TDE_x/TDE_0) \times 100\}$) 2 h after intraperitoneal injection of various doses of TDE (TDE_x). TDE_0 represents the control group (0 mg/kg of TDE). Each data point represents mean ± S.E. AChE: acetylcholinesterase; BuChE: butyrylcholinesterase, ChE: cholinesterase. $^{*}p < 0.05$ compared to the control group (0 mg/kg of TDE = TDE_0).

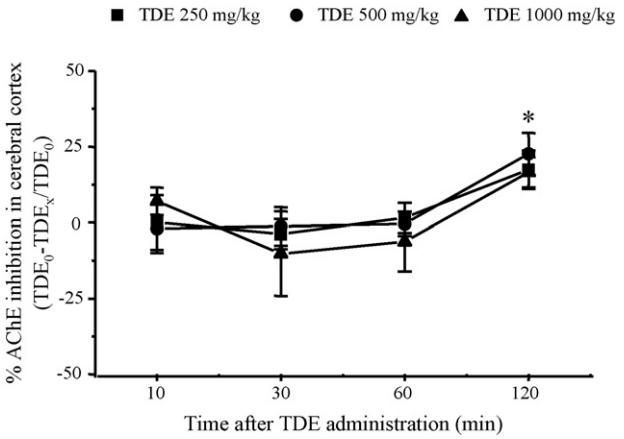

Fig. 3. Normalized % cortical AChE inhibition levels in rats (cortical AChE activity of $\{(TDE_0 - TDE_x/TDE_0) \times 100\}$) at different time points after intraperitoneal injection of various doses of TDE (TDE_x: 250, 500, 1000 mg/kg). TDE_0 represents the control group (0 mg/kg of TDE). Each data point represents mean ± S.E. AChE: acetylcholinesterase. $^{*}p < 0.05$ compared to the control group (0 mg/kg of TDE = TDE_0).

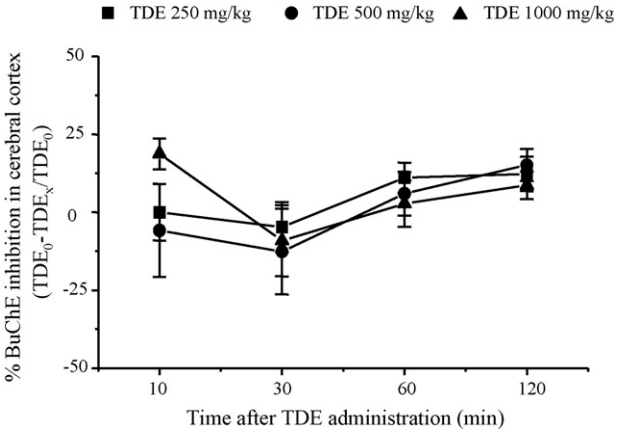

Fig. 4. Normalized % cortical BuChE inhibition levels in rats (cortical BuChE activity of $\{(TDE_0 - TDE_x/TDE_0) \times 100\}$) at different time points after intraperitoneal injection of various doses of TDE (TDE_x: 250, 500, 1000 mg/kg). TDE_0 represents the control group (0 mg/kg of TDE). Each data point represents mean ± S.E. BuChE: butyrylcholinesterase.

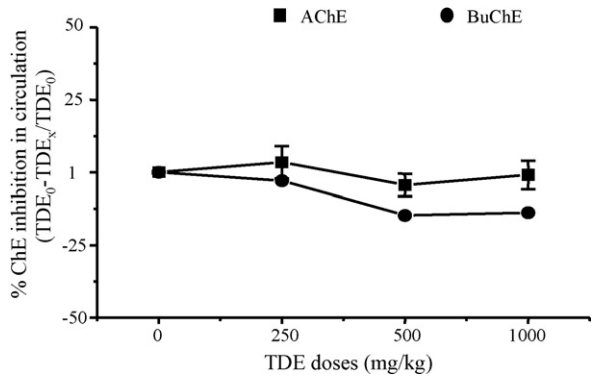

Fig. 5. Normalized % circulating ChE inhibition levels in rats (circulating ChE activity of $\{(TDE_0 - TDE_x/TDE_0) \times 100\}$) 2 h after intraperitoneal injection of various doses of TDE (TDE_x). TDE_0 represents the control group (0 mg/kg of TDE). Each data point represents mean ± S.E. AChE: acetylcholinesterase, BuChE: butyrylcholinesterase.

