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Research article

Bee venom disrupts vascular homeostasis: apitoxin and melittin trigger vascular cell toxicity and aortic dysfunction in mice

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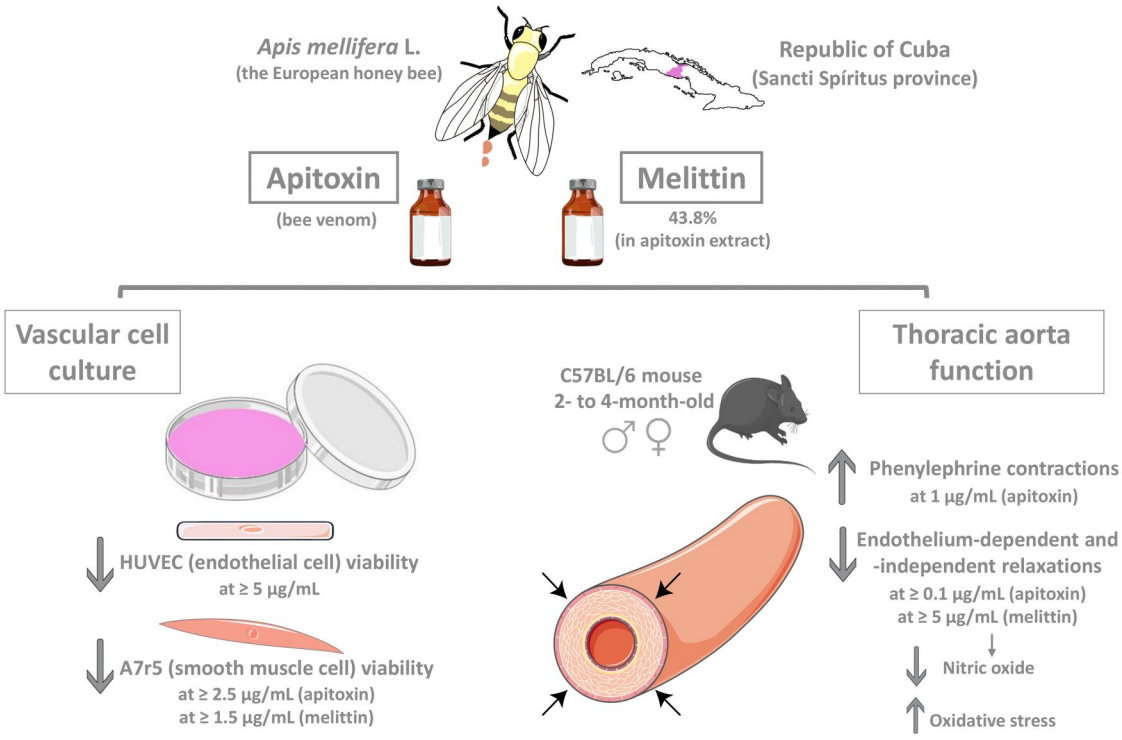
Abstract

Bee venom (apitoxin) is a mixture of bioactive molecules, with melittin as its principal component. Although its therapeutic potential is increasingly recognized, its toxic effects on vascular homeostasis remain underexplored. We investigated the impact of apitoxin and melittin on vascular cell viability and mouse aortic function. Cytotoxicity was assessed in cultured endothelial and smooth muscle cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Aortic function was evaluated by mounting thoracic aortas from young male and female C57BL/6J mice in tissue baths. Isometric tension was measured during phenylephrine-induced contractions, as well as endothelium-dependent (acetylcholine) and -independent (sodium nitroprusside) relaxations. To evaluate the roles of nitric oxide (NO) and oxidative stress, we used the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) and the antioxidant superoxide dismutase (SOD), respectively. High-performance liquid chromatography analysis revealed that melittin comprised 43.80% of apitoxin. Both apitoxin and melittin exhibited concentration-dependent cytotoxicity, significantly reducing endothelial cell viability at concentrations ≥5 μg/ml, whereas smooth muscle cells were affected at lower concentrations (≥2.5 μg/ml for apitoxin; ≥1.5 μg/ml for melittin). In functional experiments, apitoxin enhanced phenylephrine-induced contractions at 1 μg/ml and impaired both endothelium-dependent and -independent relaxations at ≥0.1 μg/ml, particularly in males. Although melittin mimicked these effects, higher concentrations (≥5 μg/ml) were required, suggesting that other venom components contribute to the vascular functional toxicity of apitoxin. L-NAME and SOD prevented apitoxin-induced vascular impairments, implicating the NO pathway and oxidative stress. These findings demonstrate that apitoxin impairs vascular cell viability and aortic function at clinically relevant concentrations, underscoring both its vascular risks and therapeutic potential.

Keywords: natural toxins; sex differences; vascular toxicity; endothelial dysfunction; nitric oxide; oxidative stress

Graphical Abstract



Apitoxin, commonly known as bee venom, is a complex mixture of bioactive molecules, including peptides, enzymes, and amines, each contributing distinct biological activities. Its principal component is melittin, complemented by other notable constituents such as apamin, adolapin, and mast cell degranulating peptide, and enzymes like phospholipase A_2 and hyaluronidase (Pucca et al. 2019; Tanuwidjaja et al. 2021). In addition, apitoxin contains vasoactive amines, including histamine and catecholamines such as dopamine and noradrenaline (Moreno and Giralt 2015). Traditionally, bee venom has been used to treat conditions like arthritis and chronic pain, and more recently, research has expanded its potential applications to diseases such as multiple sclerosis, cancer, and skin disorders (Son et al. 2007; Wehbe et al. 2019; Stela et al. 2024). These effects are largely attributed to its anti-inflammatory, immunomodulatory, and cytotoxic properties (Son et al. 2007; Moreno and Giralt 2015; Lee and Bae 2016; Wehbe et al. 2019; Gu et al. 2020; Stela et al. 2024). Despite its extensive study in inflammation-related conditions, often involving vascular processes, its direct cardiovascular effects remain insufficiently explored.

Although the therapeutic potential of apitoxin is well-recognized, its clinical application is limited by safety concerns. Beyond the well-known risks of anaphylactic shock and renal failure, stings from *Apis mellifera* L. (the European honey bee) have been associated with serious, and sometimes fatal, cardiovascular events, including hypotension, arrhythmia, and myocardial infarction (Gueron et al. 2000). Experimental studies also report that intravenous administration of bee venom induces cardiovascular depression in rats (Kang et al. 2008). These harmful effects are frequently attributed to melittin, the principal active compound in bee venom. Its vasoactive properties are associated with interactions with the endothelium, a key regulator of vascular homeostasis (Forstermann and Neufang 1985; Thomas et al. 1986; Rapoport et al. 1989; Hutcheson and Griffith 2000; Černe et al. 2010). Notably, melittin can stimulate nitric oxide (NO) production in endothelial cells, promoting vasodilation in various vascular beds (Thomas et al. 1986; Rapoport et al. 1989; Hutcheson and Griffith 2000). These vasodilatory effects are independent of cyclooxygenase activity (Forstermann and Neufang 1985; Thomas et al. 1986; Rapoport et al. 1989; Černe et al. 2010).

