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Antioxidant properties of berberine on cultured rabbit corpus cavernosum smooth muscle cells injured by hydrogen peroxide[1]

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Key words

Abstract

Aim: To investigate the antioxidant properties of berberine (Ber) on corpus cavenosum smooth muscle cells (CCSMC) in penile erectile dysfunction. **Methods:** We examined the effects of Ber on cultured rabbit CCSMC damaged by hydrogen peroxide (H_2O_2) through examining cell viability by methyl thiazolyl tetrazolium assay and assessing the level of malondialdehyde (MDA), superoxide dismutase (SOD) activity, nitric oxide (NO) products, and lactate dehydrogenase (LDH) release in cells after stimulation with H_2O_2. **Results:** Treatment with 1 mmol/L H_2O_2 significantly decreased the cell viability, NO products, and SOD activity of CCSMC from 100% to 48.57%±4.1% ($P<0.01$), 66.8±16.3 to 6.7±2.1 µmol/L ($P<0.01$), and 49.5±1.8 to 30.1±2.6 U/mL ($P<0.01$), respectively, and increased LDH release and MDA content from 497.6±69.5 to 1100.5±56.3 U/L ($P<0.01$) and 3.7±1.3 to 78.4±2.9 nmol/mg protein ($P<0.01$), respectively. However, treatment with different concentrations of Ber (10–1000 µmol/L) inhibited the damaging effects of H_2O_2, with increased cell viability ($P<0.05$ or $P<0.01$), NO production ($P<0.01$), and SOD activity ($P<0.01$) and decreased LDH release and MDA content (both $P<0.01$). **Conclusion:** Ber could produce its antioxidant action on oxidative stress-induced cultured CCSMC. These effects may be of benefit in the prevention of penile erectile dysfunction.

Introduction

Normal penile erection depends on vascular smooth muscle relaxation in erectile tissue and penile arteries, with the main mediator of relaxation being nitric oxide (NO)[1]. Evidence suggests that oxidative stress mediated through the superoxide radical (superoxide) and other reactive oxygen species (ROS) may be central to impaired cavernosal function in erectile dysfunction (ED)[2]. The inactivation of NO by superoxide results in impaired penile NO transmission and smooth muscle relaxation. Given our current understanding of ED pathophysiology, antioxidants may be beneficial in alleviating penile ED in the short and long term.

Berberine (Ber), an isoquinoline alkaloid, is a well-known component of the Chinese herb Huanglian (*Coptis chinensis* Franch) of the family Berberidaceae. The compound exhibits pharmacological activities of antioxidant action[3,4]. In our previous study, Ber was found to induce NO-mediated relaxation of rabbit corpus cavernosum[5]. Therefore, we aimed to observe the effects of Ber on rabbit corpus cavenosum smooth muscle cells (CCSMC) injured by hydrogen peroxide (H_2O_2) and to understand the role of Ber in the prevention of ED.

Materials and methods

Animals Male New Zealand rabbits (22–26 weeks old, 3–4 kg body weight; supplied by the Animal Services Center, Yunyang Medical College, Shiyan, China) were used. The rabbits had free access to a standard diet (Altromin pellets) and tap water until use. Fasting was achieved by depriving food but not drinking water 16 h before surgery.

Isolation and culture of CCSMC The CCSMC were isolated from male New Zealand rabbits as described previously[6].

Briefly, the penises were excised at the point of adhesion to the lower pubic bone. The organs were cleared from fat and connective tissue. The facia penis, glans penis, corpus spongiosum, and tunica albuginea were carefully removed. The corpus cavernosum was washed in sterile phosphate-buffered saline (PBS), cut into small strips, and placed for 12–18 h at 37 °C in a tube with 5 mL PBS-buffered minimum essential medium (MEM) containing 0.1% collagenase (type I). After incubation, the pieces were rinsed with PBS-buffered MEM and then mechanically dispersed with a pipette (tip inside diameter 0.5 mm) in the medium. Dissociated cells were collected by centrifugation at 1000 r/min for 5 min and resuspended in 5 mL complete MEM (supplemented with 20% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate). The cells were seeded in a 75 cm^2 culture flask and cultured undisturbed for 2–3 d in a humidified 5% CO_2–95% air incubator at 37 °C. After inoculation for 3 d, the anchorage-dependent cells radially spread, and part of the area was confluent. If the color of the medium became yellow (ie, pH <7), the medium was changed immediately to discard the suspension cells and broken fiber and cells. Afterward, the medium was changed every 2–3 d according to the condition.

