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Original Research

Bradykinin B1 receptor antagonist protects against cold stress–induced erectile dysfunction in rats

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Abstract

Background: Erectile dysfunction (ED) demonstrates seasonal variation with higher rates in winter, and we hypothesize that endothelial damage in erectile tissue caused by bradykinin receptor B1 (B1R) might be detrimental to this change.

Aim: To find out direct correlations between cold stress and ED, through which to further investigate the functional roles of B1R in erectile tissue and to elucidate the therapeutic roles of the B1R antagonist in a cold stress–induced ED rat model.

Methods: Cold stress rat models are established through long-term intermittent exposure to low temperature. After their erectile function was assessed, ED rats were treated with the B1R antagonist through intraperitoneal injection. Penile tissues were obtained at the end of the experiment after measurement of intracavernosal pressure/mean arterial pressure (ICP/MAP); the location and distribution of cytokine expression were determined by immunohistochemistry; cytokine levels and NOS and CD31 expression were detected by Western blotting; and collagen fibers and smooth muscles were observed through Masson staining.

Outcomes: Cold stress impairs erectile function, and the B1R antagonist protects against it.

Results: We observed decreased erection frequency, prolonged erection latency time, decreased ICP/MAP, overexpression of B1R, increased expression of cytokines on cavernous sinus endothelium, and increased levels of collagen fibers/smooth muscles on erectile tissue in response to cold stress. Also, NOS and CD31 expression was downregulated. B1R antagonist treatment shows enhanced erectile function through increased erection frequency, shortened erection latency time, and increased ICP/MAP. Also, it reduces collagen fibers/smooth muscles, TNF-α, TGF-β1, and IL-6 and upregulates the expression of nNOS and CD31.

Clinical Translation: Our findings cast new light on the correlations between cold stress and erectile function and potential new applications of existing B1R antagonist drugs in the field of ED.

Strengths and Limitations: Our data support that cold stress impairs erectile function. B1R-mediated, cytokine-induced corpus cavernosum fibrosis and endothelial damage might be the main reason behind it, and B1R inhibition protects against fibrosis and endothelial damage. Other ways of B1R antagonist blocking methods in different types of ED still need to be investigated.

Conclusion: Long-term intermittent cold stress impairs erectile function, and B1R-mediated, cytokine-induced corpus cavernosum fibrosis and endothelial damage might be the main reason behind it. B1R inhibition also protects against fibrosis and endothelial damage. Our data support the hypothesis that cold stress impairs erectile function and that B1R blockade ameliorates the symptoms of ED, possibly by reversing fibrosis and endothelial damage in erectile tissue.

Keywords: ED; cold stress; bradykinin 1 receptor; fibrosis; endothelial damage..

Introduction

Erectile dysfunction (ED), formerly termed *impotence*, is defined as the failure to achieve or maintain a rigid penile erection suitable for satisfactory sexual intercourse. While no specific time period is part of this definition, some have suggested that the condition needs to persist for 6 months.[1] Any disease process that affects penile vessels, nerves, hormone levels, smooth muscle tissue, corporal endothelium, or tunica albuginea can cause ED. ED can also be a symptom of a range of underlying pathologies, such as cardiovascular disease,[2] diabetes mellitus,[3] and hypertension.[4] Interestingly, these conditions are well associated with seasonal fluctuation with the highest peak in winter.[5,6] Similar studies on ED revealed that evidence from online search engine queries across a 10-year period in the United States demonstrated seasonal variation in ED with an increase in winter months.[7] A cross-sectional study from a tertiary university hospital across 10 years showed that ED admissions are associated with higher peaks in the winter season.[8]

This seasonal variation of ED can be explained by shared risk factors with systemic diseases that show similar seasonal variation, and endothelial dysfunction appears to be an important common pathway among these conditions. Since we have already found that the occurrence of ED demonstrates seasonal variation with higher rates in winter, direct correlations between cold weather or cold stress and ED or endothelial dysfunction is yet to be thoroughly investigated.