However, the vascular actions of melittin are complex and context-dependent, varying with both the vascular bed and its concentration. At low concentrations, melittin promotes vasorelaxation, whereas at higher concentrations, it induces vasoconstriction (Forstermann and Neufang 1985; Thomas et al. 1986; Rapoport et al. 1989; Černe et al. 2010). This duality arises from distinct mechanisms, including endothelium-dependent pathways and the activation of K_{Ca} channels in vascular smooth muscle cells (Hutcheson and Griffith 2000; Černe et al. 2010). At high concentrations (1 to 10 µg/ml), melittin exhibits irreversible cytotoxic effects on endothelial cells, impairing endothelium-dependent relaxations, likely through mechanisms involving the NO pathway (Rapoport et al. 1989). In addition to melittin, studies using isolated rat aorta have shown that whole venom induces endothelium-independent contraction, likely mediated by voltage-gated Ca^{2+} channels, angiotensin I receptors, and α-adrenergic receptors (Sousa et al. 2013). Interestingly, these effects were not observed with isolated melittin, suggesting that other components of apitoxin may interact synergistically to produce its vascular activity (Sousa et al. 2013). These findings highlight the intricate interplay among the various bioactive components of apitoxin and their collective impact on vascular tissues. However, despite these observations, the precise mechanisms underlying the vascular effects of apitoxin remain poorly characterized and warrant more detailed investigation.

In this study, we aim to deepen our understanding of the vascular effects of apitoxin by examining its impact on both cultured endothelial and smooth muscle cells, as well as on aortic reactivity in mice. We hypothesize that apitoxin exerts cytotoxic effects on vascular cells and impairs vasodilatory responses, primarily through mechanisms involving NO signaling.

Materials and methods

Bee venom harvesting and analytical standardization

Bee venom (apitoxin) used in this study was obtained from *Apis mellifera* L. apiaries located in the central region of Cuba (Sancti Spíritus province), and the collection took place in February 2022. The venom was harvested using a method first described by Marcovic and Molnar (1954), later refined by Palmer (1961), and further developed by other researchers (Benton et al. 1963; Gunnison 1966; Nobre 1990), with more recent improvements by de Graaf et al. (2021). The collection process involved introducing specialized traps into the hives, which were constructed using latex-coated glass and electrically conductive steel wires connected to units generating electrical impulses. These impulses, with a frequency of 50 to 1000 Hz, a duration of 2 to 3 s, and pauses of 3 to 6 s, stimulated the bees. A key advantage of this method is that bees release venom in response to the electrical stimulus without losing their stingers, allowing them to survive and continue their normal life cycle. The venom is deposited on the surface of the glass as the stinger passes through the latex coating. After 12 h of exposure to the electrical stimuli, the glass traps were removed from the hives. The venom deposited on the glass was carefully scraped off and collected into sterile, amber bottles. From approximately 10 active hives, about 20 glass traps with venom deposits on their surfaces can be harvested, yielding approximately 1 g of high-quality apitoxin.

High-performance liquid chromatography

The composition of melittin in the extract was determined by high-performance liquid chromatography (HPLC) with ultraviolet detection. Extract dissolved in water: 0.1% formic acid was centrifuged at 14,000 rpm. Afterwards, 25 µl supernatant was injected into the HPLC system (PerkinElmer, Madrid, Spain) comprising a 200 Pump, a 717 plus autosampler, and a 2487 dual λ absorbance detector (Waters, Barcelona, Spain). A C18 column, Kromasil 100-5-C18, 4.0 × 200 mm (Teknokroma, Sant Cugat, Barcelona, Spain) was used. The mobile phase was acetonitrile: 0.1% trifluoroacetic acid in water (40:60 vol/vol), which was run with an isocratic regular low flow rate of 1 ml/min, and the wavelength ultraviolet detector was set at 280 nm. The analytical validation was conducted using an appropriate melittin standard (Sigma-Aldrich, Saint Louis, MO, USA). The quantification was performed by external calibration.

Cell culture and treatments

The rat aortic smooth muscle cell line (A7r5) was obtained from ECACC (European Collection of Cell Cultures). A7r5 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and not used after passage 30. Primary cultures of human umbilical vein endothelial cells (HUVECs) were generously provided by Dr Ana Paula Dantas (Department of Biomedical Sciences, University of Barcelona, Barcelona, Spain). HUVEC cells were cultured on gelatin-coated plates (1/10 in water of 2% gelatin, type-B for 1 h room temperature; Sigma-Aldrich, Saint Louis, MO, USA) in endothelial cell growth medium-2 supplemented with Bullet Kit (Lonza, Basilea, Switzerland), 100 U/ml penicillin, 100 mg/ml streptomycin, and 5% FBS and used between passages 4 and 10. Both cell types were maintained at 37 °C in a humidified environment containing 5% CO_2.

For treatments, A7r5 and HUVEC cells were seeded at 40,000 cells/ml and grown for 48 h before treatments in their corresponding complete medium. A7r5 cells were starved in 0.2% FBS of their corresponding medium for 1 h before adding treatments. HUVEC cells were treated in 0.2% FBS containing the corresponding treatments following serum starvation. Serial dilutions of the treatments were prepared in 0.2% FBS medium and applied to the cultured cells. Treatments were maintained for 24 h, and then cell media was collected to measure cell viability and was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method.

Cell viability

Cell viability was determined by the MTT reduction method. Briefly, after collection of treatment media, cells were incubated in 0.5 mg/ml MTT (Sigma-Aldrich, Saint Louis, MO, USA) dissolved in 0.2% FBS medium for 90 min. Medium was then replaced by dimethylsulfoxide (DMSO; Sigma-Aldrich, Saint Louis, MO, USA) to dissolve the blue formazan precipitate, proportional to the cell viability. Absorbance was measured spectrophotometrically at 560 and 700 nm in a microplate reader (Synergy HT and data analysis software KC4, Bio-Tek Instruments Inc.). Values obtained at 700 nm were used as blank and subtracted from those at 560 nm.

Animals

We used 2- to 4-month-old male and female C57BL/6J mice ($n = 38$) obtained from Charles River (Sant Cugat del Vallès, Barcelona, Spain). The mice were housed in the animal facility at the Universitat Autònoma de Barcelona, following institutional guidelines. The environment was controlled with a constant temperature of 20 ± 2 °C, a 12/12-h light/dark cycle, 60% humidity, and ad libitum access to food and water. All experiments complied with the Spanish legislation on the protection of animals used for scientific purposes (RD 53/2013) and the European Union directive (2010/63/EU). The protocols were approved by the ethics committee of the Universitat Autònoma de Barcelona (approval code: FJA-eut/01).

Tissue preparation

Mice were euthanized using isoflurane (5% mixed with 0.8 l/min O_2) and then exsanguinated via decapitation. The descending thoracic aorta was carefully dissected and placed in ice-cold aerated (95% O_2, 5% CO_2) Krebs–Henseleit (KH) solution containing NaCl (112 mM), KCl (4.7 mM), $CaCl_2$ (2.5 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $NaHCO_3$ (25 mM), and glucose (11.1 mM). The aorta was then cleaned of fat and connective tissue.

