Doctor of Philosophy

Photothermal-mediated Local Heating Using Nano-functionalized Stent to Treat Stent-induced Tissue Hyperplasia in Rat Esophagus

The Graduate School
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Department of Medicine
Young Chul Cho

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Supervisor: Ji Hoon Shin

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Young Chul Cho

Department of Medicine
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This certifies that the dissertation
of Young Chul Cho is approved.

Committee Chair Dr. Kyung Won Kim

Committee Member Dr. Ji Hoon Shin

Committee Member Dr. Do Hoon Kim

Committee Member Dr. Heung Kyu Ko

Committee Member Dr. Wooram Park

Department of Medicine
University of Ulsan, Korea
February 2021

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입학한 지 5년 만에 '박사 학위 논문' 이라는 귀한 열매를 맺을 수 있도록 도와주신 모든 분들께 감사의 인사 올립니다.

신지훈 교수님. 항상 따뜻하게 반겨주시고 학위를 끝까지 마무리 할 수 있도록 지도의 끈을 놓지 않고 이끌어 주신 교수님의 따뜻한 배려와 조언에 깊은 감사를 드립니다.

박정훈 선생님. 첫 연구자의 길로 들어서는 순간부터 지금까지 항상 곁에 계셔주셔서 감사합니다. 선생님께서 제 선배님 인 것이 얼마나 다행스럽고 고마운 일인지 매번 깨닫는 하루입니다.

김경원 교수님. 한계에 부딪힐 때마다 교수님의 격려는 연구자로서 제 자신의 역할과 책임을 재인식 하는 계기가 되었습니다. 교수님을 통해 제 인생의 방향을 잡을 수 있었고 교수님의 열정적인 지도는 제가 항상 겸손한 사람이 될 수 있게 합니다. 존경과 감사의 마음을 드립니다.

김도훈 교수님, 고흥규 교수님, 박우람 교수님. 코로나19로 인해 어려운 상황에도 이 논문이 세상에 나올 수 있도록 귀한 시간 내주셔서 진심으로 감사드립니다. 교수님들의 세심한 제안과 깊은 관심 덕분에 논문의 완성도를 크게 높일 수 있었습니다. 교수님들의 은혜를 잊지 않겠습니다.

고민이 있을 때마다 자신의 일처럼 생각해주시고 애정 어린 조언을 해주시는 고유선 박사님, 이제는 박사 동기가 되는 신용빈 박사님, 매번 잊지 않고 저를 잘 챙겨주시는 이정현 선생님, 이지우 선생님, 김미현 선생님. 앞으로 좋은 일만 함께 하기를 항상 기원합니다.

우린 항상 철 없을 거야. 조진환, 박경훈, 임석년, 김광래, 강민국, 엄기현 모두 지금처럼 행복하자.

우리 디케이 친구들 장지성, 김관태 고맙다. 너희가 있어 가슴이 웅장해진다.

무엇보다 지금의 저를 있게 한 사랑하는 가족들. 오랜 시간 공부하는 아들 믿고 지켜봐주신 존경하는 나의 아버지, 어머니, 동생 정아, 막내 콩이, 그리고 부족한 사위 항상 예뻐해 주시는 장인어른, 장모님, 정안철, 정윤철 두 처남 모두 감사하고 사랑합니다. 고모와 명구형, 제헌이 누나, 명래형, 시은 누나에게도 감사 드립니다.

마지막으로 사랑하는 나의 아내와 우리 딸, 정혜원과 조하은에게 감사의 마음을 바칩니다. 힘들었던 그리고 좋았던 모든 찰나의 순간들을 당신과 함께할 수 있어 행복했습니다. 지금 이 순간에도, 앞으로도 모든 날들을 사랑하는 우리 가족들과 함께하고 싶습니다. 고맙고 사랑합니다.

ABSTRACT

Background and Purpose: The aim of the present study was to investigate the effectiveness of photothermal therapy (PTT) using a gold nanoparticle (AuNP)-coated stent for treating stent-induced tissue hyperplasia in the rat esophagus.

Materials and Methods: All experiments were approved by animal research committee. An AuNP-coated, self-expandable metallic stent (SEMS) was produced to conduct PTT under near-infrared laser irradiation. Forty rats were randomly divided into four groups (10 rats each). The animals in group A (non-coated SEMS) and group B (AuNP-coated SEMS with local heating at 65°C at 4 weeks) were sacrificed 4 weeks after stent placement. The rats in group C (AuNP-coated SEMS with local heating at 65°C at 4 weeks) and group D (AuNP-coated SEMS with local heating at 65°C at 4 and 8 weeks, respectively) were sacrificed 8 weeks after stent placement. The effectiveness of local heating was assessed by histopathological analysis results.

