

BASIC SCIENCE

Gosha-Jinki-Gan Improved Erectile Dysfunction Caused by Anti-Cancer Agent Oxaliplatin by Decreasing Transcriptional Expression of Phosphodiesterase-5 in Rats



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ABSTRACT

Background: A platinum-containing anti-cancer agent, oxaliplatin (L-OHP), is known to induce peripheral neuropathy, including erectile dysfunction (ED) as a side effect, while Gosha-jinki-gan (GJG) is a traditional Japanese herbal medicine mainly used for peripheral neuropathy.

Aim: To investigate the effect of GJG on L-OHP-induced ED in rats.

Methods: Twelve-week-old male Wistar/ST rats were categorized into the following groups: Sham, Sham+GJG, L-OHP, and L-OHP+GJG (each n = 10). The L-OHP and L-OHP+GJG groups were injected intravenously with L-OHP (4 mg/kg) for 2 consecutive days in the first week. Statistical significance was determined using Bonferroni's multiple comparison test.

Outcomes: At the end of the study period, erectile function was evaluated by measuring intracavernosal pressure (ICP) and mean arterial pressure (MAP) after cavernous nerve stimulation. Western blot analysis was used to assess the neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) levels, and quantitative polymerase chain reaction was used to assess the expression of phosphodiesterase-5 (PDE-5) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-1.

Results: The ICP/MAP ratio of L-OHP rats (0.34 ± 0.06) was significantly lower than that of Sham rats (0.67 ± 0.03 , $P < .01$), however, the ICP/MAP ratio of L-OHP+GJG rats (0.55 ± 0.01) was significantly higher than that of L-OHP rats ($P < .01$). There were no significant differences in the nNOS and eNOS protein expression between both groups ($P > .05$). GJG administration significantly decreased PDE-5 and NADPH oxidase-1 messenger RNA expressions in the L-OHP+GJG group.

Clinical Translation: This animal model study suggests that GJG might be effective for erectile function in cancer survivors.

Strengths & limitations: Our study identified that GJG had no notable side effects in the treated group. Further investigation of the cavernous nerve would also help elucidate the mechanism of GJG effect, which is a limitation of this study.

Conclusion: We found that GJG administration improved L-OHP-induced ED by improving transcriptional PDE-5 expression. **Kataoka T, Kawaki Y, Kito Y, et al. Gosha-Jinki-Gan Improved Erectile Dysfunction Caused by Anti-Cancer Agent Oxaliplatin by Decreasing Transcriptional Expression of Phosphodiesterase-5 in Rats. Sex Med 2022;10:100484.**

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Key Words: Gosha-Jinki-Gan; Anticancer Agents; Erectile Dysfunction; Phosphodiesterase-5

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INTRODUCTION

There are many cancer patients worldwide, and several chemotherapeutic agents are administered in their treatment. Oxaliplatin (L-OHP), a third-generation platinum-containing anti-cancer drug, is widely used for colorectal cancer, and colorectal cancer is the third most common type of cancer among adults in the Western world.¹⁻² We previously reported that L-OHP caused erectile dysfunction (ED) in rats,³ while neuropathy is a common adverse effect of the platinum agent.⁴ The development of neuropathy is a major concern that may negatively influence a patient's quality of life. Mols et al. reported that 42% of men who had been treated with L-OHP complained of erectile problems.⁵ ED also influences a patient's quality of life, and appropriate treatment is required.

Gosha-jinki-gan (GJG) is a Japanese herbal medicine (Kampo) that contains a mixture of extracts from 10 raw botanical materials in fixed proportions.⁶ In Japan, GJG has been widely used for the treatment of diabetic neuropathy without severe adverse events.⁷ GJG was also effective in treating chemotherapy-induced peripheral neuropathy.⁸ GJG was reported to prevent neuropathy without weakening the efficacy of anti-cancer agents.⁹ Therefore, GJG may be safely used for the treatment of ED associated with anti-cancer drug treatment. However, the effectiveness of GJG for ED has not been clarified. Our hypothesis is "Is GJG effective for the ED caused by the anti-cancer agent oxaliplatin?". In this study, we investigated the effect of GJG on erectile function after the administration of the anti-cancer agent oxaliplatin using a rat model.