Aorta reactivity

Aorta segments (1.5 mm in length) were mounted onto a four-channel wire myograph (model 620 M; Danish Myo Technology, Aarhus, Denmark) under isometric conditions, and filled with ice-cold, aerated KH solution (5% O_2 and 5% CO_2), as previously described (Jiménez-Altayó et al. 2017). After a 30-min stabilization period at 37 °C, arteries were stretched to a resting tension of 6 mN. The arteries were then allowed to equilibrate for an additional 45 min and were exposed twice to a K^+-enriched KH solution (containing 100 mM KCl). After washing, vessels were preincubated for 30 min with different concentrations of apitoxin (0.1 to 1 µg/ml) or

melittin (0.1 to 10 μg/ml). Endothelial-dependent vasodilations to acetylcholine (ACh; 1 nM to 100 μM; Sigma-Aldrich, Saint Louis, MO, USA) were assessed in vessels precontracted with 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_2\alpha$ (U46619; Calbiochem-Merck KGaA, Darmstadt, Germany) to achieve 70% to 100% of the KCl-induced contraction. Contractile responses mediated by α_1-adrenoceptor stimulation were studied by evaluating phenylephrine (Phe; 1 nM to 10 μM; Sigma-Aldrich, Saint Louis, MO, USA)-induced contractions. To assess smooth-muscle sensitivity to NO (endothelium-independent responses), arteries were exposed to increasing concentrations of the NO donor sodium nitroprusside (SNP; 0.1 nM to 100 μM; Sigma-Aldrich, Saint Louis, MO, USA). The effects of the non-selective NO synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 300 μM; Sigma-Aldrich, Saint Louis, MO, USA) or the superoxide anion scavenger superoxide dismutase (SOD; 200 U/ml; Sigma-Aldrich, Saint Louis, MO, USA) were determined by adding each treatment 30 min before the concentration-dependent responses.

Measurement of 2-hydroxyethidium

The analysis of circulating 2-hydroxyethidium (Noxygen Science Transfer & Diagnostics GmbH, Germany, Cat#: NOX-14.1) was conducted using HPLC with fluorescence detection, serving as a quantitative measure of superoxide anion levels, as previously described (Laurindo et al. 2008; Samhan-Arias et al. 2018; Jiménez-Xarrié et al. 2020). To obtain the samples, after carefully removing adipose tissue and blood, aortas were placed in cold KH solution supplemented with gentamicin (30 mg/l) (Genta-gobens, Laboratorios Normon SA, Tres Cantos, Madrid, Spain) and amphotericin B (15 mg/l) (Biowhittaker, Lonza, Basel, Switzerland). Vessels were then seeded into wells of a 96-well plate (Costar, Corning Inc., Corning, NY, USA) containing 50 μl of Matrigel (BD Bioscience, San Jose, CA, USA) at 4 °C (Costar, Corning Inc., Corning, NY, USA). After 30 min at room temperature, 200 μl of microvascular endothelial cell growth medium (Labclinics, Barcelona, Spain), supplemented with 5% FBS, gentamicin (30 μl/ml), and amphotericin B (0.015 μl/ml), were added to each well along with the treatments: either vehicle (distilled water) or apitoxin (1 μg/ml). Plates were incubated for 24 h at 37 °C and 5% CO_2, after which the medium was collected and frozen at −70 °C until analysis. The presence of 2-hydroxyethidium in the samples was quantified by comparison with a calibration curve generated from the xanthine–xanthine oxidase reaction, following the method previously described (Michalski et al. 2014).

Statistical analyses

All results are expressed as mean ± SEM of the number (n) of animals or independent experiments in each group, as indicated in the figure legends. Vasodilator responses to ACh and SNP are expressed as a percentage of the tone generated by U46619-induced pre-contractions. Contractile responses to Phe are expressed as a percentage of the contractions induced by KCl (100 mM). Normality testing was systematically performed before selecting the appropriate statistical test. The Mann–Whitney U test was used to compare 2 groups. For comparisons involving more than 2 groups, one-way or two-way ANOVA, followed by Tukey's post hoc test, was applied, depending on the experimental design. Statistical analysis was conducted using GraphPad Prism version 8.3 (San Diego, California, USA) software, and statistical significance was set at $P < 0.05$.

Results

Apitoxin is rich in melittin

The melittin content in the apitoxin extract batch used in this study was analyzed using HPLC. Figure 1 highlights the specific HPLC peak corresponding to melittin. Melittin eluted approximately 3 min after injection, with a measured content of 43.80% in the extract.

Apitoxin and melittin reduce the survival of endothelial and smooth muscle cells

To study the direct effects of apitoxin and its main component, melittin, on vascular cell viability, we assessed their effects on the viability of endothelial and smooth muscle cells using the MTT assay in cultured HUVEC and A7r5 cells, respectively. In endothelial cells, a 24-h incubation with either apitoxin or melittin led to a significant reduction in cell survival at concentrations of ≥5 μg/ml (Fig. 2A). In smooth muscle cells, apitoxin significantly decreased survival at concentrations of ≥2.5 μg/ml, whereas melittin at ≥1.5 μg/ml, with both reaching their maximum effect at 5 μg/ml (Fig. 2B). Thus, both apitoxin and melittin exhibited a concentration-dependent cytotoxic effect in both vascular cell types.

Apitoxin impairs both endothelium-dependent and -independent relaxations in the mouse aorta

We investigated the effects of apitoxin on mouse aortic function. Initially, we assessed how apitoxin (0.1 to 10 μg/ml) influenced aortic tone. At concentrations of 1 and 10 μg/ml, apitoxin caused a significant increase in vascular tone compared with the control (data not shown). This high response at 10 μg/ml made it difficult to continue studying vascular responses at this concentration. Therefore, subsequent studies were limited to concentrations of 0.1 and 1 μg/ml. Next, we evaluated the concentration-dependent relaxation response to ACh, an endothelium-dependent agonist. Apitoxin at 0.1 to 1 μg/ml significantly reduced ACh-induced relaxations (Fig. 3A). Similarly, endothelium-independent relaxations to SNP were also significantly diminished by apitoxin (Fig. 3B). In addition, apitoxin at 1 μg/ml increased contractions induced by Phe (Fig. 3C). These findings indicate that apitoxin significantly impairs both endothelium-dependent and -independent vasodilation in the mouse aorta, while also enhancing Phe-induced vasoconstrictive responses.

Increased susceptibility to aortic vasodilator dysfunction induced by apitoxin in males

We then separated the mice by sex to investigate whether apitoxin affects aortic vasodilation differently in males and females (Fig. 4). Apitoxin significantly impaired ACh-induced relaxation in both sexes (Fig. 4A and B). However, while apitoxin significantly reduced SNP-induced vasorelaxation in males (Fig. 4C), it had no significant effect in females (Fig. 4D). These results suggest that the impact of apitoxin on aortic vasodilation may be more pronounced in males than in females.