were in the range of 7–25%. Our findings indicate that TDE is a reversibly selective AChE inhibitor.

In this study, it was demonstrated that TDE could induce Fos-like immunoreactivity (FLI) in the nuclei of the cerebral cortex 2 h after TDE administration. No FLI-positive neurons were found in the cortical sections, in which the primary antibody was omitted (Fig. 6a). The FLI-positive neurons were scattered all over the cerebral cortex in all TDE-treated groups (Fig. 6b–e). The number of FLI-positive neurons per animal in the cerebral cortex was 101 ± 14, 124 ± 19 and 108 ± 22 in the groups administered intraperitoneally with 250, 500 and 1000 mg/kg of TDE, respectively. The numbers of cortical FLI-positive neurons in all three TDE-treated groups were greater ($P < 0.05$) than those in the control group (49 ± 9, Fig. 7). However, FLI-positive neurons in the cerebral cortex among the three TDE-treated groups were not significantly different ($P = 0.1$). Furthermore, galantamine (10 mg/kg, intraperitoneal injection; Fig. 6g) induced greater FLI in the cerebral cortex than in the control (NSS, intraperitoneal injection; Fig. 6f). These results suggest that TDE can enhance neuronal activity in the cerebral cortex similarly to galantamine (an AChE inhibitor) and that its enhancement of neuronal activity is dose-independent for the doses used in this study.

4. Discussion

The major finding of this study was that TDE can inhibit neuronal AChE activity in an animal model, as it does *in vitro*. This study also demonstrated, for the first time, that TDE has cortical AChE inhibitory effects and can enhance neuronal activity in an animal model.