METHODS

Animals

Twelve-week-old male Wistar and/or ST rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All experimental protocols were approved by the ethics review board of Nagoya City University and conducted in accordance with our institutional standards for the care and use of animals (H25-P-09).

Treatment Protocols

In total, 40 rats were assigned to 4 groups (each $n = 10$): L-OHP, L-OHP with GJG (L-OHP+GJG), Sham, and Sham with GJG (Sham+GJG). L-OHP (Yakult Honsha Co., Ltd. Tokyo, Japan) was dissolved in a 5% glucose solution just before administration. The rats were administered a dose of 4 mg/kg body weight of oxaliplatin intravenously on days 1 and 2. In the Sham rats, the same volume of the 5% glucose solution was injected with the same dosing schedule. GJG is composed of 10 herbal medicines: Rehmanniae radix (5.0 g), Achyranthis radix (3.0 g), Corni fructus (3.0 g), Moutan cortex (3.0 g), Alismatics rhizome (3.0 g), Dioscoreae rhizome (3.0 g), Plantaginis semen (3.0 g), Hoelen (3.0 g), processed Aconiti tuber (1.0 g), and Cinnamomi

cortex (1.0 g). This drug was prepared as a spray-dried powder from a hot-water extract (yield 16%) and was obtained from Tsumura & Co. (Tokyo, Japan). In the L-OHP and Sham groups, CE-2 was administered to the rats, which is a standard feed for rats (CLEA Japan, Inc., Tokyo, Japan). In the L-OHP+GJG and Sham+GJG groups, rats were fed CE-2 containing 1% GJG. After 4 weeks, the rats were evaluated for erectile function.

Measurement of Biological Parameters

At the end of the treatment period, blood samples were obtained from the rats via the vena cava. After coagulation and centrifugal separation at 800 g for 20 minutes at 4°C, serum samples were stored at -80°C until analysis. The biological parameters were analyzed by Fujifilm Vet Systems Co., Ltd. (Chofu, Tokyo, Japan).

Examination of Erectile Function

Intracavernous pressure (ICP) was measured by electrical stimulation as previously reported.^{3,10-14} Rats from each group were briefly anesthetized using isoflurane (Mylan, Canonsburg, PA, USA) using inhalation anesthesia apparatus (Nakazawa Seisakusho, Funabashi, Japan). The carotid artery was cannulated for continuous monitoring of the mean arterial pressure (MAP), and the left crus of the corpus cavernosum was cannulated using a 23-G needle for continuous ICP monitoring. The pressure transducer was connected through an amplifier to a data acquisition board (PowerLab 2/26; ADInstruments Pty. Ltd., Bella Vista, Australia). Stainless steel bipolar wire electrodes (Unique Medical, Osaka, Japan) and a pulse generator (Nihon Kohden, Tokyo, Japan) were used for penile stimulation with the following parameters: 1 minute at 5 V, 1–16 Hz, and a square wave duration of 5 minutes. Erectile function was evaluated using the maximum ICP/MAP ratio, as ICP is influenced by systemic arterial pressure.

Western Blot Analysis

Western blot analysis was performed as previously reported.^{3,15-16} We extracted protein from the corpus cavernosum using Cell Culture Lysis Reagent (Promega Corp., Madison, WI, USA) in accordance with the manufacturer's instructions. Total protein concentration was quantified using BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL, USA). Samples containing 30 mg of total protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto polyvinylidene difluoride membranes (ImmobilinTM; Millipore Corp., Bedford, MA, USA). The membranes were then blocked for 1 hour using 5% skim milk in Tris-buffered saline (pH 7.5)–Tween 20 (TBST) at room temperature, and they were sequentially incubated with mouse monoclonal anti-neuronal nitric oxide synthase (nNOS) (1:100;

Abcam, Tokyo, Japan), anti-endothelial nitric oxide synthase (eNOS) (1:100; Abcam), and mouse monoclonal anti- β -actin (1:5,000; Sigma, Saint Louis, MO, USA) in 5% skim milk at 4°C overnight. After washing with TBST, anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (1:5,000; GE Healthcare, Little Chalfont, UK) was added for 1 hour at room temperature, and the membranes were washed again with TBST. Protein bands were visualized using enhanced chemiluminescent (Amersham Biosciences, Little Chalfont, UK) and ImageQuant LAS 4000 mini (GE Healthcare). Semiquantitative densitometry was performed using ImageJ software (NIH, Bethesda, MD, USA).