Melittin impairs both endothelium-dependent and -independent relaxations in the mouse aorta

We subsequently investigated whether melittin, the main component of apitoxin, could mimic the apitoxin-induced aortic vasodilator dysfunction (Fig. 5). We first observed that at concentrations of 5 to 10 μg/ml, melittin reduced ACh-induced relaxations (Fig. 5A), whereas only at 10 μg/ml did it induce a significant impairment of SNP-induced relaxations (Fig. 5B). These

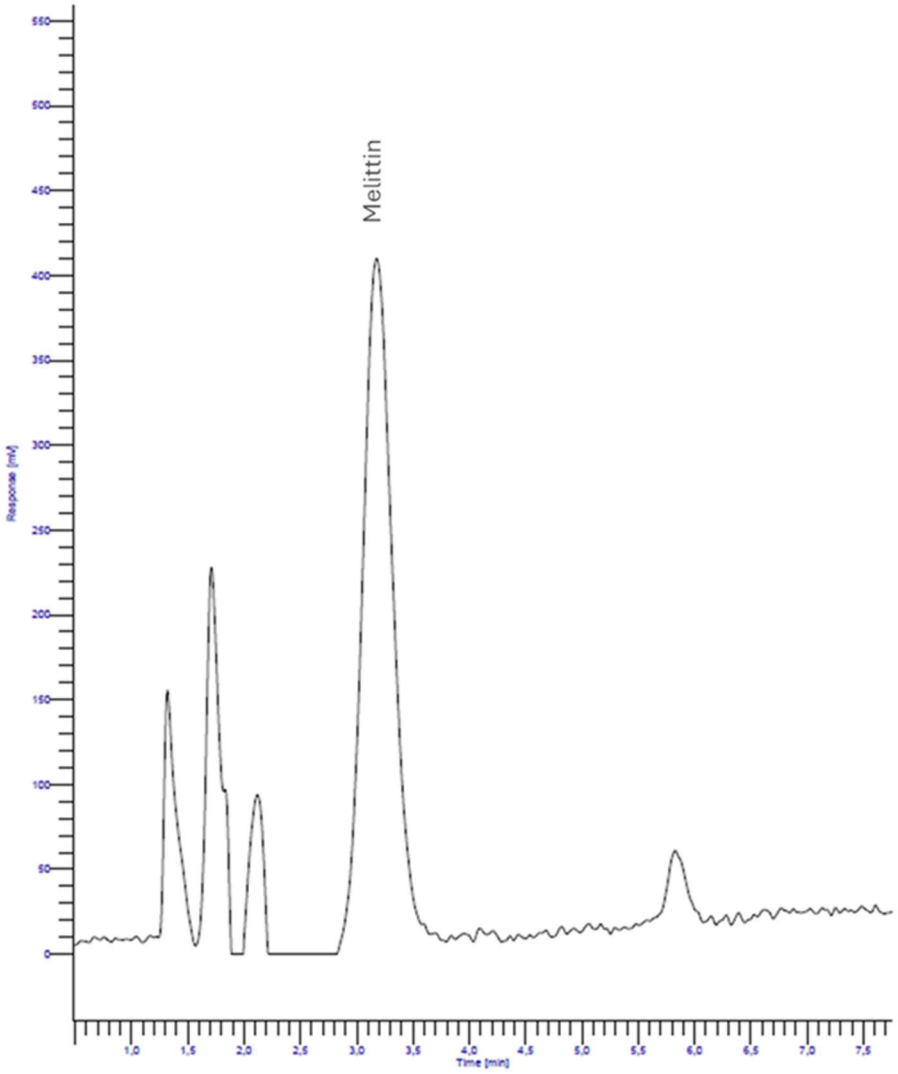


Fig. 1. Detection of melittin levels. Representative HPLC trace showing the peak used for quantifying melittin concentration with a standard. Melittin elutes at 3.09 min post-injection.

results highlight that melittin, as the primary component of apitoxin, plays a crucial role in mediating the vasodilator dysfunction associated with apitoxin exposure, although its effects are observed at higher concentrations.

NO is involved in the apitoxin-induced impairment of endothelium-dependent and -independent relaxations

NO is a major contributor to aortic relaxation, and decreases in NO availability have been associated with vasodilator dysfunctions (Tousoulis et al. 2012; Costa et al. 2021). In addition, previous studies have implicated the NO pathway in the impairment of endothelium-dependent relaxations induced by melittin in the rat thoracic aorta (Rapoport et al. 1989). To investigate the role of NO as a potential mechanism underlying apitoxin-induced aortic vasodilator dysfunction, aortas were incubated with L-NAME (300 μM), a non-selective NOS inhibitor (Fig. 6). In the presence of L-NAME, aortic relaxations to ACh were significantly decreased, regardless of apitoxin exposure, thereby abolishing the apitoxin-induced impairments in relaxation (Fig. 6A). Similarly, although L-NAME per se significantly improved relaxations to SNP, it also prevented the impairment induced by apitoxin (Fig. 6B). These findings suggest that the vasodilator dysfunction caused by apitoxin is closely linked to NO signaling pathways, as inhibiting NO synthesis prevented the effects of apitoxin on aortic relaxation.

Superoxide dismutation prevents apitoxin-induced impairment of endothelium-dependent and -independent relaxations

Excessive production of superoxide anions may contribute to reduced NO availability (Matsubara et al. 2015; Costa et al. 2021). To assess whether increased superoxide anion production is involved in the vascular effects of apitoxin, we first incubated aortas in culture medium with or without apitoxin (1 μg/ml). This treatment exhibited a trend ($P = 0.0571$) toward elevated superoxide anion release from the aorta (Fig. 7A). To further evaluate the role of superoxide in apitoxin-induced vasodilator dysfunction, aortas were incubated with SOD (200 U/ml), an enzyme that catalyzes the dismutation of superoxide anions into oxygen and hydrogen peroxide (Fig. 7). Incubation with SOD significantly decreased aortic relaxations to ACh, and the addition of apitoxin did not exacerbate this dysfunction (Fig. 7B). In addition, SOD

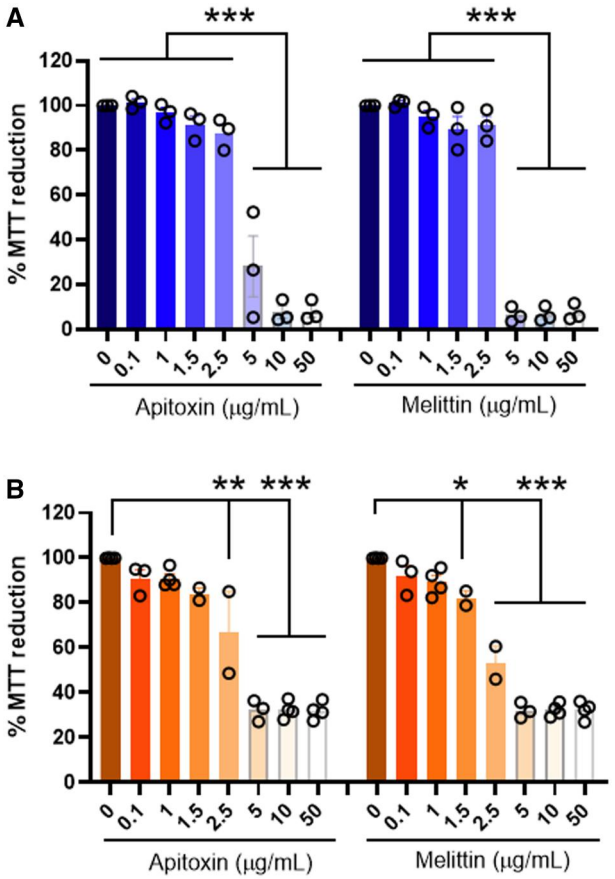


Fig. 2. Apitoxin and melittin reduce vascular cell survival in a concentration-dependent manner. Effects of apitoxin (0.1 to 50 µg/ml) and melittin (0.1 to 50 µg/ml) on % MTT reduction in HUVEC (A) and A7r5 (B) cells. Results are the mean ± SEM from $n = 3$ to 4 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by one-way ANOVA with Tukey's *post hoc* test.

incubation effectively restored the impaired relaxations to SNP (Fig. 7C). These findings suggest that superoxide anion formation plays a key role in the mechanism by which apitoxin disrupts vasodilatory responses.