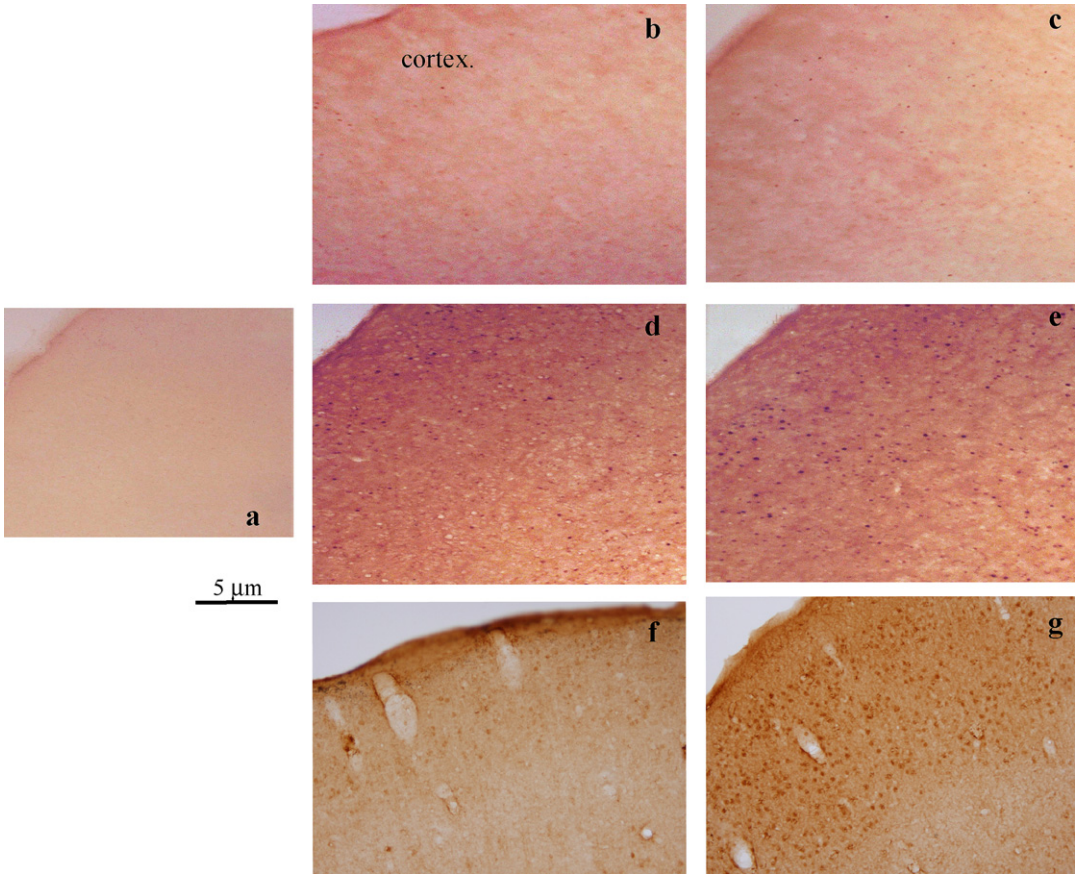

Fig. 6. Representations of Fos-positive neurons in the cerebral cortex 2 h after intraperitoneal injection of various doses of TDE. (a) Negative control represents the cortical tissue from the 1000 mg/kg TDE-treated group with the primary Fos antibody omission. (b–e) Representation of the cortical tissue 2 h after intraperitoneal TDE injection (0 mg/kg (b), 250 mg/kg (c), 500 mg/kg (d) and 1000 mg/kg (e)). (f–g) Representation of the cortical tissue 2 h after intraperitoneal galantamine injection (0 mg/kg (f), 10 mg/kg (g)).

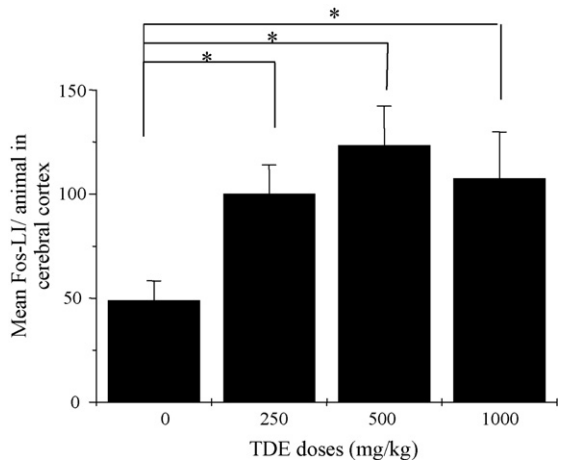

Fig. 7. Mean Fos-positive neurons per animal in the cerebral cortex 2 h after intraperitoneal injection of various doses of TDE ($n = 6$ per group). $^{*}p < 0.05$ compared to the control group (0 mg/kg of TDE).

Our finding demonstrated that TDE could inhibit cortical AChE activity in a dose-independent manner 2 h after administration, but had no effect on cortical BuChE at that time point. These results are consistent with the study's *in vitro* finding, in which TDE was shown to inhibit AChE activity more than BuChE activity. These findings suggest that TDE may be a selective AChE-I similar to donepezil and galantamine (Ballard, 2002). In contrast to the *in vitro* study, in which the AChE inhibitory effect of TDE was ten times less than that of galantamine, it is important to note that the cortical AChE inhibitory effect in the *in vivo* study, 2 h after TDE administration, was the same as that of galantamine. The percentage inhibition of cortical AChE activity following galantamine administration in our study is consistent with that in reports from previous animal studies (Bores et al., 1996; Geerts et al., 2005). Therefore, the differences between *in vitro* and *in vivo* results may possibly be due to those variations in the bioavailability of active compounds in TDE and galantamine in animal models.