Results: All procedures were successful in all of the animals. Seven rats were excluded because of stent migration (n = 2) and death (n = 5). Tissue hyperplasia-related variables were significantly higher in group A than in groups B-D (all $p < 0.05$). HSP70 and TUNEL expression were significantly lower in group A than in groups B-D (all $p < 0.05$). Tissue hyperplasia -related variables were significantly higher in group C than in groups B and D (all $p < 0.05$).

Conclusion: PTT using AuNP-coated SEMS successfully treated tissue hyperplasia after stent placement in the rat esophagus.

Key words: Self-expandable metallic stent, Photothermal therapy, Gold nanoparticle, Tissue hyperplasia

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LIST OF ABBREVIATIONS

SEMS	self-expandable metallic stent
HSP	heat shock protein
AuNP	gold nanoparticle
NIR	near-infrared
PTT	photothermal therapy
BPEI	branched polyethylenimine
PDA	polydopamine
RT	room temperature
DW	deionized water
PEI	polyethylenimine
SEM	scanning electron microscopy
EDS	energy-dispersive X-ray spectroscopy
CCK-8	Cell Counting Kit-8
TH	tissue hyperplasia
H&E	hematoxylin and eosin
MT	Masson's trichrome
IHC	immunohistochemistry
SD	standard deviation

INTRODUCTION

Esophageal stenting using a self-expandable metallic stent (SEMS) is currently the most common therapeutic strategy for treating malignant and benign diseases (1-3). The use of SEMS devices is significantly limited however by malignant and/or benign tissue growth through the stent meshes after placement, which can lead to newly developed stricture formation and recurrent symptoms (2-5). Drug-eluting, biodegradable, and nano-functionalized stents have been investigated as possible avenues to overcome this complication but have not been successful in treating stent-induced malignant and/or benign tissue formation (6-12).

Controlled local heat treatments have been introduced for targeted cancer therapy and management of tissue hyperplasia (13-15). It has been demonstrated that moderate heat (41–50°C) treatments can not only decrease in-stent restenosis caused by neointimal hyperplasia in animal artery models (15-17), but can also inhibit granulation tissue formation with minimal damage to surrounding structures in the rat skin (14). Local application of moderate temperatures reduces collagen accumulation, increases cell apoptosis, alters cell metabolism, and activates a family of heat shock proteins (HSPs) (15-16). Thermal therapy using various nanoparticles such as different shaped gold nanoparticles (AuNPs) and magnetic nanoparticles has been studied extensively for inducing controlled heat treatments with high efficiency and accuracy (18-19).

AuNPs have excellent absorption properties in the near-infrared (NIR) spectrum of biological tissues and their photothermal-converting efficiency has been studied previously as a possible biomedical application for targeting different diseases (15-19). Recently, AuNP-based functionalized stents were introduced to locally treat cancer cells or tissue hyperplasia adjacent to stented non-vascular luminal organs (13, 14, 20, 21). Park et al. have reported in rat models that locally

applied temperature increases (50°C to 65°C) successfully suppressed tissue hyperplasia after esophageal and gastroduodenal stent placement (13, 21). We hypothesized that AuNP-coated SEMS could be used for localized photothermal therapy (PTT) under NIR laser irradiation. We speculated that this approach would treat stent-induced tissue hyperplasia after SEMS placement through the induction of PT-induced cell apoptosis and activation of HSPs. We here investigated the feasibility of this therapy in the rat esophagus.

MATERIALS AND METHODS

Fabrication of AuNP-coated SEMS

The SEMS (diameter, 5 mm; length, 15 mm) was knitted from a single, 0.127-mm-thick nitinol wire filament (S&G biotech, Seongnam, Korea). Two radiopaque markers at each end of the stent were attached and two barbs to the middle of the stent were attached to prevent migration of the stent.

Branched polyethyleneimine (bPEI, Mw; 600 Da), dopamine hydrochloride, hydrogen tetrachloroaurate (III) trihydrate (HAuCl4, 99.9%), silver nitrate (AgNO3, 99%), L-ascorbic acid (≥98%), sodium borohydrate (98%), and sodium deoxycholate (≥97%) were used (Sigma–Aldrich, St. Louis, MO, USA) to fabricate AuNP-coated SEMSs.