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Endothelial dysfunction is generated when there is an imbalance in the production or bioavailability of endothelium-derived nitric oxide, generating a decreased vasodilator response and a prothrombotic and proinflammatory endothelium.[9] Bradykinin is a potent short-lived vasoactive peptide, which acts as an inflammatory mediator; it is released in pathologic conditions such as trauma and inflammation, which bind to its kinin receptors. The action of bradykinin is mediated through an interaction with cell surface bradykinin receptors. The 2 subtypes of bradykinin receptors are B1 and B2 (B1R and B2R), which belong to the G protein–coupled receptor family.[10] Given the important role for B1R in mediating inflammatory response and immune cell infiltration, B1R-mediated signaling mechanisms play a role in several cardiovascular diseases, including hypertension, heart failure, stroke, diabetes, and atherosclerosis.[11] Multiple studies have confirmed that activation of B1R can directly onset the inflammatory response in different types of endothelial cells[12,13] through upregulating cytokines such as TNF-α,[14] TGF-β1,[15] and IL-6[13] and cause endothelial tissue damage. Studies on B1R and its role in fibrosis of different organs suggest that deletion or inhibition of B1R reduces fibrosis in kidney,[16] skin,[17] and the paranasal sinuses,[18] and in terms of treatment for renal fibrosis, B1R antagonists have become the most promising drug target. Hence, in addition to the endothelial dysfunction previously mentioned, fibrosis of the erectile tissue is one of the key factors contributing to ED.[19,20]

However, the functional roles of B1R in ED, especially in the erectile tissue, have not been fully elucidated. Therefore, in this study, we aim to investigate the therapeutic role of the B1R antagonist in a cold stress–induced rat model of ED. The hypothesis is that the B1R antagonist improves endothelial dysfunction and reduces fibrosis by downregulating the expression of inflammatory cytokines in erectile tissue.

Methods

Ethical approval

Protocols for rats used in this study were approved by our university's Animal Use for Research and Education Committee.

Animals

Animal experiments were conducted on male Sprague-Dawley rats, 6 to 8 weeks old, weighing 180 to 220 g.

Cold stress–induced rat model of ED

After the rats were allowed to acclimate for 3 weeks, apomorphine (APO)–induced penile erection tests were performed to confirm normal erectile function. Rats were randomly divided into control (n = 10) and cold stress (n = 20) groups. Animals from the control group were housed in an environment of 22 ± 1 °C with humidity of $55\% \pm 5\%$ under a 12-hour light-dark cycle and received filtered water and standard food. Animals from the cold stress group were housed in cages (n = 5 per cage) in a ventilated temperature- and humidity-controlled environment of 4 °C for 8 h/d with constant humidity of $55\% \pm 5\%$ under a 12-hour light-dark cycle and received filtered water and standard food for 4 weeks. All animals were maintained on their original environment and diet until the end of this study.

Evaluation of erectile function

APO-induced penile erection

APO was dissolved in saline with ascorbic acid (0.2 mg/mL) at a concentration of 10 mg/mL. After the rats were put into roofless high glass enclosures in a dark and quiet room for 10 minutes, APO solution was given through subcutaneous injection at a dosage of 90 mg/kg; then, the rats were put into the roofless high glass enclosures again for 30 minutes for observation and assessment, and the numbers of erections and the time for first erection were observed and recorded. A full erection is noted as the emergence and licking of the engorged glans penis.[21]

ICP/MAP measurement

In this experiment, erectile function was assessed by the ratio of intracavernosal pressure (ICP) to mean arterial pressure (MAP).[22,23] Rats were put under anesthesia by 5% sodium pentobarbital, and the pelvic ganglion and cavernous nerve were exposed (Figure 1A) at the lateral side of the base of the prostate by midline laparotomy. The penis was exposed by removing overlying skin and ischiocavernosus muscle. Two 24-gauge needles were connected to PE-50 tubes with heparinized saline (250 IU mL^{-1}): one was inserted into the right carotid artery to measure MAP; the other was inserted into the corpus cavernosum (CC) to measure ICP. PE-50 tubes were connected to the data acquisition system (BL420; Techman Software Co., Ltd, Chengdu, China). The cavernous nerve was stimulated with a stainless-steel bipolar hook electrode with the following parameters: 15 Hz, pulse width of 1 millisecond, and 7.5 V for 60 seconds. The ratio of maximal ICP (mm Hg) to MAP (mm Hg) was recorded.

Treatment with the B1R antagonist

After 4 weeks of exposure to cold stress, 10 rats were randomly picked from the cold stress group and given the B1R antagonist Lys-(des-Arg9, Leu8)-bradykinin trifluoroacetate salt (CAS 71800-37-8) through an intraperitoneal injection at the dosage of 500 μg/kg/d for 3 weeks, while the rest of cold stress group was still exposed to cold stress. During treatment, rats in the B1R antagonist treatment group were still exposed to cold stress and received filtered water and standard food. The rats from the control group were given the same amount of saline solution at the same dosage.