We also found that TDE could inhibit neuronal AChE activity about 20%, suggesting that TDE may not be a potent AChE-I compared to others commercially available AChE-Is (donepezil, tacrine, rivastigmine) (Kosasa et al., 1999). Kosasa and colleagues reported that the percentage inhibition of neuronal AChE activity in an *ex vivo* study in rats was 30–46%. Their finding was detected by using microdialysis and high performance liquid chromatography (HPLC) with electrochemical detection of neuronal AChE activity 0.5–2 h after a single administration of several AChE-Is, such as 2.5 mg/kg of donepezil, 10 mg/kg of tacrine and 2.5 mg/kg of rivastigmine. Therefore, the low potency of TDE in the neuronal AChE inhibition from this study might have been for several reasons. First, the method we used in the analysis of ChE activity in the brain was less sensitive in detecting AChE activity than the microdialysis and HPLC method. Second, the TDE was in the form of a crude extract, which, naturally, contained all of the alkaloids of TDE. The pharmacological properties of these alkaloids have been previously identified. Although several alkaloids may act as AChE-Is, some may not (van Beek et al., 1984). Thus, the inhibitory effect of TDE that results from mixed-compounds acting as AChE-Is may be less potent than specific therapeutic drugs for Alzheimer's disease. Finally, a consideration in the use of several medications in the treatment of neurodegenerative disease is the capacity of the drug to pass the blood–brain barrier (BBB). Our findings show that TDE has an inhibitory effect on neuronal AChE activity, suggesting that it can cross the BBB. However, the neuronal AChE inhibitory effect of TDE could not be detected until 2 h after administration. This suggests that the efficiency of TDE in crossing the BBB may be low. The efficiency of TDE in crossing the BBB is neither known nor has it been compared with available AChE-Is. It is possible that TDE may have a lower efficacy in crossing the BBB compared to other inhibitors, causing the AChE activity measured in this study to be less than those observed in other studies. Future studies on the inhibitory effect of purified alkaloids from TDE on AChE activity, as well as the efficacy of TDE in passing the BBB, will be needed to prove this hypothesis.

There are several possible active compounds in TDE that could play a role as AChE-Is. The *Tabernaemontana divaricata* specimens used in this study were in the form of crude extracts. These crude extracts consisted of at least forty-four alkaloids (Rastogi, 1980; Sharma and Cordell, 1988; Van Der Heijden, 1989), and non-alkaloid constituents; such as triterpenoids (Rastogi, 1980; Sharma and Cordell, 1988; Van Der Heijden, 1989), steroids (Sharma and Cordell, 1988; Dagino, 1991), flavonoids (Henriques et al., 1996), phenyl propanoids (Henriques et al., 1996) and phenolic acids (Henriques et al., 1996). Previous studies have shown that several alkaloids in TDE, such as coronaridine (Andrade et al., 2005), voacangine (Andrade et al., 2005) and isovoacristine (Raymond-Hamet, 1962), have anti-AChE activity *in vitro*. Therefore, the inhibitory effects of AChE activity in our animal model could be due to the effect of mixed alkaloids in TDE. The inhibitory AChE effects of each pure alkaloid in TDE also need to be determined in future studies.

The results of this study also demonstrated the enhancement of cortical neuronal activity 2 h following the administration of TDE, as indicated by an increase in Fos-positive neurons. The cerebral cortex was chosen to determine neuronal activity and ChE activity, since it plays an important role in learning and memory, and is generally the main representative of the central cholinergic innervation (Warburton et al., 2003). In the analysis of the immediate early gene (IEG), Fos induction is a useful tool for investigating activated neuronal populations. Fos acts as a messenger in coupling short-term neuronal activity with changes at the level of gene transcription and, as such, should serve as a marker for those neurons undergoing some modification as a result of learning (Morgan and Curran, 1990; Curran and Morgan, 1995; Hughes and Dragunow, 1995). In normal physiological states, the basal level of IEG expression in the brain is low. However, different stimuli can induce IEG in neurons of CNS structures known to be involved in the processing of these stimuli.