The cells were subcultured after attaining >90% confluency (at approximately 10–14 d). The previous medium was decanted from the culture flask, PBS was added to wash the cells, and the cells were detached with 0.25% trypsin for 30–60 s. When the most of cells became round and the intracellular space became broader as seen on inverted microscopy, complete Dulbecco's modified Eagle's medium (DMEM) was added to stop the reaction of trypsin. Then the cells on the wall of the culture flask were mechanically dispersed with a pipette and subcultured at a ratio of 1:2 or 1:3 in the culture flask. The cells from the second to fourth passage were used.

Immunohistochemical staining for α-smooth muscle actin The laboratory procedure for SP immunostaining involved a streptomycin avidin-peroxidase immunochemistry kit (Zhongshan Co, Beijing, China). Briefly, the anchorage CCSMC were digested with 0.25% trypsin for 3–5 min and suspended. The suspended cells were placed in 24-well culture plates, some with coverslips, in complete growth medium and cultured at 37 °C. After 2–3 d, the cells were washed twice with PBS, fixed with 4% formaldehyde-PBS for 10 min, and washed twice with PBS. The cells were permeated with 0.5% Triton-X 100 for 10 min and washed twice with PBS. After being blocked with 5% normal goat serum for 30 min, the cells were incubated with a monoclonal antibody against α-smooth muscle actin (1:100,

Chemicon International, Temecula, CA, USA) at 4 °C overnight. After being washed 3 times with 0.01 mmol/L PBS, the cells were incubated with biotin-labeled goat anti-mouse immunoglobulin G (IgG, 1:400) at 37 °C for 30 min. After 3 washings with 0.01 mmol/L PBS, the cells were incubated with horseradish peroxidase-labeled streptomycin avidin (1:400) at 37 °C for 20–30 min, and finally diaminobenzidine (DAB) was added for staining. To ensure the reliability and specificity of the results of the immunohistochemical staining, goat serum and PBS were used to replace the first antibody in our control test.

Effect of Ber on cultured CCSMC injured by H_2O_2

Cell viability assays In this experiment, the groups of cells included the control group (cultured in medium only), model group (cultured in 1 mmol/L H_2O_2), and test groups (cultured in different concentrations of Ber and 1 mmol/L H_2O_2).

A methyl thiazolyl tetrazolium (MTT) assay (Duchefa, The Netherlands) to measure cell viability was performed as described previously[7]. The CCSMC were digested with 0.25% trypsin and seeded at 2×10^4 cells/well in 200 μL in 96-well culture plates. The cells were first cultured for 2–3 d in MEM medium supplemented with 10% fetal bovine serum under normal conditions, then 24 h in medium containing 5% fetal bovine serum. Subsequently, the cells were incubated with Ber (0.1–1000 μmol/L) for 24 h, followed by 4 h stimulation with 1 mmol/L H_2O_2, and then incubated for an additional 4 h at 37 °C with 20 μL 0.5 mg/mL MTT. The medium was removed and 150 μL/well DMSO was added to resolve the formazan salts. The plates were shaken for 10 min to allow formazan dissolution and then absorbance was measured on a microplate reader at 490 nm. The cell survival rate was calculated: (absorbance of treated group–absorbance of blanks)/(absorbance of control–absorbance of blanks)×100 as a percentage.

Measurement of NO products NO undergoes a series of reactions with several molecules present in biological fluid, leading to the accumulation of the final products, nitrate, and nitrite (NO_2^- and NO_3^-). Thus, the index of total production is the sum of both NO_2^- and NO_3^- accumulated in the tissue samples. The CCSMC were seeded at 2×10^4 cells/well in 96-well culture plates and treated as described above. The cell supernatant was collected and the NO products were determined according to the manufacturer's (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) recommendation on a microplate reader at 550 nm.

Lactate dehydrogenase release Cell injury induced by H_2O_2 was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) released from damaged or

destroyed treated cells, and an aliquot of bathing media was combined with NADH and pyruvate solutions. LDH activity proportional to the rate of pyruvate loss was assayed according to the manufacturer's (Nanjing Jiancheng Bioengineering Institute, China) recommendation on a microplate at 440nm.

Determination of superoxide dismutase activity　The superoxide dismutase (SOD) activity of the treated cell supernatant was determined on a microplate at 550 nm. One unit of SOD was defined as the amount required to inhibit the rate of reduction of cytochrome c by 50%.