Discussion

In this study, we investigated the effects of apitoxin and its primary active component, melittin, on vascular cell survival and agonist-induced aortic function in mice. HPLC analysis confirmed that melittin accounted for 43.80% of the extract, consistent with previous reports (Wehbe et al. 2019; Carpena et al. 2020; Tanuwidjaja et al. 2021). Both apitoxin and melittin significantly reduced the viability of cultured endothelial and smooth muscle cells and impaired endothelium-dependent and -independent vasorelaxation in the mouse aorta. These effects were more pronounced in male mice. Notably, apitoxin impaired relaxations at lower concentrations than melittin, suggesting that additional venom components contribute to the vascular functional toxicity of this toxin. Inhibition of NO synthesis with L-NAME and neutralization of superoxide anions by SOD effectively prevented apitoxin-induced vasodilatory dysfunction, underscoring the roles of NO signaling and oxidative stress. Although these findings highlight the vascular risks associated with apitoxin exposure, they also point to its potential for therapeutic use in vascular-related conditions.

The cytotoxic effects of apitoxin and melittin are well-documented, particularly regarding their potential as anticancer agents (Son et al. 2007; Moreno and Giralt 2015; Carpena et al. 2020; Gu et al. 2020). However, fewer studies have investigated their harmful effects on vascular cells. In this study, we assessed the cytotoxicity of apitoxin and melittin using cultured endothelial (HUVEC) and smooth muscle (A7r5) vascular cell models. Twenty-four hours of incubation with apitoxin and melittin induced cytotoxicity and significantly reduced cell survival in both types of vascular cells. These effects were observed at concentrations of 5 µg/ml in endothelial cells and 1.5 to 2.5 µg/ml in smooth muscle cells. These results align with previous studies reporting similar cytotoxic effects of melittin at 10 µg/ml in HUVEC (Černe et al. 2013), 1 to 10 µg/ml in rat aortic endothelial cells (Rapoport et al. 1989), and both melittin and apitoxin at 0.4 to 0.8 µg/ml in rat aortic smooth muscle cells (Son et al. 2006). These findings are consistent with the well-established membrane-disrupting properties of bee venom, which contains lytic components such as phospholipase A2 and melittin itself (Moreno and Giralt 2015; Wehbe et al. 2019; Carpena et al. 2020).

The modulatory effects of apitoxin on vascular tone were also investigated. In the mouse aorta, apitoxin significantly increased arterial tone at concentrations as low as 1 µg/ml. These findings align with previous studies, though the concentration required to produce these effects varies. For instance, Sousa et al. (2013) reported a concentration-dependent vasoconstrictor effect of apitoxin starting at 10 µg/ml in the rat aorta, indicating that our extract modifies arterial tone at lower concentrations (1 µg/ml) in the mouse aorta. To further investigate the impact of apitoxin and melittin on vascular function, we examined their effects on agonist-induced responses. Notably, apitoxin impaired both endothelium-dependent and -independent relaxations to ACh and SNP, respectively, at concentrations ranging from 0.1 to 1 µg/ml. Melittin exhibited a similar inhibitory effect, though it became significant only at concentrations of 5 µg/ml or higher, which partly aligns with previous studies suggesting that melittin (1 to 10 µg/ml) impairs ACh-induced relaxations in the rat aorta (Rapoport et al. 1989). These findings suggest that additional bioactive components of apitoxin, beyond melittin, may contribute to the overall inhibitory effects on aortic function. Of note, apitoxin also impaired Phe-induced contractions, demonstrating its broad influence on vascular function. Taken together, these results underscore the potential pathological impact of apitoxin exposure on vascular function. Remarkably, female mice exhibited slightly lower susceptibility to apitoxin-induced vascular dysfunction compared with males. This protective effect may be attributed to estrogens, which are well known for their cardioprotective properties and their role in reducing overall cardiovascular risk (Iorga et al. 2017; Xiang et al. 2021). These findings suggest that females may require higher doses of apitoxin to exhibit effects comparable to those observed in males. This reinforces the importance of considering sex as a biological variable in studies of vascular toxicology.

Our findings underscore the key role of NO in the apitoxin-induced inhibition of vascular relaxation, as this effect was abolished by L-NAME, a NOS inhibitor. In addition, significant endothelium-independent effects were also observed, consistent with the observed cytotoxic actions of apitoxin on cultured smooth muscle cells. Previous studies have demonstrated that melittin exerts its effects on vascular tone regulation through both endothelium-dependent and -independent mechanisms