The cationic polymer was coated on SEMS surface through polydopamine (PDA) coating before depositing AuNPs on the surface. Dopamine hydrochloride (1 mg/mL) was dissolved in 15 mL of 5 mM Tris buffer (pH 8.5), in which the stent was immersed. The coating process was performed at room temperature (RT) with magnetic stirring (12 h). The PDA-coated SEMS was washed with deionized water (DW), and the coating was repeated twice under the same conditions. Next, for coating PDA-coated SEMS surface with a cationic polymer, it was immersed in polyethyleneimine (15 mL; PEI, 600 Da) solution (10 mg/mL) and was stirred at RT (12 h). Finally, it was washed with DW. AuNPs were coated on the surface of a PEI-coated SEMS by modifying a reported procedure (11). The PEI-coated SEMS was immersed in 0.5 mM sodium cholate solution (15 mL). Then, 90 μL of 50 mM AgNO3, 3 mL of 10 mM HAuCl4 solution, and 0.96 mL of 100 mM ascorbic acid solution were sequentially added to the previous solution. The reaction proceeded at RT (12 h). After reaction completion, the solution turned dark purple. After the reaction, the AuNP-coated SEMS was washed with DW and dried at RT (24 h) (**Figure 1**).

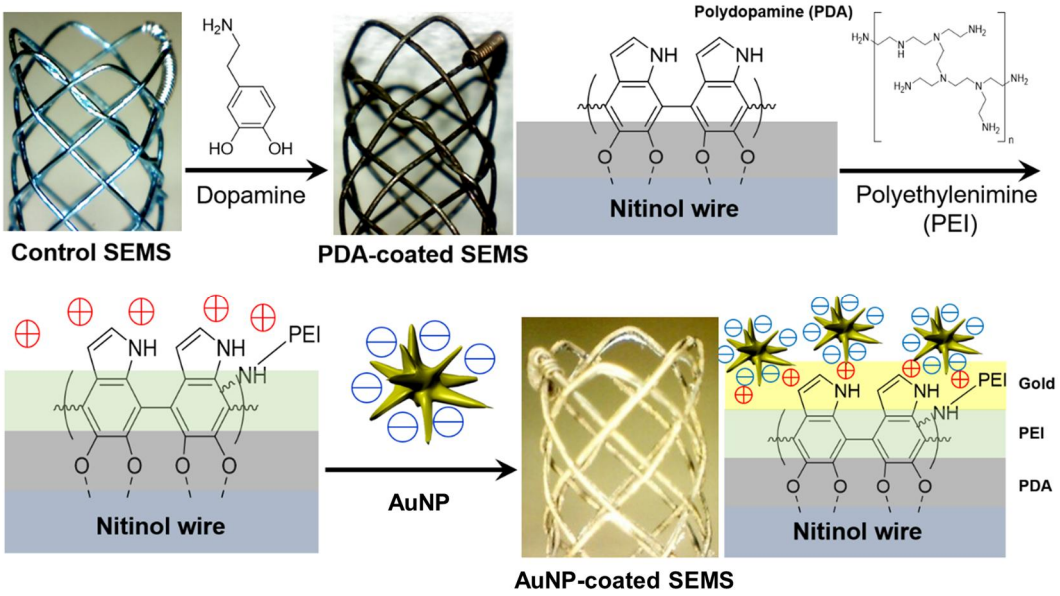


Figure 1. Schematic illustration of preparation process for AuNP-coated SEMS: The cationic polymer was coated on the surface of SEMS through PDA-coating and AuNPs were sequentially deposited on the polymer layer.

Note. SEMS, self-expandable metallic stents; PDA, polydopamine; PEI, polyethylenimine; AuNP, gold nanoparticles

Characterization and NIR laser-induced PT properties

The surface characteristics of control (nitinol), PDA-coated and AuNP-coated SEMSs were evaluated using scanning electron microscopy (SEM) (JSM-820, JEOL Ltd., Tokyo, Japan) with energy-dispersive X-ray spectroscopy (EDS) (INCAx-sight, Oxford Instruments, Abingdon, Oxfordshire, UK). Each SEMS was fixed on aluminum pin stubs using a carbon tape, and the surface analysis was conducted for elemental mapping of gold.

To investigate PT characteristics in vitro, the control, PDA-coated, and AuNP-coated SEMSs were irradiated (60 s) with a 1-mm diameter fiber-coupled NIR (808 nm) diode laser (OCLATM LASER, NDLUX Inc., Anyang, Korea) at 1.27 W/cm2. Thermal images were obtained using an infrared thermal camera (ICI9320P, Infrared Cameras Inc., Beaumont, TX, USA) while increasing the temperature, and electronic files were acquired every 5 seconds for 70 seconds. All studies were repeated ten times to determine statistical reproducibility.