Assessment of malondialdehyde content　Malondialdehyde (MDA), an end product of lipid peroxidation, reacts with thiobarbituric acid to form a colored substance. The CCSMC were seeded at 2×10^4 cells/well in 96-well culture plates and treated. Then the medium was discarded and the cells were washed twice with PBS. Subsequently, a solution of 1 mL 0.1 mmol/L PBS-0.05 mmol/L EDTA (pH 8.0) and 50 µL 1% Triton-X 100 was added into the well to lyse the cells. The MDA content of the cells was determined on a microplate reader at 532 nm. The protein concentrations were determined by Coomassie brilliant blue method.

Drugs and chemicals　Ber (chemical purification>98%) was purchased from Sigma (St Louis, MO, USA). DMEM (high glucose) medium and collagenase type I were from Gibco (USA). Trypsin (1:250) and *L*-glutamine were from Amresco (Solon, Ohio, USA). MTT was from Duchefa (The Netherlands). The detection kits for SOD, MDA, NO, LDH, and Coomassie brilliant blue G250 were from Nanjing Jiancheng Bioengineering Institute (China). Fetal bovine serum was from Hangzhou Sijiqing Biological Engineering Materials (Hangzhou, China).

Statistical analysis　Data are expressed as mean±SEM. Differences between groups were assessed by the Student's *t*-test. *P*<0.05 was considered significant.

Results

Morphology and verification of CCSMC　After culture for approximately 10–14 d, the CCSMC grew radially and a group of cells grew in parallel along their longitudinal axis, which showed obvious orientation (Figure 1). The CCSMC were verified as spindle-shaped cells by anti-α-actin monoclonal antibody immunohistochemical staining (Figure 2).

Effects of Ber on CCSMC injured by H$_2$O$_2$　Pretreatment with 1 mmol/L H$_2$O$_2$ decreased the cell viability from 100% for control cells to 48.57%±4.1% (*P*<0.01), and treatment with Ber (10–1000 µmol/L) inhibited the effect of H$_2$O$_2$ (*P*<
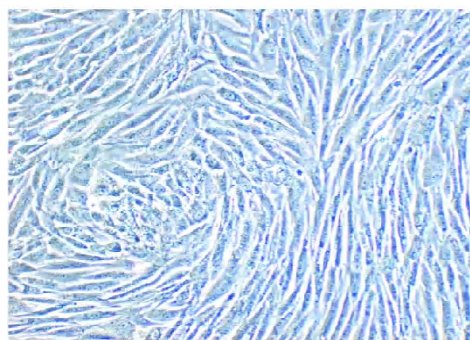

Figure 1.　Primary cultured rabbit corpus cavernosum smooth muscle cells (×100). Confluent cells were parallel or swirling and demonstrated the characteristic peak and vallecular growth of smooth muscle cells.
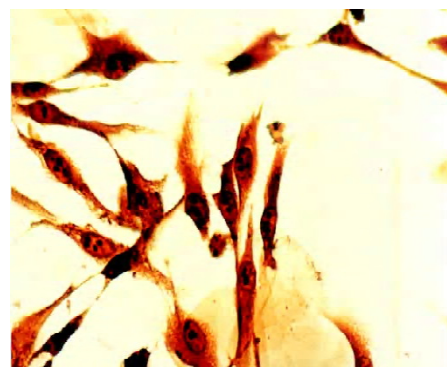

Figure 2.　Immunohistochemistry staining for α-smooth muscle actin of CCSMC. Cells were incubated with monoclonal antibody against α-smooth muscle actin (1:100) at 4 °C overnight. After 3 washings with 0.01 mmol/L PBS, the cells were incubated with biotin-labeled goat antimouse IgG (1:400) at 37 °C for 30 min. After 3 washings with 0.01 mmol/L PBS, the cells were incubated with horseradish peroxidase-labeled streptomycin avidin (1:400) at 37 °C for 20–30 min and finally DAB was added for staining (×100).

0.05 or *P*<0.01; Figure 3).

The NO level in the CCSMC was far lower (66.8±16.3 µmol/L) than that in the endothelial cells (569±20.3 µmol/L). Treatment with 1 mmol/L H$_2$O$_2$ decreased the level of NO from 66.8±16.3 to 6.7±2.1 (*P*<0.01). However, incubation with 1–1000 µmol/L Ber in a concentration dependent manner increased the level of NO (*n*=8, *P*<0.01; Figure 4).