Western blot analysis

The middle part of the penile shaft was stored at −80 °C until Western blotting. The penile tissue of Sprague-Dawley rats from different groups was placed in a radioimmunoprecipitation assay buffer, lysed for 1 hour, and centrifuged at 120,00 *g* at 4 °C for 10 minutes. Total protein extraction and Western blot analysis were quantified via the BCA Protein Analysis Kit (PC0020; Solarbio). Protein samples (10 μg/well) were separated by 12% SDS-PAGE, transferred onto PVDF membranes, and blocked with 5% skim milk powder at room temperature for 2 hours. Membranes were incubated overnight at 4 °C with the following primary antibodies: anti–TNF-α (ab6671, 1:500; Abcam), anti–TGF-β1 (ab92486, 1:500; Abcam), anti-nNOS (SC-398843, 1:500; Santa Cruz Biotechnology), anti-eNOS (76198, 1:1000; Abcam), anti-CD31 (281583, 1:2000; Abcam); anti–IL-6 (9324, 1:1000; Abcam); anti-GAPDH (AF7821, 1:6000; Affinit), and anti–bradykinin receptor B1 polyclonal (BML-SA632, 1:500; Enzo Life Sciences).

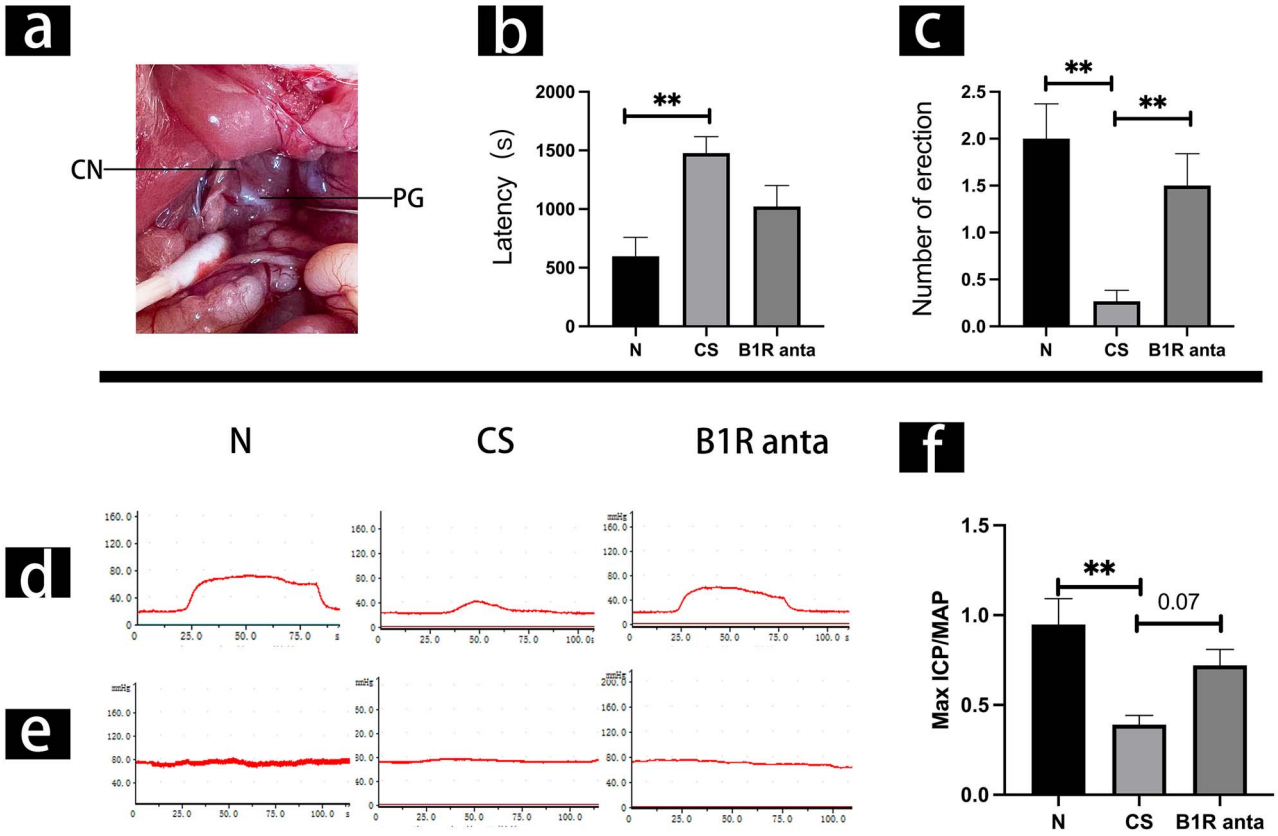

Figure 1. Different evaluations of erectile function before and after treatment. (A) Superolateral view of the pelvis of the rats subjected to intracavernosal pressure measurement, illustrating the cavernous nerve (CN) and pelvic ganglion (PG). Comparison of (B) erection latency time and (C) number of erections between groups in the APO-induced penile erection test. APO, apomorphine. Representative (D) ICP curves and (E) MAP curves in response to cavernous nerve electric stimulation. (F) Comparison of ICP/MAP ratios. $^{*}P < .05$. $^{**}P < .001$. B1R anta, bradykinin B1 receptor antagonist treatment group; CS, cold stress group; ICP, intracavernous pressure; MAP, mean arterial pressure; N, normal control group.