Cytotoxicity of control, PDA-coated, and AuNP-coated wires

The cytotoxicity of the control, PDA coated, and AuNP coated wires was analyzed using a standard Cell Counting Kit-8 (CCK-8). L929 and 293 cells were seeded into 96-well plates at a concentration of 1 x 104 cells/well, respectively. After incubation at 37 °C for 24h, each wire was placed on the different well for 1, 12, 24, 36, and 48 h, respectively, and 10 µl of CCK-8 solution was added to the 96-well plate and incubated.

Cell viability was evaluated by measuring the absorbance of each well at 450 nm using a microplate reader. All experiments were repeated three times and results were averaged.

Animal study

The Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences (Seoul, Korea) approved all of the experiment protocols and animals used in this study (2019-13-056). In total, 40 Sprague-Dawley male rats (300–350 g; Orient Bio, Seongnam, Korea) underwent SEMS placement into the esophagus. Rats were randomly divided into four groups using Random Allocation software (version 2.0; Microsoft, Seattle, WA, USA). Group A (n = 10) received non-coated SEMS. Group B (n = 10) received AuNP-coated SEMS with local heating at 65°C at 4 weeks. All rats in groups A and B were sacrificed 4 weeks after stent placement. Two additional groups were included to evaluate any rebound effects of local heating. Group C (n = 10) received AuNP-coated SEMS with local heating at 65°C at 4 weeks. Group D (n = 10) received AuNP-coated SEMS with local heating at 65°C at both 4 and 8 weeks. All rats in groups C and D were sacrificed 8 weeks after SEMS placement for histopathological analysis (**Figure 2**). All animals were sacrificed by administering inhalable pure dioxide. The body weight of each rat was measured weekly until they were sacrificed. All animals were housed at one per cage in a room with a 12-hour contrast cycle at 24 ± 1 °C with a relative humidity of 55 ± 10%. Standard rodent chow and water were provided ad libitum. All animals were acclimated for at least 1 week prior to conducting the experiments.

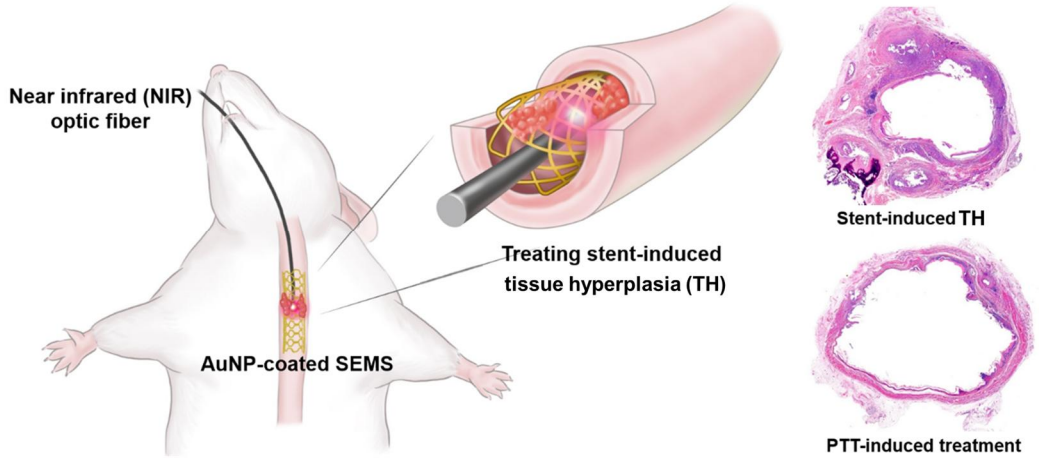


Figure 2. Schematic illustration of the photothermal therapy (PTT) method using a gold nanoparticle (AuNP)-coated self-expandable metallic stent (SEMS)

Stent placement

For stent placements in the esophagus, the rats were anesthetized via an intramuscular injection of 50 mg/kg zolazepam and 50 mg/kg tiletamine (Zoletil 50; Virbac, Carros, France) and 10 mg/kg xylazine (Rompun; Bayer HealthCare, Leverkusen, Germany). Under fluoroscopic guidance, a 0.014-inch guidewire (Transcend; Boston Scientific, Watertown, MA) was inserted through the mouth and passed through the stomach. A customized 6-Fr sheath and dilator were then advanced into the lower esophagus through the guide wire. With the sheath left in place, the dilator and the guidewire were removed. A stent in a compressed state was loaded into the sheath and placed in the esophagus using a pusher catheter. The stent was deployed at the level of the mid-thoracic esophagus under continuous fluoroscopic monitoring. After the procedure, esophagography was performed to confirm the location of the stent.