Real-Time Quantitative Polymerase Chain Reaction

Real-time quantitative polymerase chain reaction (qRT-PCR) analysis was performed as previously reported.^{10,13,17} Total RNA was extracted from corpus cavernosum samples using TriPure Isolation Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Using a ReverTra Ace- α kit (Toyobo, Osaka, Japan), 1 μ g of total RNA was reverse-transcribed into complementary DNA, which served as the template for qRT-PCR performed using the KAPA SYBR Fast qPCR Kit (Roche, Pleasanton, CA, USA). The primer sequences are shown in Table 1. Amplification and detection were performed using the CFX96 Real-time System (Bio-Rad, Hercules, CA, USA). The thermal cycler conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles each of 95°C for 15 seconds and 60°C for 1 minute, 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds to analyze the dissociation curve. Primer specificity was verified via analysis of

Table 1. Real-time quantitative polymerase chain reaction primer sequences

mRNA		Sequence
NADPH oxidase-1	Forward	5'-GCTGGCCTTACT GGAGTGGTC-3'
	Reverse	5'-TCCTGCCGATAAA CTCCATAGC-3'
PDE-5	Forward	5'-CACAGTGCATGTTT GCTGGTCTGA-3'
	Reverse	5'-CAATCAGCAATGCCAA GGTCTCCA-3'
β -actin	Forward	5'-TGTGTGGSTTGG TGGCTATC-3'
	Reverse	5'-CATCGTACTCCT GCTTGCTGATC-3'

mRNA = messenger RNA; NADPH = nicotinamide adenine dinucleotide phosphate; PDE-5 = phosphodiesterase-5.

The thermal cycler conditions were as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C, 15 s at 95°C, 15 s at 60°C, and 15 s at 95°C to analyze the dissociation curve. Primer specificity was verified via analysis of the dissociation curve.

the dissociation curve. Target gene expression was quantified relative to β -actin expression by using the comparative C_T method. All measurements were performed in triplicate.

Statistical Analyses

Results were expressed as box-and-whisker plots and the mean \pm standard error of the mean (SEM). Statistical significance was determined by a Bonferroni's multiple comparison test using in