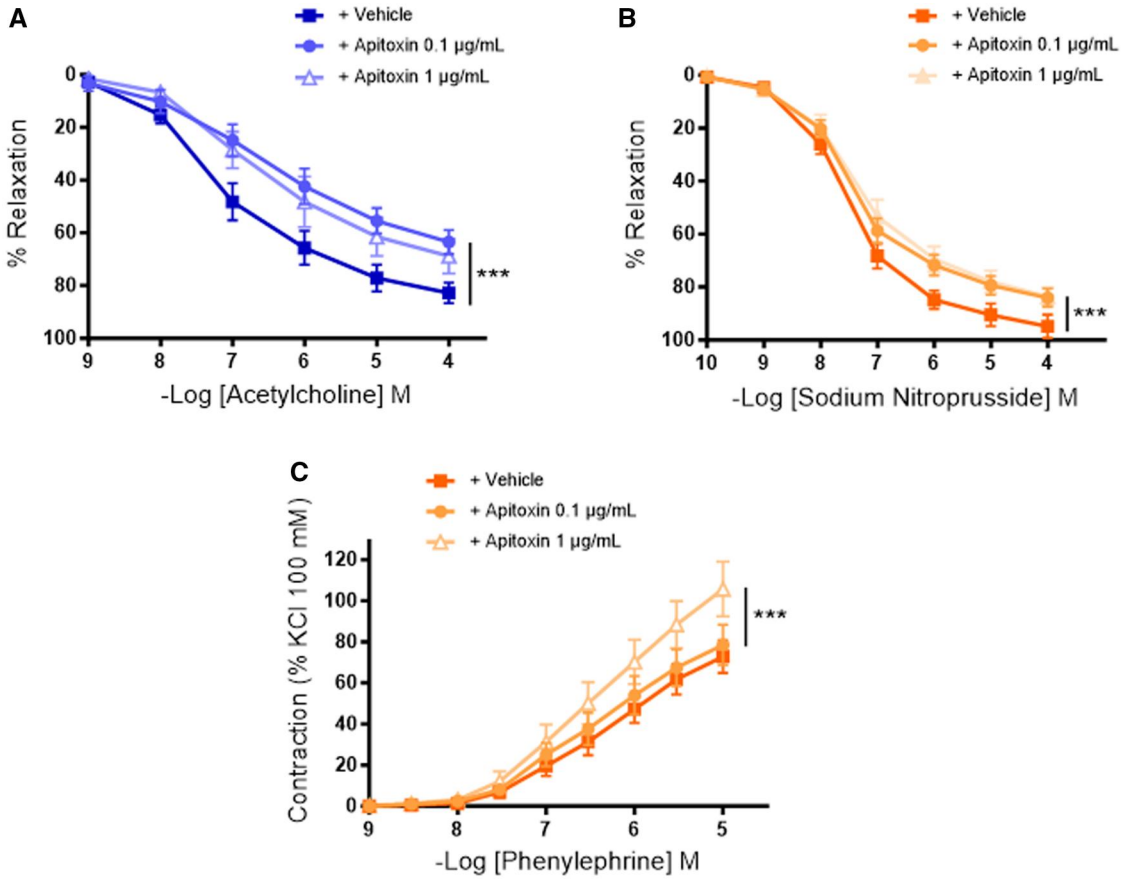


Fig. 3. Apitoxin impairs vascular relaxation responses induced by acetylcholine and sodium nitroprusside, as well as vasoconstriction induced by phenylephrine. Effects of apitoxin (0.1 to 1 µg/ml) on mouse aorta concentration-dependent relaxations induced by acetylcholine (A) and sodium nitroprusside (B), and concentration-dependent contractions induced by phenylephrine (C). Results are the mean ± SEM from $n = 15$ (A), $n = 16$ (B), and $n = 16$ (C) male and female mice. ***$P < 0.001$ by two-way ANOVA with Tukey's post hoc test.

(Forstermann and Neufang 1985; Thomas et al. 1986; Rapoport et al. 1989; Hutcheson and Griffith 2000; Černe et al. 2010). Similarly, apitoxin impacts vascular function by modulating smooth muscle activity (Sousa et al. 2013). These findings emphasize the multifaceted nature of their actions on the vascular system, involving a combination of effects on endothelial function and smooth muscle dynamics. The prevention of SNP-induced relaxation impairment by L-NAME suggests that endogenous NO plays a critical role in the effects of apitoxin on endothelium-independent vascular relaxation. A potential mechanism involves the interaction of NO with other free radicals, such as superoxide anions, leading to the formation of peroxynitrite, a highly reactive compound known to damage smooth muscle cells (Kagota et al. 2009). Consistent with this, previous studies have shown that oxidative stress contributes to the DNA-damaging effects of bee venom in human peripheral blood leukocytes (Gajski et al. 2012). In line with these findings, we observed a clear trend toward increased superoxide anion release in the mouse aorta following apitoxin exposure. Furthermore, we evaluated the impact of oxidative stress inhibition using SOD, an antioxidant enzyme that mitigates superoxide anion-induced damage. Our results demonstrate that SOD pretreatment effectively prevented the vascular relaxation impairments induced by apitoxin. These findings suggest that oxidative stress, triggered by elevated superoxide anion production and/or the subsequent generation of reactive oxygen and nitrogen species, plays a central role in mediating the vascular effects of apitoxin.

Although the present results emphasize the vascular toxicity of apitoxin, they do not preclude its beneficial properties in other contexts (Coulter-Parkhill et al. 2021; Stela et al. 2024). Some studies have reported both pro-oxidant (Han et al. 2002; Kamel et al. 2024) and antioxidant (Suh et al. 2006; Badr et al. 2016; Kocyigit et al. 2019; Kim et al. 2021; Martinello and Mutinelli 2021) properties of apitoxin, and our findings highlight the critical role of oxidative stress in apitoxin-induced vascular dysfunction. Notably, some mechanisms underlying the toxicity observed in our research, such as NO modulation, could also play a role in the protective effects reported in chronic settings. These findings emphasize the dual nature of the biological activity of apitoxin. Future research should explore how these seemingly opposing effects can be leveraged for therapeutic benefit.

A single bee sting delivers approximately 0.14 to 0.30 mg of venom, whereas the median lethal dose (LD50) for an adult is estimated at 2.8 to 3.5 mg of venom per kg of body weight (Schumacher et al. 1994; Carpena et al. 2020). For a 70 kg individual, this corresponds to a total venom requirement of 196 to 245 mg. Accordingly, approximately 817 to 1,750 stings would be required to reach the LD50, resulting in 50% mortality (Schumacher et al. 1994; Dos Santos-Pinto et al. 2018; Pucca et al. 2019; Carpena et al. 2020). However, severe systemic

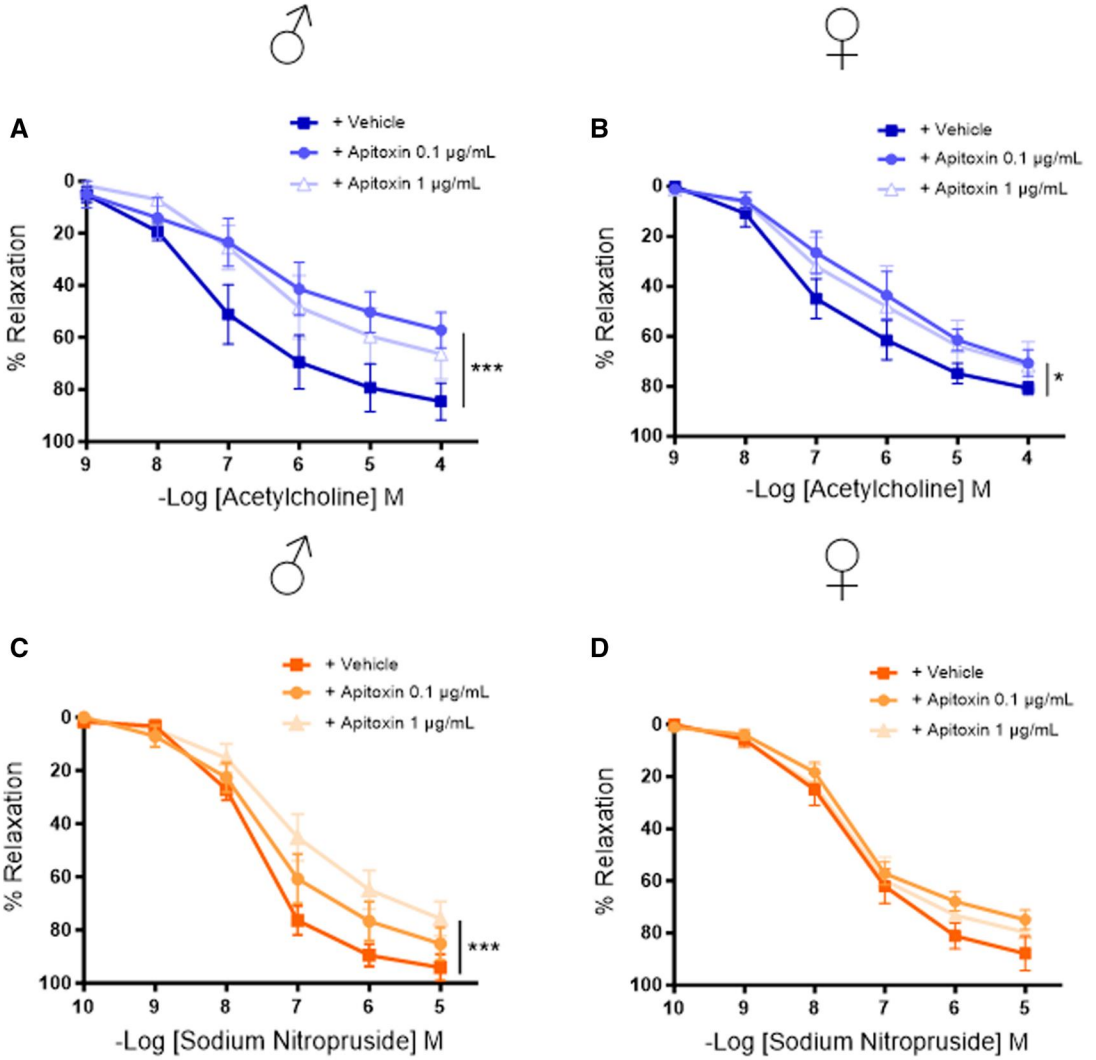


Fig. 4. Apitoxin impairs vascular relaxation responses induced by acetylcholine and sodium nitroprusside, particularly in males. Effects of apitoxin (0.1–1 µg/ml) on mouse aorta concentration-dependent relaxations induced by acetylcholine (A and B) and sodium nitroprusside (C and D) in male (A and C) and female (B and D) mice. Results are the mean ± SEM from $n = 7$ to 8 male and $n = 7$ to 9 female mice. $^{*}P < 0.05$, $^{***}P < 0.001$ by two-way ANOVA with Tukey's post hoc test.