Photothermal therapy under near-infrared irradiation

PTT under NIR irradiation was performed at both 4 weeks (groups B, C, and D) and 8 weeks (group D) after stent placement in the experimental rats. For NIR laser irradiation, a 1-mm diameter fiber-coupled NIR (808 nm) diode-laser (OCLATM LASER, NDLUX Inc., Anyang, Korea) was inserted into the sheath by fluoroscopy. Using fluoroscopic guidance, the sheath and optic fiber were advanced through the mouth into the middle portion of the stented area. NIR laser was irradiated for 60 seconds in all rats. After stent placement and local heating, 0.05 mg/kg of buprenorphine (Renophan; Hanlim Pharmaceutical, Seoul, Korea) were intramuscularly injected to all rats every 6 hours for pain control for 48 hours. All rats were monitored until recovery from anesthesia and were then returned to their cages.

Histological analysis

Surgical exploration of the entire esophagus and stomach was followed by gross examination to determine possible esophageal injury after stent placement or irradiation. Tissue samples were fixed in 10% neutral buffered formalin for 24 hours, then embedded in paraffin and sectioned. The stented esophagus was sectioned transversely in the middle area. The slides were stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT).

Histological evaluation using H&E was based on the degree of submucosal inflammatory cell infiltration, the number of epithelial layers, the thickness of the submucosal fibrosis, and the tissue-hyperplasia-related percentage of the esophageal cross-sectional area of stenosis calculated as 100 × (1 − [stenotic stented area/original stented area]) (6, 11). The degree of inflammatory cell infiltration was subjectively determined according to the distribution and density of the inflammatory cells, i.e. graded as 1, mild when there was occasional visible infiltration of single leukocytes; 2, mild-to-moderate when there was patchy infiltration of leukocytes; 3, moderate when coalescing leukocytes made individual loci indistinguishable; 4, moderate-to-severe when there was diffuse infiltration of leukocytes throughout the submucosal layer; and 5, severe when there was diffuse infiltration with multiple necrotic foci (21). The collagen-deposition level was subjectively determined with the following scores: 1 = mild, 2 = mild to moderate, 3 = moderate, 4 = moderate to severe, and 5 = severe. The esophagus was analyzed histologically using a BX51 microscope (Olympus, Tokyo, Japan). Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) was used for the measurements. The histological findings were based on the consensus of three observers who were blind to the experimental groups.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections using TUNEL (Apoptotag kit, Biogene, Darmstadt, Germany) and HSP70 (1:1000; Abcam, Cambridge, UK) primary antibodies. The sections were visualized using a BenchMark XT IHC automated immunohistochemical stainer (Ventana Medical Systems, Tucson, AZ). The degree of TUNEL and HSP70 positive deposition was determined subjectively (1 = mild, 2 = mild to moderate, 3 = moderate, 4 = moderate to severe, and 5 = severe). IHC findings were also based on the consensus of three observers of three observers who were blind to the experimental groups.

Statistical analysis

Data were expressed as a mean ± standard deviation (SD). Differences between the groups were analyzed using Kruskal–Wallis or Mann–Whitney U test, as appropriate. A p-value of <0.05 was considered statistically significant. Statistical analyses were performed using SPSS software (version 24.0; SPSS, IBM, Chicago, IL).

RESULTS

Characterization of AuNP-coated SEMS

AuNP-coated SEMSs were successfully fabricated using a two-step synthesis process. Firstly, a PDA coating process converted the hydrophobic surface of nitinol SEMSs to help AuNPs adhere to the surface of the nitinol stent. Then, AuNPs were successfully coated on the surface of stent. SEM images showed that the AuNPs were uniformly coated by the PDA-mediated cationic polymer coating layer. Surface analysis showed that gold signal was significantly increased in the AuNP-coated SEMS. Titanium and nickel signals were also mainly detected due to the nitinol wires. PDA-coated SEMS resulted an enhanced carbon signal compared to the control SEMS (**Figure. 3**).

The cytotoxicity results showed that cell death was not observed, which indicated that the control, PDA-coated, and AuNP-SEMSs was nontoxic, as shown in **Figure 4**.