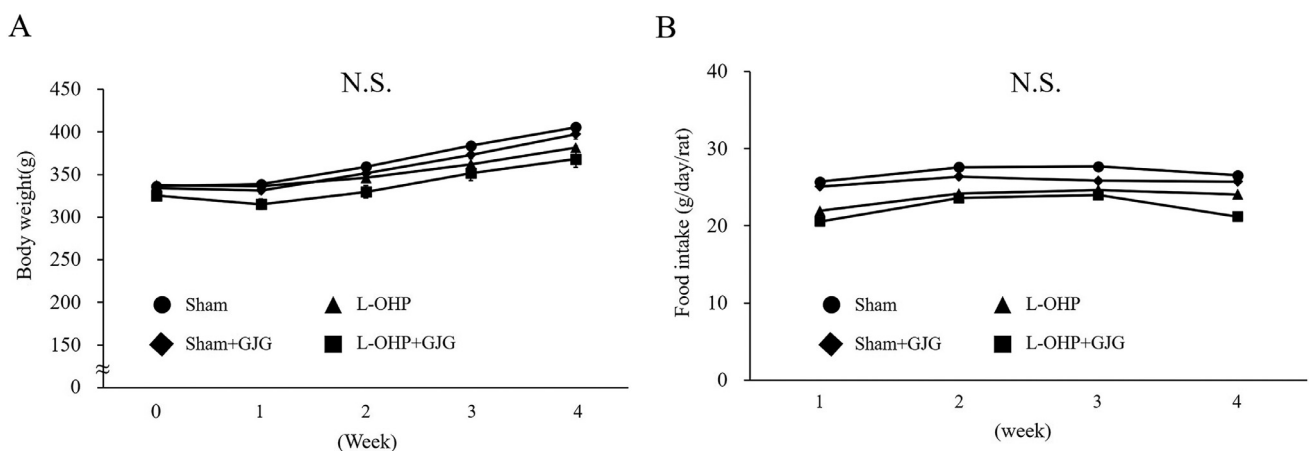


Figure 1. (A) Changes in the bodyweight of the Sham, Sham with Gosha-jinki-gan (Sham+GJG), Oxaliplatin (L-OHP), and L-OHP with GJG (L-OHP+GJG) rats. (B) Changes in the food intake of the Sham, Sham with Gosha-jinki-gan (Sham+GJG), Oxaliplatin (L-OHP), and L-OHP with GJG (L-OHP+GJG) rats.

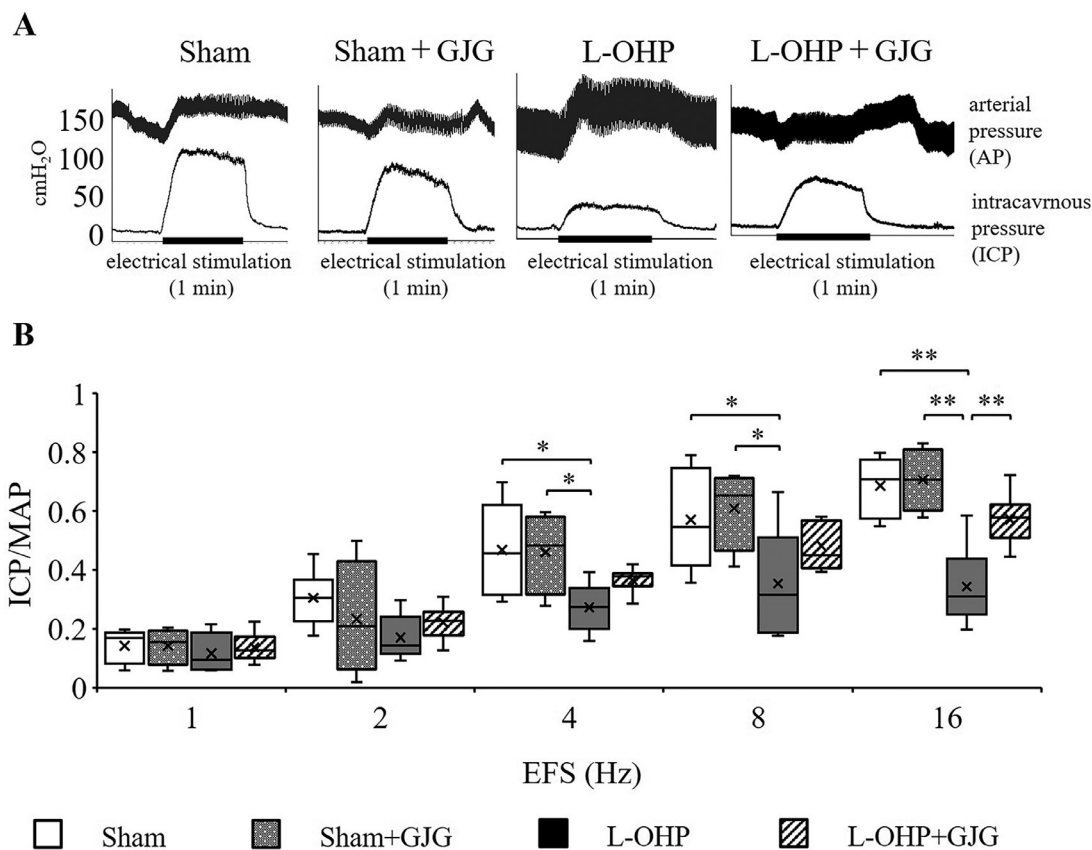
Data are reported as means \pm standard error of the mean ($n = 10$ per group). N.S. indicates not significant using Bonferroni's multiple comparison test for analysis.