Following the primary antibody incubation, membranes were incubated with horseradish peroxidase–conjugated secondary antibody (ZB2301, ZB2305, 1:6000; ZSGB-BIO, Inc) for 2 hours at room temperature. Protein bands were visualized with ECL reagent (BioSharp), and Image J (version 1.53a; National Institutes of Health) was used for densitometry analysis. Protein expression was presented as the ratio of the absorbance value of the targeted protein to the internal reference absorbance value of the internal control. All experiments were repeated at least 3 times.

Masson staining

Masson staining with a trichrome stain kit (Sigma-Aldrich Co) was performed to visualize fibers in tissues, following the manufacturer's instructions. Briefly, the tissue slides were deparaffinized, stained in preheated Bouin solution, and washed in running tap water to remove the yellow color from sections. Slides were then stained in Working Weigert's Iron Hematoxylin Solution, Biebrich Scarlet-Acid Fucshin, Working Phosphotungstic/Phosphomolybdic Acid Solution, and Aniline Blue Solution. The stained slides were observed under an optical microscope (10× magnification).

Histology

For immunohistochemistry, the middle part of the penile shaft was fixed in 4% paraformaldehyde in phosphate-buffered saline overnight and stored in 70% ethanol at 4 °C until sectioning. Tissue slices (5 μm) were deparaffinized in xylene and rehydrated in 100%, 95%, 70%, and 50% ethanol and water. A solution of 3% H_2O_2 was used to inhibit endogenous peroxidase. After blocking with 5% bovine serum albumin (Keygen Biotech) for 30 minutes, the tissue was incubated with the following antibodies at 4 °C overnight: anti–TNF-α (AF7014, 1:200; Affinity), anti–TGF-β1 (BF8012, 1:200; Affinity), and anti–IL-6 (9324, 1:500; Abcam). Subsequently, the tissue was incubated with horseradish peroxidase–conjugated goat anti-rabbit secondary antibody (1:1000, ab6721; Abcam) for 2 hours at room temperature. 3,3′-Diaminobenzidine and hematoxylin were used for target protein and nuclear staining, respectively. The primary antibody was replaced with 5% bovine serum albumin for the negative control. Images acquired from 5 fields of each section at 400× magnification were used for analysis (5 sections from each subject). Images were captured via a Nikon microscope (Eclipse Ti-S; Nikon Instruments Co, Ltd).

Data analysis and statistics

Quantitative data are reported as mean ± SE. Means of 2 groups were analyzed with the Student t test, and >2 group means were analyzed by 1-way analysis of variance, followed by the Duncan multiple-comparison test of significance via a commercial software package (SPSS 28.0; IBM). $P < .05$ was considered statistically significant.

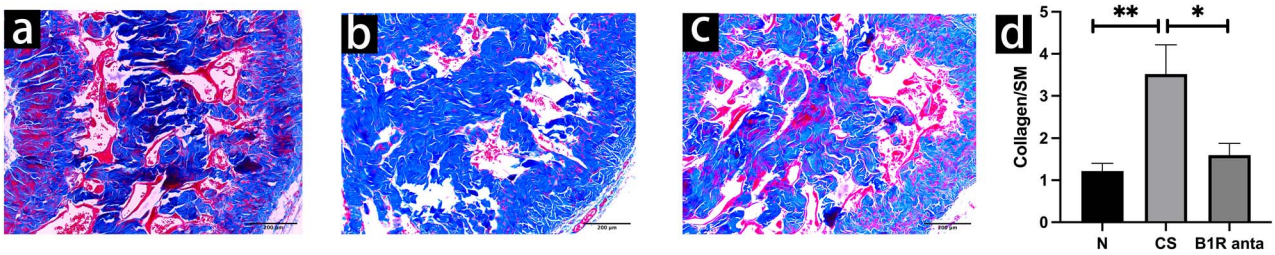

Figure 2. Masson staining results show collagen fibers, smooth muscles, and their ratio before and after treatment. Representative Masson staining results of (A) normal control group, (B) cold stress group, and (C) bradykinin B1 receptor antagonist treatment group. (D) Ratio of collagen fibers to smooth muscle cells by expression level per group. $^{*}P < .05$. $^{**}P < .001$. Red, smooth muscle cells; blue, collagen fibers. Scale bar = 200 μm.