H$_2$O$_2$ treatment increased LDH release, as compared with the control CCSMC, from 497.6±69.5 to 1100.5±56.3 U/L (*P*<0.01), and treatment with 0.1–1000 µmol/L Ber inhibited this effect (*n*=8, *P*<0.05 or *P*<0.01; Figure 5). SOD activity was decreased with H$_2$O$_2$ treatment from 49.5±1.8 to 30.1±2.6 U/mL (*P*<0.01), and treatment with 1–1000 µmol/L Ber re-
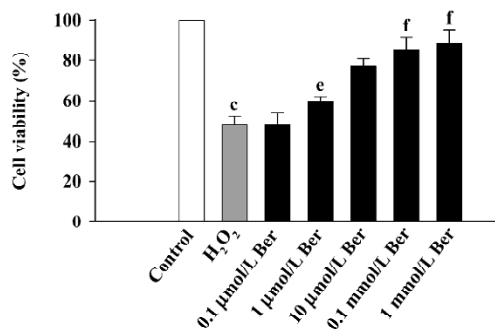
Figure 3. Effect of Ber (0.1−1000 μmol/L) on cell viability of corpus cavernosum smooth muscle cells (2×10^4 cells) injured by 1 mmol/L H_2O_2. MTT assay was used to measure cell viability. Cells were seeded at 2×10^4 cells/well in 200 μL in 96-well culture plates and first cultured for 2−3 day in MEM medium supplemented with 10% fetal bovine serum under normal conditions, then cultured 24 h in medium containing 5% fetal bovine serum. Subsequently, cells were incubated with Ber (0.1−1000 μmol/L) for 24 h, followed by 4 h stimulation with 1 mmol/L H_2O_2, and then incubated for an additional 4 h at 37 °C with 20 μL 0.5 mg/mL MTT. Medium was removed and 150 μL/well DMSO was added to resolve the formazan salts. Plates were shaken for 10 min to allow formazan dissolution and then absorbance was measured on a microplate reader at 490 nm. Cell survival rate was calculated: (absorbance of treated group-absorbance of blanks)/(absorbance of control-absorbance of blanks)×100 as a percentage. *n*=8. Mean±SD. $^cP<0.01$, compared with the control group; $^eP<0.05$, $^fP<0.01$, compared with the H_2O_2 group.
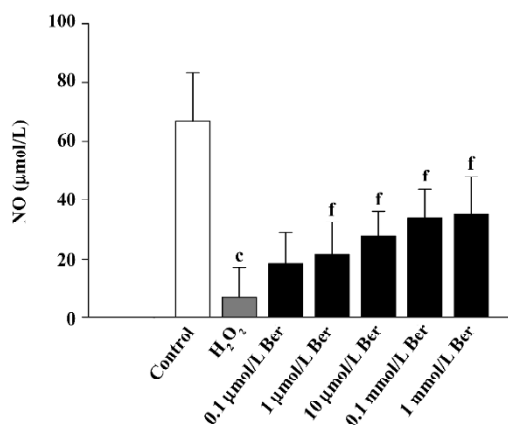

Figure 4. Effect of Ber (0.1−1000 μmol/L) on NO products of the corpus cavernosum smooth muscle cells (2×10^4 cells) injured by 1 mmol/L H_2O_2. Cells were seeded at 2×10^4 cells/well in 96-well culture plates and treated as described. Then the supernatant was collected and NO products were determined on a microplate at 550 nm. *n*=8. Mean±SD. $^cP<0.01$, compared with the control group; $^fP<0.01$, compared with the H_2O_2 group.

stored the activity (*n*=8, *P*<0.05 or *P*<0.01; Figure 6).

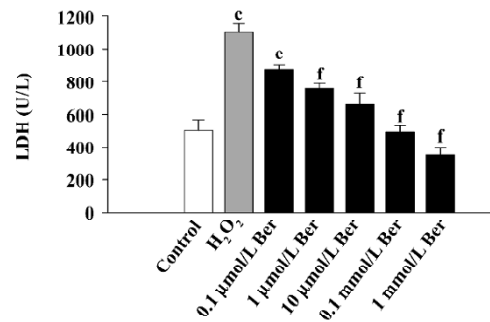However, 1 mmol/L H_2O_2 treatment significantly increased

Figure 5. Effect of berberine (0.1−1000 μmol/L) on LDH release of the corpus cavernosum smooth muscle cells (2×10^4 cells) injured by 1 mmol/L H_2O_2. Cells were treated as described and LDH was assayed on a microplate at 440 nm. *n*=8. Mean±SD. $^cP<0.01$, compared with control group; $^eP<0.05$, $^fP<0.01$, compared with H_2O_2 group.
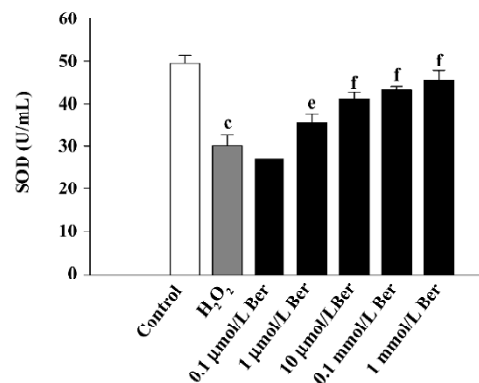