Table 2. Biological parameters of Sham, Sham with Gosha-jinki-gan (Sham+GJG), Oxaliplatin (L-OHP), and L-OHP with GJG (L-OHP+GJG) rats

Biological parameter	Unit	Sham	Sham+GJG	L-OHP	L-OHP+GJG	P-value
Na	$\mu\text{Eq/L}$	142.5 \pm 1.7	142.0 \pm 1.1	143.0 \pm 1.9	142.5 \pm 2.3	> 0.05
Cl	$\mu\text{Eq/L}$	95.3 \pm 0.6	95.3 \pm 1.0	96.8 \pm 1.1	92.5 \pm 1.7	> 0.05
K	$\mu\text{Eq/L}$	7.2 \pm 0.7	7.1 \pm 0.3	6.1 \pm 0.4	6.3 \pm 0.3	> 0.05
Ca	mg/dL	11.9 \pm 0.4	12.2 \pm 0.2	11.9 \pm 0.3	11.6 \pm 0.3	> 0.05
P	mg/dL	10.5 \pm 0.6	10.0 \pm 0.2	9.7 \pm 0.9	11.0 \pm 1.9	> 0.05
Total protein	g/dL	6.0 \pm 0.2	6.1 \pm 0.2	6.1 \pm 0.2	6.0 \pm 0.1	> 0.05
Albumin	g/dL	3.4 \pm 0.1	3.4 \pm 0.1	3.4 \pm 0.1	3.4 \pm 0.1	> 0.05
GOT (AST)	IU/L	114.3 \pm 18.7	104.8 \pm 5.6	111.0 \pm 18.9	116.5 \pm 18.9	> 0.05
GPT (ALT)	IU/L	84.5 \pm 14.9	76.5 \pm 4.5	74.5 \pm 12.5	71.8 \pm 8.8	> 0.05
LDH	IU/L	460.0 \pm 99.3	293.5 \pm 33.8	568.0 \pm 181.0	775.5 \pm 439.7	> 0.05
ALP	IU/L	1359 \pm 65.6	1205 \pm 106	1258 \pm 109	1216 \pm 116	> 0.05
Amylase	IU/L	3382 \pm 128	3220 \pm 111	3588 \pm 133	3263 \pm 78	> 0.05
Lipase	IU/L	9.8 \pm 0.3	9.5 \pm 0.5	9.8 \pm 0.5	9.8 \pm 0.3	> 0.05
BUN	mg/dL	19.0 \pm 1.2	18.8 \pm 1.0	19.8 \pm 0.3	17.8 \pm 1.0	> 0.05
Creatinine	mg/dL	0.33 \pm 0.02	0.33 \pm 0.03	0.35 \pm 0.03	0.30 \pm 0.00	> 0.05
Total cholesterol	mg/dL	69.0 \pm 5.7	68.8 \pm 1.8	77.3 \pm 3.9	75.3 \pm 3.6	> 0.05
Triglyceride	mg/dL	99.3 \pm 13.3	82.0 \pm 7.9	61.8 \pm 5.6	83.0 \pm 7.8	> 0.05

ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase; LDH = lactate dehydrogenase.

Data are reported as the mean \pm standard error of the mean (n = 4 per group). P-values were analyzed by Bonferroni's multiple comparison test.

**Figure 2.** Measures of erectile function in Sham, Sham with Gosha-jinki-gan (Sham+GJG), Oxaliplatin (L-OHP) and L-OHP with GJG (L-OHP+GJG) rats.

(A) Representative tracings of intracavernous pressure (ICP) and arterial pressure changes during electrical field stimulation (EFS, 16 Hz) of the cavernous nerve. (B) Erectile function according to the ICP and/or mean arterial pressure (MAP) ratio. Data are presented as box-and-whisker plots (n = 6 per group). *P < .05, **P < .01 vs each group using Bonferroni's multiple comparison test for analysis.