Results

Impaired erectile function in response to cold stress was enhanced by B1R antagonist treatment

To determine whether cold stress caused ED, we assessed erectile function using an APO-induced penile erection test (Figure 1B and 1C) and ICP/MAP (Figure 1D-F). After cold stress, erection latency time was significantly increased, while the number of erections and maximum ICP/MAP were significantly decreased. After rats received the B1R antagonist treatment, we observed a shortened erection latency time, a greater number of erections, and an increasing trend for maximum ICP/MAP.

Treatment with the B1R antagonist reduced cold stress–induced fibrosis in the CC

Masson staining showed that the area of collagen fibers (in blue) was increased while the area of smooth muscles (in red) was decreased in response to cold stress, and this effect was reversed after treatment with the BR1 antagonist. In all, collagen fibers and smooth muscle were significantly increased in rats from the cold stress group and significantly reduced after treatment with the B1R antagonist (Figure 2).

Cold stress upregulated B1R expression in the CC

Western blotting analysis showed that after being exposed to cold stress for 4 weeks, the expression of B1R was significantly increased in the CC, as we expected (Figure 3).

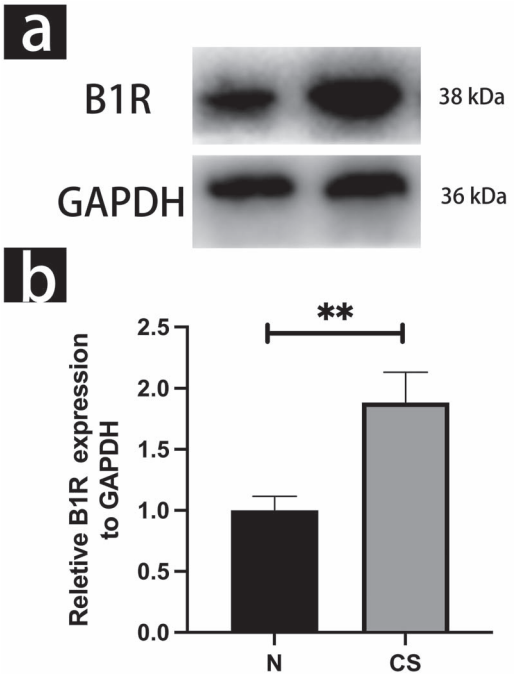

Figure 3. B1R expression in the corpus cavernosum in response to cold stress. (A) Representative Western blotting result of B1R. (b) B1R expression level examined by Western blotting. $^{*}P < .05$. $^{**}P < .001$. B1R, bradykinin B1 receptor; CS, cold stress group; N, normal control group.

Treatment with the B1R antagonist prevented diminished NOS and CD31 expression in the CC in cold stress–induced ED rats

In Western blotting analysis, we observed significantly reduced eNOS and nNOS expression in the CC in response to cold stress. The treatment with the B1R antagonist partially reversed this effect (Figure 4). CD31 expression was downregulated after exposure to cold stress and reversed by treatment with the B1R antagonist (Figure 4).

BIR blockage decreased local cytokine expression in the CC

Immunohistochemistry results showed increased levels of TGF-β1, TNF-α, and IL-6 expression on the cavernous sinus endothelium, and this change was reversed after B1R treatment. In Western blotting analysis, TGF-β1, TNF-α, and IL-6 expression was significantly elevated in response to cold stress, and these effects were reversed by B1R antagonist treatment (Figures 5 and 6).

Discussion

We report decreased erectile function (reduced erection frequency, prolonged erection latency time, lower ICP/MAP) and overexpression of B1R on erectile tissue in response to cold stress, suggesting possible involvement of this receptor in penile erection mechanism. Thus, we assessed the therapeutic roles of the B1R antagonist Lys-(des-Arg9, Leu8)-bradykinin trifluoroacetate salt (CAS 71800-37-8) on ED. Our results showed that the B1R antagonist enhances erectile function through increased erection frequency, shortened erection latency time, and higher ICP/MAP. Also, it reduces fibrosis in penile tissue by inhibiting TGF-β1, TNF-α, and IL-6. It improves endothelial function of the erectile tissue by upregulating the expression of nNOS and CD31.