manifestations and fatalities have been reported following fewer stings, likely due to individual susceptibility and age-related vulnerability (Franca et al. 1994; Schumacher et al. 1994). Notably, venom concentrations observed in patients stung by swarms fall within the range shown to cause significant vascular effects in experimental studies. For example, serum venom concentrations of 1.9 to 3.8 µg/ml (Franca et al. 1994) are comparable to the levels of whole venom and melittin shown to affect vascular tone in porcine left anterior descending coronary arteries (Černe et al. 2000, 2010) and in the rat aorta (Rapoport et al. 1989; Sousa et al. 2013). Although such levels are typically associated with multiple stings, our findings in the mouse aorta demonstrate that even lower concentrations, such as 0.1 µg/ml, can significantly impair vasorelaxation. Based on average human blood volume, this concentration could plausibly be reached transiently following a single sting, especially in smaller individuals or under conditions of rapid systemic absorption. Therefore, our results suggest that even a single bee sting, in the absence of massive envenomation, can disrupt vascular tone and impair vasorelaxation, particularly in individuals with pre-existing cardiovascular or allergic conditions. In addition, the high concentration of venom at the sting site can cause significant effects in the local environment, contributing to localized cytotoxicity and vascular dysfunction. Taken together, the severity of these outcomes likely depends on factors such as individual sensitivity (e.g. rate of systemic absorption, immune status, comorbidities, previous sensitization), age, body weight, number of stings, venom load, among others.

This study has some limitations that warrant consideration. Although we employed both cultured vascular endothelial and smooth muscle cells and ex vivo functional assessments of mouse aorta to investigate the vascular effects of apitoxin, these models may not fully replicate the complexity of the in vivo environment. Although they provide valuable insight into the cytotoxic and vasoactive effects of bee venom, future in vivo studies are necessary to confirm the systemic relevance of these findings. For example, previous reports indicate that both bee venom and melittin can induce cytogenetic and oxidative damage in human peripheral blood cells (Garaj-Vrhovac and Gajski 2009; Gajski et al. 2012, 2016; Sjakste and Gajski 2023), underscoring the need to further assess their systemic toxicity in non-target cells. This potential for cyto/genotoxicity in normal tissues remains one of the major challenges for therapeutic applications. Notably, some studies suggest that such toxicity could

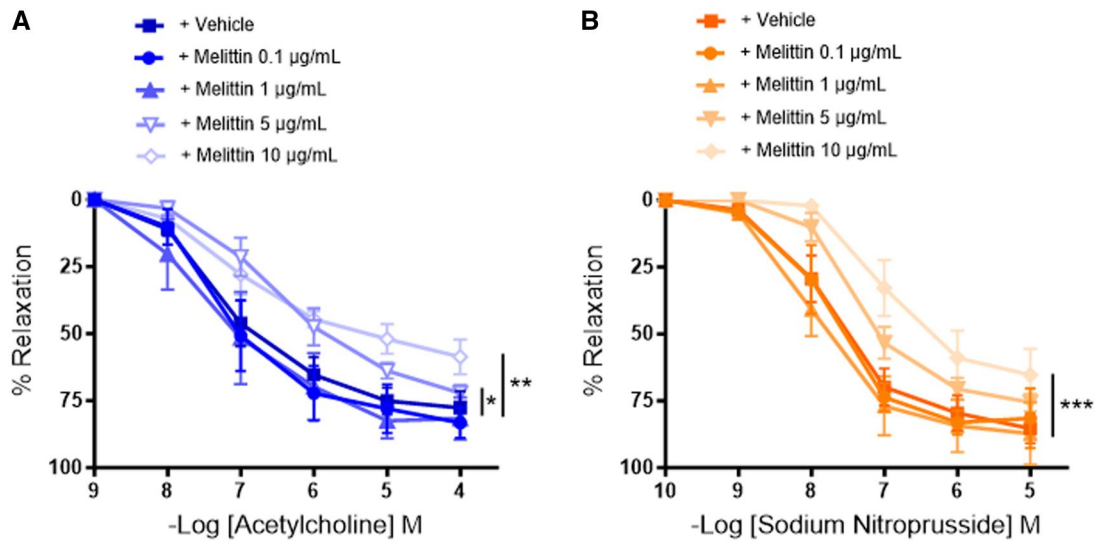


Fig. 5. Melittin impairs vascular relaxation responses induced by acetylcholine and sodium nitroprusside. Effects of melittin (0.1 to 10 µg/ml) on mouse aorta concentration-dependent relaxations induced by acetylcholine (A) and sodium nitroprusside (B). Results are the mean ± SEM from $n = 4$ to 7 (A) and $n = 4$ to 8 (B) male mice *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by two-way ANOVA with Tukey's post hoc test.

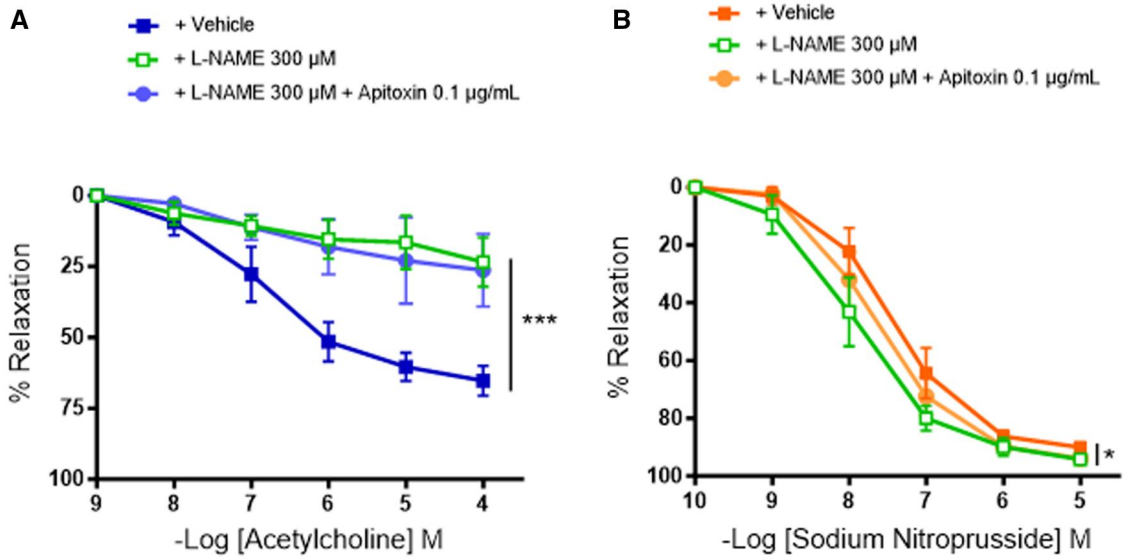


Fig. 6. Incubation with L-NAME abolishes the apitoxin-induced impairments of acetylcholine and sodium nitroprusside relaxations. Mouse aorta concentration-dependent relaxations induced by acetylcholine (A) and sodium nitroprusside (B) in the presence of L-NAME (300 µM) or L-NAME + Apitoxin (0.1 µg/ml). Results are the mean ± SEM from $n = 5$ (A) and $n = 5$ (B) male (60%) and female (40%) mice. *$P < 0.05$, ***$P < 0.001$ by two-way ANOVA with Tukey's post hoc test.