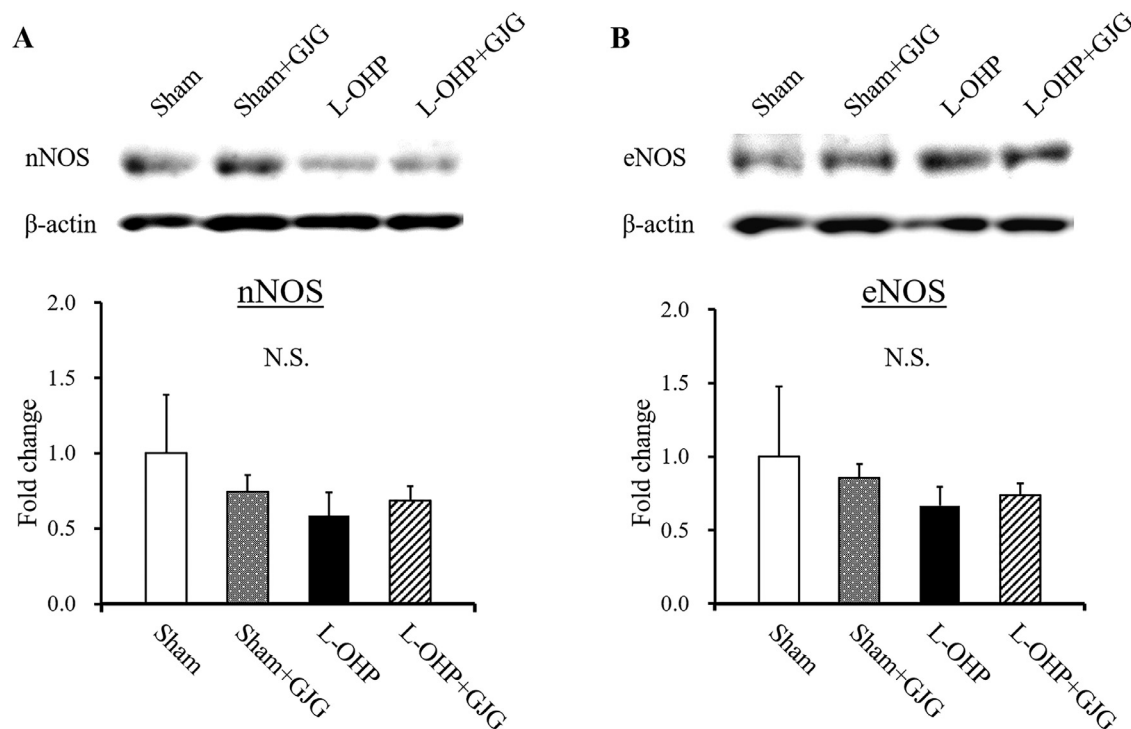


Figure 3. Western blot analysis of the expression of nitric oxide synthase in the corpus cavernosum of Sham, Sham with Gosha-jinki-gan (Sham+GJG), Oxaliplatin (L-OHP), and L-OHP with GJG (L-OHP+GJG) rats.

Representative immunoblots of (A) nNOS and (B) eNOS. Densitometric quantification of the corresponding bands. The data were normalized by β -actin expression and presented as fold changes over sham. Data are reported as the mean \pm standard error of the mean ($n = 3$ per group). N.S. means not significant using Bonferroni's multiple comparison test for analysis.

EZR on R commander ver 1.41.¹⁸ P -values $< .05$ were considered statistically significant.

RESULTS

Biological Parameters

Figure 1A shows body weight changes during the study. There was no statistically significant difference in mean body weights at 4 weeks (Sham, 405.5 ± 2.5 g; Sham+GJG, 397.5 ± 5.9 g; L-OHP, 381.5 ± 2.8 g; L-OHP+GJG, 368.3 ± 10.0 g; $P > .05$). Figure 1B shows food intake during the study. There was no significant difference in food intake in any of the observation periods. Table 2 shows the biological parameters of the rats. There were no significant differences in electrolyte levels, liver function, renal function, and lipid levels ($P > .05$).

Erectile Function

Figure 2A shows representative tracings of the ICP and atrial pressure changes during electrical stimulation of the cavernous nerve. ICPs in the L-OHP group appeared to be lower than those in the Sham group. Figure 2B shows the ICP/MAP ratios for different stimulation frequencies. The L-OHP group (0.34 ± 0.06) exhibited significantly lower ratios than the Sham group (0.67 ± 0.03 ; $P < .01$) at 16Hz. The L-OHP+GJG group (0.55

± 0.01) exhibited significantly higher ratios than the L-OHP group ($P < .01$). There was no significant difference between the Sham and Sham + GJG groups (0.70 ± 0.05 ; $P > .05$).

nNOS and eNOS Protein Analysis

Figure 3 shows the nNOS and eNOS protein expression levels. There were no significant differences in nNOS and eNOS protein expression between the groups ($P > .05$).