Previous studies on cold weather or cold stress and ED have ascribed plausible explanations to lifestyle or behavioral changes in winter months, including seasonal affective disorder, increased hours spent indoors, and winter mood

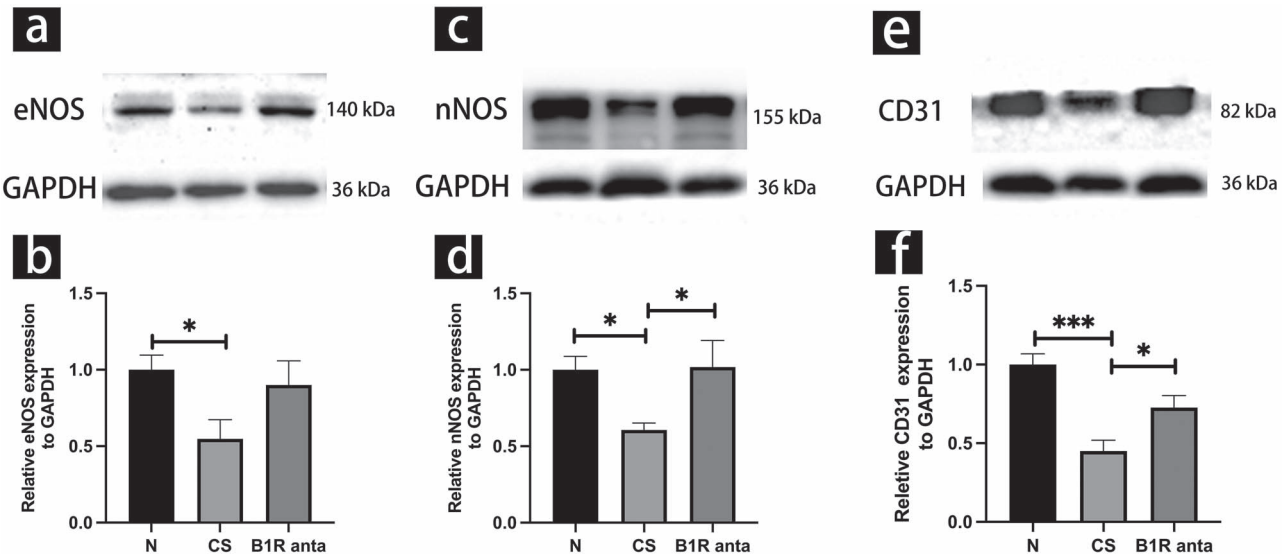

Figure 4. Western blotting shows eNOS, nNOS, and CD31 expression before and after treatment. Representative Western blotting results: (A) eNOS and (B) nNOS. Expression levels examined by Western blotting: (C) eNOS and (D) nNOS. Representative Western blotting (E) result and (F) expression level: CD31. $^{*}P < .05$. $^{**}P < .001$. $^{***}P < .0001$. B1R anta, bradykinin B1 receptor antagonist treatment group; CS, cold stress group; eNOS, endothelial nitric oxide synthase; N, normal control group; nNOS, Neuronal nitric oxide synthase