The augmentation of neuronal activity following TDE administration could result from at least two possible mechanisms. First, TDE acts as an AChE-I in animals as it does *in vitro*.

AChE is an esterase critical in the metabolism of ACh at central and peripheral synapses (Koelle, 1963). If AChE activity is inhibited, ACh levels should be increased. ACh is plentiful and widely distributed in the brain. The cholinergic system is capable of keeping the neocortex operative (Giacobini, 2003a). Therefore, high ACh levels in the cerebral cortex could lead to an increase in cortical neuronal activity. The evidence that TDE acts as a cortical AChE-I in an animal model as demonstrated in this study support this mechanism. Moreover, a previous study demonstrated that the administration of AChE-Is could lead to endogenous acetylcholine (ACh)-induced Fos expression in the supra-optic nucleus of the rat hypothalamus (Shen and Sun, 1995). Therefore, the enhancement of Fos expression in cortical neurons following TDE administration, as shown in this study, suggests that TDE may cause an increase of endogenous ACh in the cerebral cortex, resulting in an increase in cortical activity, similar to that observed in other AChE-Is. The number of Fos-positive cortical neurons in this study was quite similar to that of Fos expression in the supra-optic neurons after the administration of AChE-Is (Shen and Sun, 1995) and that of cortical neurons expressing Fos after animals had been undergoing the learning process in a previous study (Tronel and Sara, 2002). Furthermore, the amount of Fos neurons in the cerebral cortex following TDE administration was much less than the expression of Fos following organophosphate exposure (Zimmer et al., 1998). Therefore, the expression of Fos following TDE administration should not be due to the possible excitotoxicity of TDE.

In the enhancement of cortical neuronal activity, another possible mechanism of TDE could be its part as an allosteric potentiating ligand (APL) binding at the nicotinic ACh receptor (nAChR), and directly potentiating neuronal activity. It has been shown that galantamine is an APL at the nAChR (Woodruff-Pak et al., 2001). Since TDE can inhibit AChE activity and show characteristics similar to galantamine, it is, therefore, possible that TDE could enhance cortical neuronal activity via this mechanism. However, further investigations are needed to justify this possible mechanism.

We showed that both the effect of TDE on the cortical AChE activity and cortical neuronal activity lacks dose-dependent effects. The possible explanation of these findings may be that TDE acts as an AChE inhibitor. Thus, the action of this TDE may depend upon the amount of AChE level in the brain. If the 250 mg/kg of TDE inhibits all of the AChE activity in the brain, increasing the dose of TDE should not further inhibit AChE activity. Therefore, the effect of TDE may not be dose-dependent.

Our study demonstrated that the mean percentage of ChE inhibition from TDE in the circulation was insignificant 2 h after TDE administration, but was significant at 10, 30 and 60 min after TDE administration. These results were similar to those previously observed after galantamine administration (Sweeney et al., 1989; van Beijsterveldt et al., 2004; Geerts et al., 2005). Those previous studies have shown that the inhibition of enzyme activity following galantamine administration reduces over time. We found that the inhibition of AChE activity by TDE is also time-dependent and reversible. The rapid clearance of TDE may cause the short term-inhibition of circulating AChE activity following a single-dose administration of TDE. Future studies are needed to investigate the bioavailability of TDE.

5. Conclusions

Preclinical analyses using *in vivo* enzymatic techniques in this study demonstrated that TDE is a reversibly selective AChE inhibitor and can enhance neuronal activity, suggesting that it may be a candidate for the treatment of AD. Future investigations such as behavioral studies, therapeutic indices, pharmacokinetics and complete toxicological evaluation of TDE are necessary to determine its therapeutic benefits.

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