Biomarker Messenger RNA Expression Analysis

Figure 4 shows the messenger RNA (mRNA) expression levels for biomarkers related to erectile function. Expression analysis of the oxidative stress marker nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-1 revealed that the expression of NADPH oxidase-1 was significantly higher in the L-OHP group than in the Sham group ($P < .01$). This expression significantly decreased in the L-OHP+GJG group compared to the L-OHP group ($P < .05$). The expression of phosphodiesterase (PDE)-5 was significantly higher in the L-OHP group than in the Sham group ($P < .01$), whereas the expression was significantly lower in the L-OHP + GJG group than in the L-OHP group ($P < .01$).

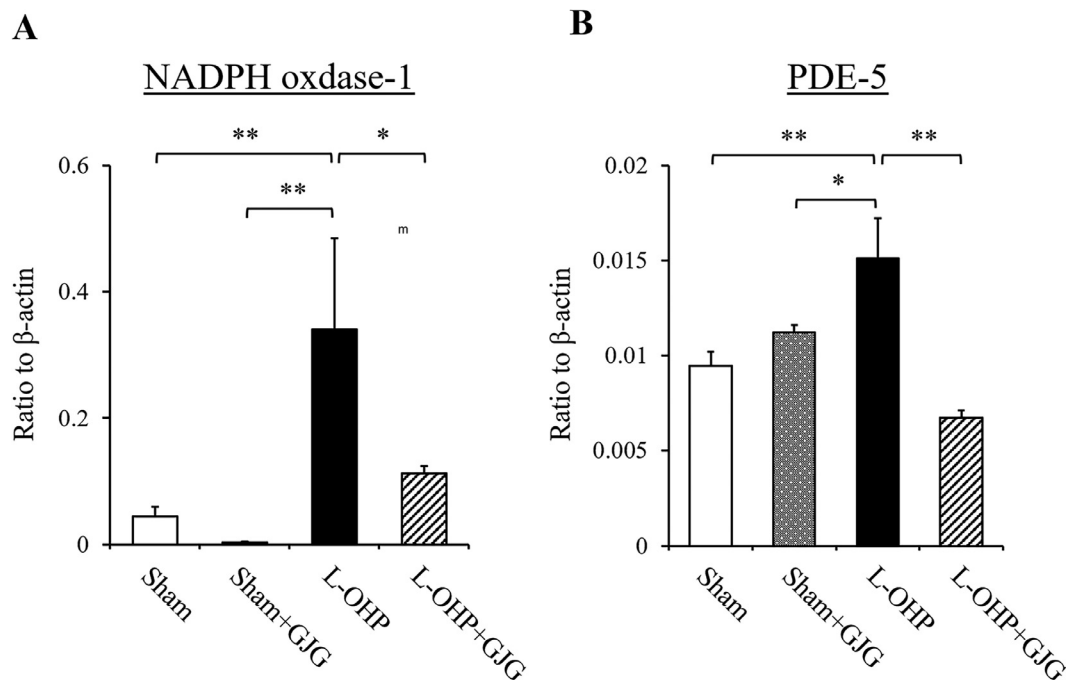


Figure 4. Biomarker messenger RNA expression in the corpus cavernosum of Sham, Oxaliplatin (L-OHP), and L-OHP with Goshajinkigan (L-OHP+GJG) rats.

(A) Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-1. (B) Phosphodiesterase (PDE)-5. Target gene expression was quantified relative to β -actin using the comparative CT method.

Data are reported as the mean \pm standard error of the mean ($n = 6$ per group). * $P < .05$, ** $P < .01$ vs each group using Bonferroni's multiple comparison test for analysis.

DISCUSSION

In this study, the Japanese traditional medicine GJG improved the ED caused by the administration of an anti-cancer agent, L-OHP, in rats (Figure 2). Electrolyte abnormalities were not seen in the L-OHP + GJG administration group (Table 2). Hepatic dysfunction and renal dysfunction were also not observed in the L-OHP + GJG administration group. These data showed that no notable side effects were observed in the treated group.