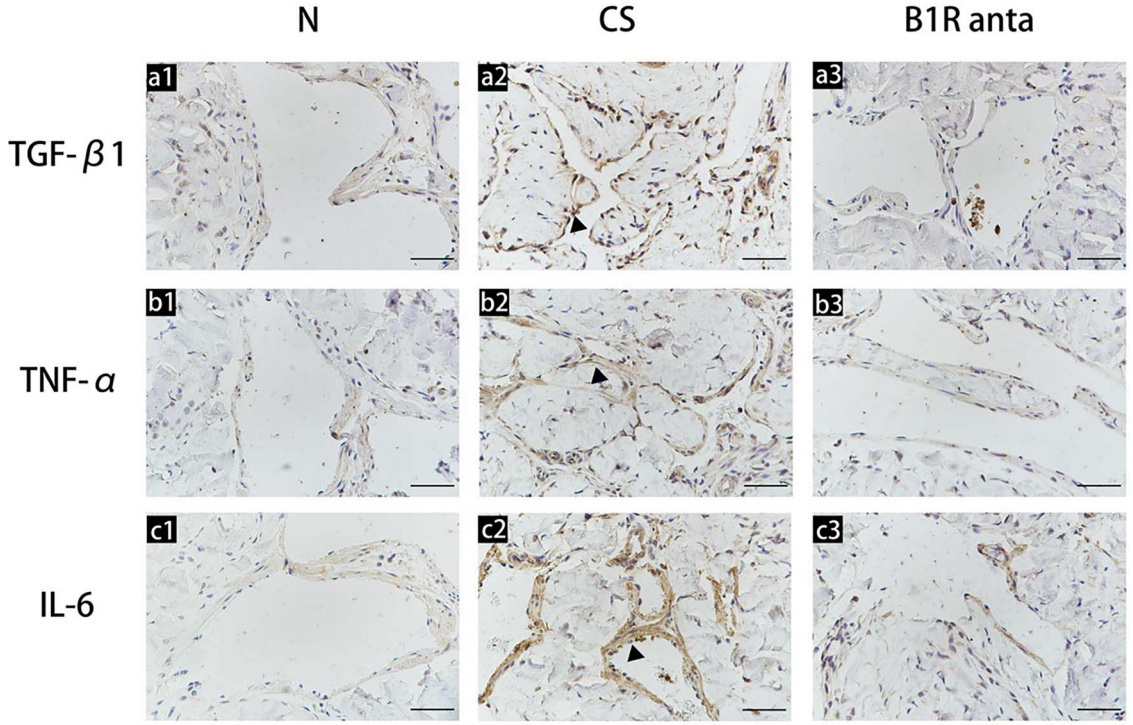

Figure 5. Immunohistochemistry results show the distribution of TGF-β1, TNF-α, and IL-6 in the cavernous sinus before and after treatment. Representative expression in each group: (A1-A3) TGF-β1, (B1-B3) TNF-α, and (C1-C3) IL-6. B1R anta, bradykinin B1 receptor antagonist treatment group; CS, cold stress group; N, normal control group. Scale bar = 20 μm. ▲, cavernous sinus endothelium.

disorders, among others. In our study, we aimed to identify a more direct correlation between cold stress and ED, and we observed decreased erection frequency, prolonged erection latency time, and decreased ICP/MAP in response to cold stress, meaning that cold stress is indeed correlated with ED. Additionally, in response to cold stress, expression of B1R and levels of TNF-α, TGF-β1, and IL-6 in the endothelium of penile tissue were significantly elevated, and penile tissue indicated elevated collagen fibers and smooth muscle, suggesting possible fibrotic activity in erectile tissue. Other studies on B1R indicated that its activation is critically involved in the inflammatory response in different organs, such as the brain, kidney, and cardiovascular system,[24] and the increased levels of TNF-α, TGF-β1, and IL-6 in the CC in this study might be the result of an activated bradykinin system. Numerous studies on TNF-α and ED have revealed

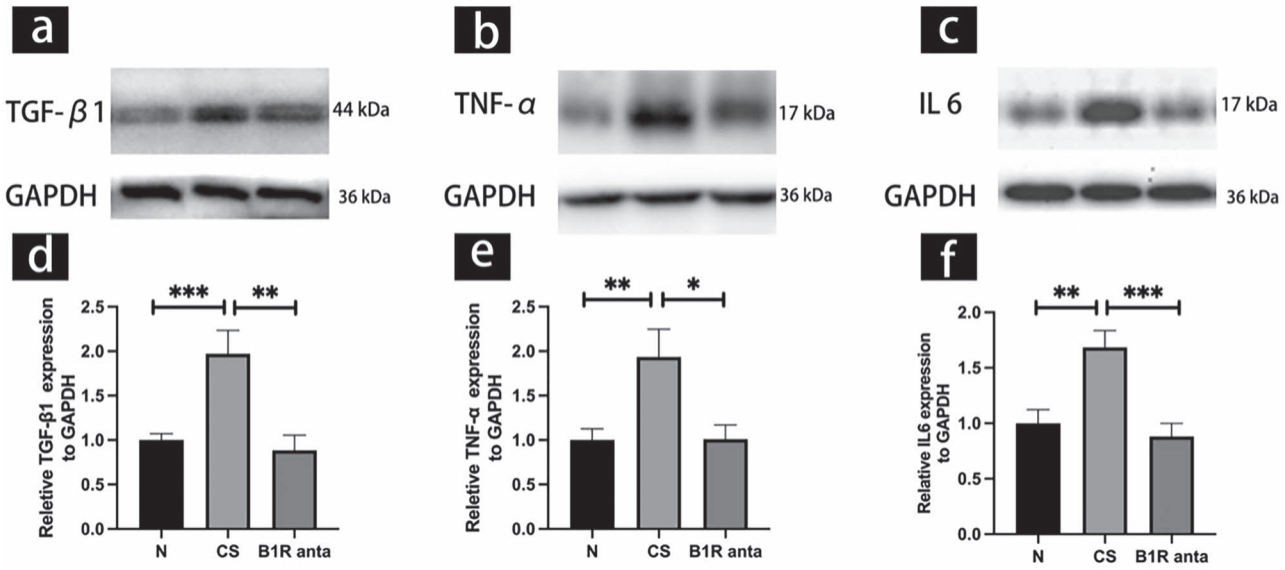

Figure 6. Western blotting showing cytokine expression before and after treatment. Representative Western blotting results: (A) TGF-β1, (B) TNF-α, and (C) IL-6. Expression levels examined by Western blotting: (D) TGF-β1, (E) TNF-α, and (F) IL-6. $^{*}P < .05$. $^{**}P < .001$. $^{***}P < .0001$. B1R anta, bradykinin B1 receptor antagonist treatment group; CS, cold stress group; N, normal control group.