Figure 6. Effect of berberine (0.1−1000 μmol/L) on SOD activities of the corpus cavernosum smooth muscle cells (2×10^4 cells) injured by 1 mmol/L H_2O_2. Cells were treated as described. The SOD activity of cell supernatant was determined on a microplate at 550 nm. *n*=8. Mean±SD. $^cP<0.01$, compared with control group; $^eP<0.05$, $^fP<0.01$, compared with H_2O_2 group.

the MDA content from 3.7±1.3 to 78.4±2.9 nmol/mg protein (*P*<0.01), and treatment with 0.1−1000 μmol/L Ber could inhibit this effect (*n*=8, *P*<0.01; Figure 7).

Discussion

Oxidative stress, resulting from a disturbance in the balance between the formation of free radicals in the body and their scavenging, is believed to affect the development of endothelial dysfunction and neuropathy within erectile tissues. High levels of ROS in both blood and tissues act as profibrotic factors that stimulate collagen deposition and reduce the ratio of smooth muscle to collagen, thus impairing tissue compliance to relaxation by "hardening" the arterial media[8]. Oxygen-free radicals exert their cytoplasmic
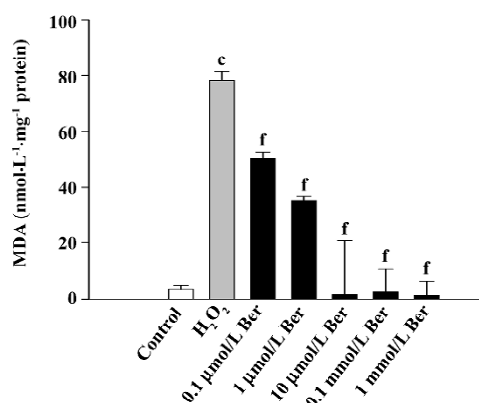
Figure 7. Effect of berberine (0.1–1000 µmol/L) on MDA content of the corpus cavernosum smooth muscle cells (2×10^4 cells) injured by 1 mmol/L H_2O_2. Cells were treated as described. Then, the medium was discarded and cells were washed twice with PBS. Subsequently, 0.1 mol/L PBS–0.05 mmol/L EDTA (pH 8.0) 1 mL and 50 µL 1% Triton-X 100 was added into the well for lysis. The MDA content of the cells was determined on a microplate at 532 nm. Protein concentrations were determined by Coomassie brilliant blue method. n=8. Mean±SD. [c]$P<0.01$, compared with control group; [f]$P<0.01$, compared with H_2O_2 group.

effect by peroxidation of membrane phospholipids, which leads to a change in permeability and loss of membrane integrity[9]. Drugs with multiple mechanisms of protective action, including antioxidant properties, may be one way to minimize tissue injury in human diseases.

Our study provides evidence supporting the paradigm of antioxidant therapy for the prevention of ED. We established a H_2O_2 injured model with cultured rabbit CCSMC and measured cell viability, NO production, and SOD activity; then MDA, a lipid peroxidation product, as an index of oxidative stress; and LDH release, to assess the change in membrane permeability induced by radicals. We found that 1 mmol/L H_2O_2 significantly increased MDA content and LDH release and decreased cell viability, NO production, and SOD activity. However, treatment with 10–1000 µmol/L Ber inhibited the damage induced by H_2O_2 by improving cell viability ($P<0.05$ or $P<0.01$) and decreasing MDA content ($P<0.01$) and LDH release ($P<0.01$).

NO is a mediator of penile erection, and reduced NO levels in the penile corpora is associated with ED[1,9]. With excessive oxidative stress, the reaction of ROS with NO to form peroxynitrite reduces NO concentrations in tissues, which would lead to ED by impairing NO-dependent smooth muscle cell relaxation. Therefore, the effect of Ber on increasing NO production would be beneficial for the prevention of penile ED.

SOD, an antioxidant enzyme, also has an important role in protecting against free radicals by reducing ROS level. We showed that SOD activity was significantly decreased after H_2O_2 exposure. However, treatment with Ber restored SOD activity.

Our results show that Ber has antioxidant action in CCSMC injured by H_2O_2 through increasing cell viability, NO production, and SOD activity and decreasing MDA content and LDH release. These effects of Ber can be of benefit in the prevention of ED.

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