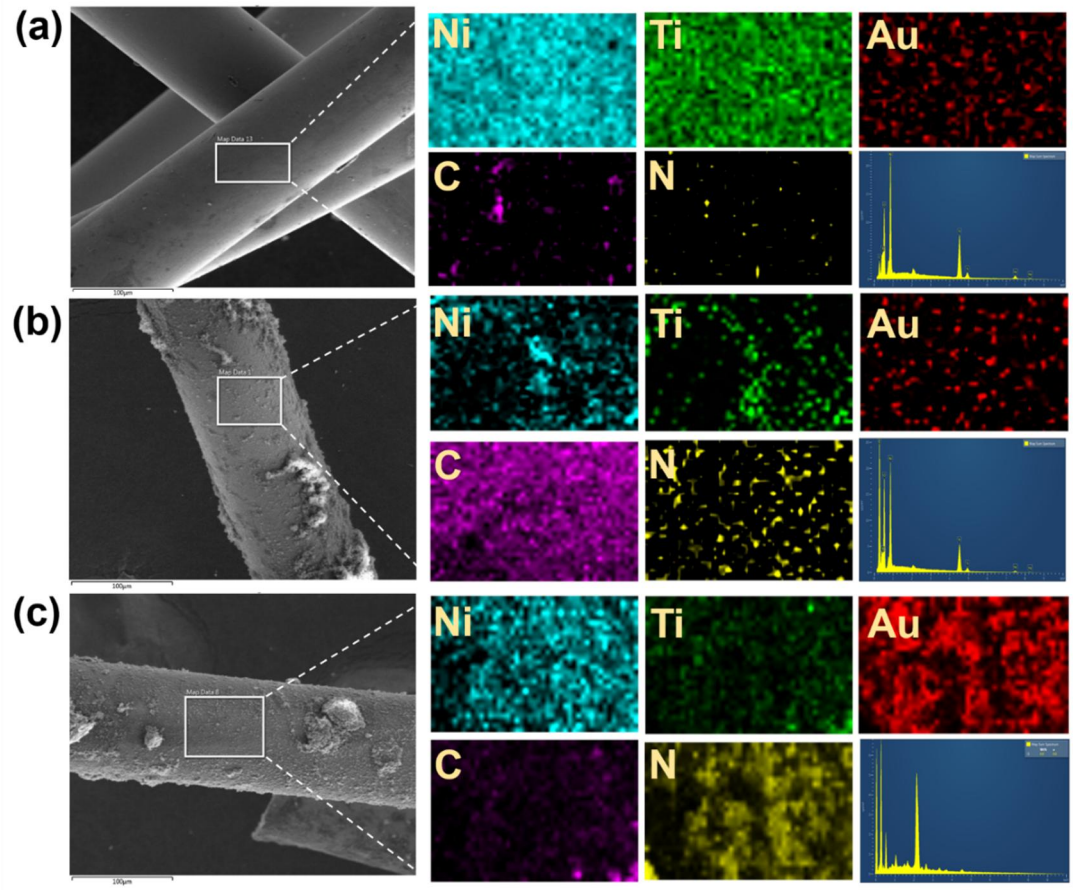


Figure 3. Scanning SEM with EDS mapping analysis of SEMS (a) nitinol SEMS, (b) PDA-coated SEMS, and (c) AuNP-coated SEMS (blue, green, red, pink, and yellow colors indicate nitinol, titanium, gold, carbon, and nitrogen, respectively).

Note. SEM, scanning electron microscopy; EDS, energy dispersive spectroscopy; SEMS, self-expandable metallic stents; PDA, polydopamine; AuNP, gold nanoparticles

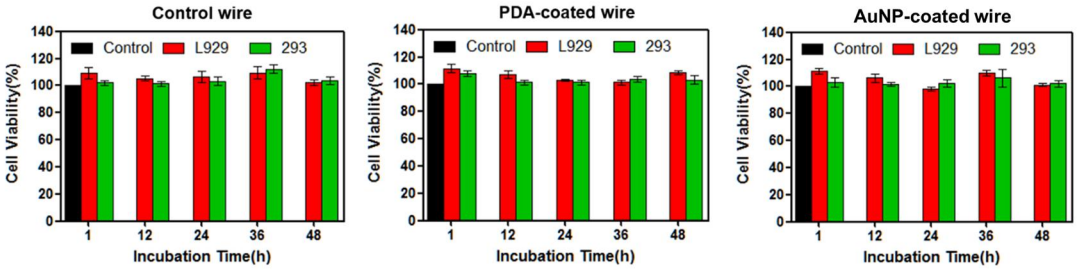


Figure 4. Cytotoxicity analysis of control nitinol wire, PDA-coated wire, and AuNP-coated wire after incubation with L929 and 293 cells at different period of time.