Peripheral neuropathy is a known side effect of L-OHP.² We investigated nNOS protein expression in the corpus cavernosum (Figure 3A). The expression of nNOS protein in the L-OHP group had not changed. However, L-OHP has been reported to reduce the number of nNOS-immunoreactive neurons.¹⁹ Song et al. reported that cavernous nerve injury decreased eNOS protein levels in a mouse model.²⁰ In this study, the expression of eNOS did not change in the L-OHP group; moreover, GJG administration did not affect NOS expression (Figure 3B). In our previous study, L-OHP significantly decreased the eNOS protein levels,³ but in this study, the eNOS expression remained unchanged in the L-OHP group. After 2-3 days of receiving oxaliplatin, the food intake also decreased. So, administering the Goshajinkigan in a stable dose was difficult. After this experiment, we conducted additional experiments by changing the frequency and duration of oxaliplatin administration. These

additional analyses showed that ED develops even with 4 mg/kg L-OHP administered on day1 and 2 for 4 weeks. Whereas, the food intake during the experimental course was stable, so we conducted another experiment with this dose, in which the conditions were milder than those in previous studies that demonstrated no change in the eNOS protein expression. However, GJG had no effect on the Nitrous oxide production. Hence, more investigation would be necessary for other target.

To investigate other mechanisms underlying the effects of GJG, we examined inflammation in the corpus cavernosum (Figure 4). Nakanishi et al. reported that GJG suppressed inflammation in the spinal cord of the mouse.²¹ Our analyses revealed that L-OHP increased the expression of the pro-oxidant NADPH-oxidase 1. However, GJG decreased NADPH-oxidase 1 in the corpus cavernosum of rats. It was possible that the dose of 1% GJG was effective for the inflammatory response caused by L-OHP in the corpus cavernosum. Further investigation using the cavernous nerve would also help elucidate the mechanism of GJG, which is a limitation of this study.

L-OHP can induce oxidative stress and cause dysfunction of the spinal nitric oxide and/or cyclic guanosine monophosphate (cGMP) pathway.^{22, 23} In the smooth muscles of the corpus cavernosum, the increased cGMP relaxes the smooth muscle and causes an erection.^{24, 25} In contrast, PDE-5 works to metabolize cGMP, so when PDE-5 increases, erectile function

declines.^{26, 27} In this study, L-OHP increased PDE-5 mRNA expression in the corpus cavernosum, while GJG decreased PDE-5 expression (Figure 4). Therefore, GJG improved the erectile function of rats by inhibiting cGMP metabolism and increasing the relaxation of smooth muscle. In the arterial smooth muscle cells, high oxygen concentrations can increase the PDE-5 expression.^{28, 29} Muzaffar S et al. reported that a superoxide from NADPH oxidase upregulates type-V PDE in the human vascular smooth muscle cells.³⁰ In this study, we speculated that the expression of PDE-5 increased because of the increasing superoxide levels by the upregulation of NADPH-oxidase 1 by L-OHP; however, GJG suppressed the PDE-5 expression by suppressing the activity of NADPH-oxidase-1. In this study, the protein levels of PDE-5 were not found; hence, future studies are warranted that measured the protein level and activity of PDE-5. PDE-5 inhibitors have been widely used for treating ED.^{31, 32} Our previous study reported that chronic PDE-5 inhibitor administration was effective for refractory ED.^{11, 33} Because GJG decreased PDE-5 expression, GJG might be effective for other types of ED. Moreover, PDE-5 inhibitors costs about approximately US\$10-20 the United States dollars per dose, while GJG costs about approximately US\$0.5 dollars per dose. Therefore, GJG is a economical cost-effective treatment choice and could be useful for chronic administration in cancer survivors.

GJG is composed of 10 herbal medicines; however, the specific component of GJG that was effective for improving the erectile function of rats has not been clarified, which we consider as a limitation. The second limitation of this study was that the dose of GJG was not changed and that we did not observe a washout period because GJG was fed to the rats in a mixed diet. The 1% GJG we used for our experiments can be converted to indicate GJG 1 g/kg/day.³⁴ In the future studies, researchers should consider changing the dose and including a washout period.

CONCLUSION

We found that administering GJG improved the L-OHP-induced ED in rats by improving the transcriptional expression of PDE-5. This animal model study indicated that GJG might be effectively improve the erectile function for male cancer survivors.

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