be mitigated through nanoformulations, which may improve treatment selectivity and reduce adverse effects on healthy cells (Pan et al. 2011). In addition, while our study identified melittin as the principal active component of apitoxin and explored its vascular effects, we did not fully dissect the contributions of other venom constituents, which may also play a role in the observed vascular toxicity. Specifically, components such as apamin, phospholipase A2, and mast cell degranulating peptide were not quantified or evaluated in this study, and their individual or synergistic effects warrant further investigation. Moreover, all experiments were conducted in young, healthy mice, which may not fully capture the range of responses in individuals with pre-existing cardiovascular risk factors; therefore, studies in aged animals or models of cardiovascular disease are warranted to explore whether such conditions influence susceptibility to the vascular effects of apitoxin. Lastly, although both male and female mice were included, the sex-specific differences observed in response to apitoxin highlight the need for further investigation into potential sex-dependent susceptibilities to venom-induced vascular dysfunction, potentially mediated by the protective vascular effects of estrogens.

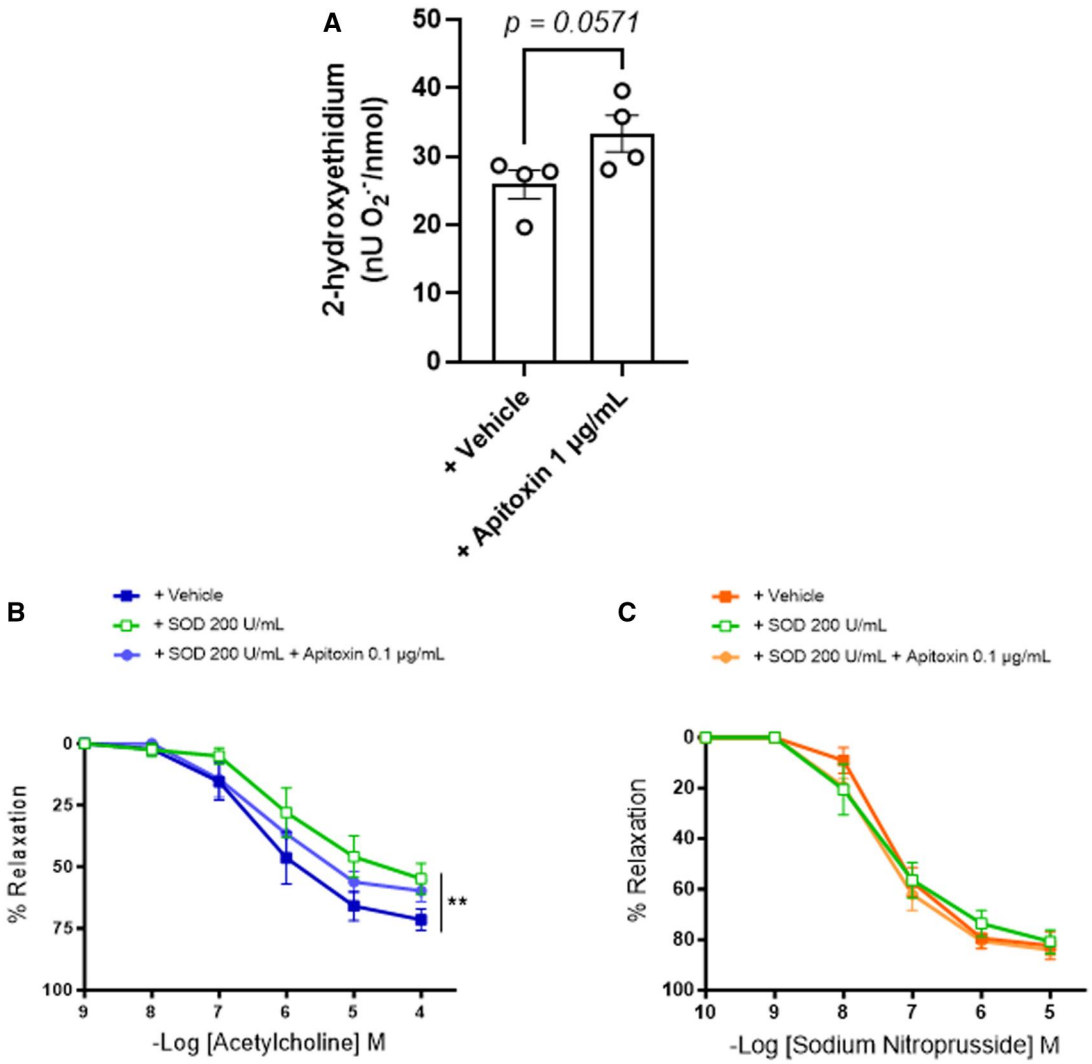


Fig. 7. Incubation with apitoxin increases the release of superoxide anion from the aorta, and SOD abolishes the apitoxin-induced impairments of acetylcholine and sodium nitroprusside relaxations. A) Analysis of 2-hydroxyethidium levels by HPLC in the presence of vehicle or Apitoxin (1 µg/ml). Results are the mean ± SEM from $n = 4$ male mice. $P = 0.0571$ by the Mann–Whitney U test. Mouse aorta concentration-dependent relaxations induced by acetylcholine (B) and sodium nitroprusside (C) in the presence of SOD (200 U/ml) or SOD + Apitoxin (0.1 µg/ml). Results are the mean ± SEM from $n = 4$ (A) and $n = 4$ (B) male (50%) and female (50%) mice. $^{**}P < 0.01$ by two-way ANOVA with Tukey's post hoc test.

Conclusion

These findings demonstrate that apitoxin causes significant cytotoxicity in vascular cells and alters aortic function. Although melittin reproduced these effects, it required higher concentrations in functional studies, suggesting that additional venom components contribute to the vascular functional toxicity of apitoxin. Notably, vascular impairments occurred at concentrations that may be reached following multiple bee stings and could plausibly occur even after a single sting, particularly in vulnerable individuals. Mechanistically, the observed impairment in vascular relaxation appears to involve NO depletion and oxidative stress. These results highlight the potential risks associated with bee venom exposure, even at low doses, and underscore the need for further research into its potential therapeutic applications in vascular disorders.

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Conflicts of interest. The authors declare no competing interests.

Data availability

Data are available upon reasonable request to the corresponding author.

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