Note. PDA, polydopamine; AuNP, gold nanoparticles

Characterization of NIR Photothermal Heating of AuNP-coated SEMS

Strong NIR absorption induced by the AuNPs formed on the stent produces significant heating directly upon NIR 808 nm irradiation. AuNP-coated SEMS reached relatively high temperatures compared to control and PDA-coated SEMS. The temperature of AuNP coated stent rapidly reached relatively a high steady-state temperature compared to the control and PDA-coated SEMSs by 50 seconds under NIR laser irradiation and cooled rapidly after irradiation was stopped. The mean steady-state temperature (± standard deviation [SD]) of the control, PDA-coated, and AuNP-coated SEMSs were 39.65 (± 0.46), 42.92 (± 0.31), and 51.02 (± 0.46) °C under 1.27 W/cm^2 NIR laser, respectively. The maximum steady-state temperature of the AuNP-coated SEMS was increased in proportion to the NIR intensity. After applying the four laser energy levels, the mean steady-state surface temperature (± SD) of the AuNP-coated SEMSs after application of the four different laser energy levels were 35.82 (± 0.44) °C at 0.64 W/cm^2, 51.02 (± 0.68) °C at 1.27 W/cm^2, 65.10 (± 0.82) °C at 1.91 W/cm^2, and 80.49 (± 0.59) °C at 2.55 W/cm^2, respectively (**Figure. 5**).

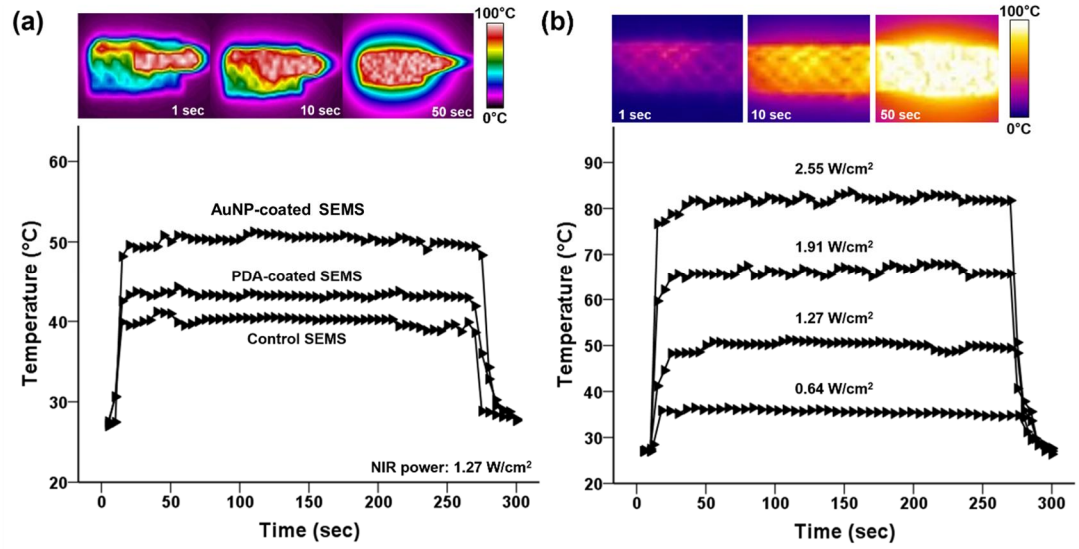


Figure 5. Temperature change measurement of (a) Control SEMS, PDA-coated SEMS, and AuNP-coated SEMS under irradiation with an 808 nm laser at 1.27 W/cm2 and (b) AuNP-coated SEMS under irradiation at four different laser powers (0.64, 1.27, 1.91, and 2.55 W/cm^2).

Note. SEMS, self-expandable metallic stents; PDA, polydopamine; AuNP, gold nanoparticles

Stent placement and PT-mediated local heating

Stent placement and PTT were technically successful in all the experimental rats. Five of 40 (12.5%) rats (one each in groups A-C and two in group D) died after stent placement due to a hemorrhage caused by the stent barbs at 1-3 days after placement. The AuNP-coated SEMS migrated into the stomach in two rats (one each in groups A and C) within 10 days of placement. The remaining 33 (82.5%) rats survived until the end of the study without stent-related complications (**Figure 6**). Although the body weights of the rats decreased at 1 week after stent placement and the PTT procedure, this did not significantly affect any of these animals in terms of their condition and behavior. There were also no significant differences between the groups in terms of the body weights after stent placement and the PTT procedure ($p > 0.05$) (**Figure 7**).