that overexpression of TNF-α in penile tissue impairs corpora cavernosa reactivity[25,26] through decreased NOS expression. TNF-α can also suppress testosterone release, which in turn results in ED.[27] We additionally found that eNOS, nNOS, and CD31 expression in penile tissue decreased in response to cold stress, indicating possible damage to endothelium. A recent study on interactions among various cytokines suggested that TNF-α enhances TGF-β1–induced endothelial-to-mesenchymal transition via TGF-β1 signal augmentation.[28] TGF-β1 is also extensively implicated in the pathogenesis of fibrosis through Smad-dependent or non-Smad pathways.[29] Furthermore, studies on IL-6 suggested that its upregulation promotes renal,[30] muscular,[31] and skin fibrosis[32] and their blockage protects against it. Thus, we evaluated the fibrosis activity in the CC through Masson staining and found that collagen fibers and smooth muscle were significantly elevated in rats from the cold stress group, suggesting possible fibrotic activity, which might be detrimental to the pathogenesis of ED.

The B1R antagonist has proven to show antifibrotic and anti-inflammatory effects in different types of cells,[33,34] but its role in the CC remains understudied. In our study, after treatment with the B1R antagonist, we observed increased erectile function (more frequent erections, shortened erection latency time, and higher ICP/MAP), which prevented overexpression of the cytokines and upregulated nNOS and CD31 expression in the CC. It also lowered collagen fibers and smooth muscle in the CC. These results along with functional studies suggest that the B1R antagonist enhances erectile function by promoting anti-inflammatory and antifibrotic activity in the CC. Furthermore, the downregulation of TNF-α, TGF-β1, and IL-6 caused by the B1R antagonist might be responsible for the antifibrotic activity, while the upregulation of nNOS and CD31 is critical to endothelial function. In addition to its role as an endothelial marker, CD31 is highly expressed at endothelial cell-cell junctions, where it functions as an adhesive stress response protein to maintain endothelial cell junctional integrity and speed restoration of the vascular permeability barrier following inflammatory or thrombotic challenge.[35,36] Accordingly, the increased expression of CD31 after cold stress suggests compromised endothelial cell integrity and permeability, which subsequently lead to fibrotic activity in the CC. Altogether, these results support the hypothesis that B1R is present in the CC and plays a part in pathologic mechanisms of fibrosis caused by cytokine overexpression and that its inhibition protects against fibrosis and endothelial damage.

This animal model might seem clinically irrelevant because in real life, it is highly unlikely that a person is constantly at 4 °C without variation in temperature. However, there are still some occupations and situations in which people are constantly subjected to a cold environment, such as cold storage workers, arctic workers, winter swimmers, and those taking cold showers in winter. If the frequency of the cold exposure is not properly controlled, it might put those at the risk of ED. On the similar topic, we found research that reported decreased infertility in adult rats after cold water immersion.[37] Therefore, these findings not only add to the debate over cold stress and ED but also cast new light on the roles that the B1R plays in mechanisms of ED.

The major limitation of this study is that we studied the role of B1R only in cold stress–induced ED rats. However, ED has other risk factors, such as diabetes, hypertension, and hyperlipidemia. Whether B1R plays the same role in other types of ED still needs to be investigated. Last, the B1R antagonist that we used in this study is the intraperitoneally injected Lys-(des-Arg9, Leu8)-bradykinin. However, there are other ways of blocking this receptor, such as oral administration of SSR240612 and genetic knockout, and we plan to use genetic knockout rats in the next phase of our study to obtain more certain results.

Nonetheless, our data support the hypothesis that cold stress impairs erectile function and B1R blockade ameliorates the symptoms of ED, possibly by reversing fibrosis and endothelial damage in erectile tissue. This may suggest a new application for existing B1R antagonist drugs.

Acknowledgments

Conceptualization: S.A. and A.R. Methodology: B.W., P.H., J.J., S.A. Investigation: B.W., P.H., J.J., S.A. Writing–original draft: A.R. Writing–review and editing: A.R., D.T. Supervision: S.A. Funding acquisition: S.A.

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Conflicts of interest None declared.

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