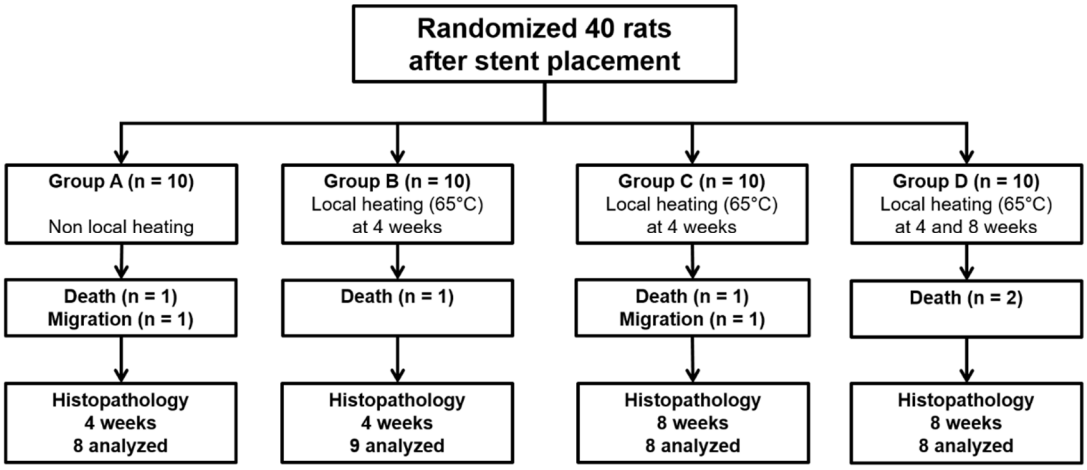
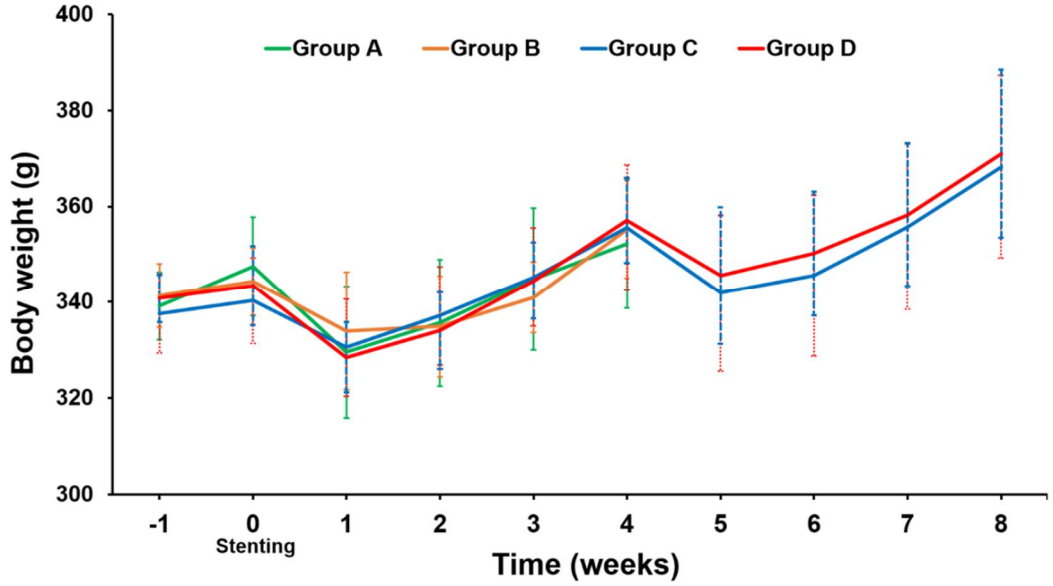


Figure 6. Flow diagram showing the randomization process and study follow-ups. Note. PTT; photothermal therapy

Figure 7. Effects of stent placement and photothermal therapy on body weight changes



	Group A	Group B	Group C	Group D	*p-value
Week 0	339.1 ± 7.1	341.3 ± 6.6	337.5 ± 8.2	340.8 ± 5.0	1.000
Week 1	347.4 ± 10.3	344.3 ± 7.1	340.3 ± 9.0	343.4 ± 8.2	1.000
Week 2	329.5 ± 13.8	334.0 ± 12.2	330.5 ± 10.1	328.4 ± 7.3	1.000
Week 3	344.8 ± 14.8	330.5 ± 7.3	345.2 ± 10.2	344.4 ± 7.9	1.000
Week 4*	352.2 ± 13.4	328.5 ± 10.2	355.6 ± 13.1	357.1 ± 8.9	1.000
Week 5	-	-	341.9 ± 16.2	345.6 ± 14.2	1.000
Week 6	-	-	345.6 ± 16.8	350.1 ± 12.9	1.000
Week 7	-	-	355.7 ± 17.2	358.3 ± 15.0	1.000
Week 8**	-	-	368.2 ± 19.1	370.9 ± 17.5	1.000

Note. Data are means ± standard deviations. Kruskal–Wallis test. Local heating procedures indicated as * *Group B, C and D,* ** *Group D.*

Histological findings

The mean percentage of the tissue-hyperplasia area, the mean thickness of submucosal fibrosis, the mean number of epithelial layers, the mean degree of the collagen deposition, and the mean degree of the TUNEL, HSP70-positive deposition were significantly different between the groups (all variables, $p < 0.001$, Kruskal–Wallis test).

The mean percentage of tissue-hyperplasia area, mean number of epithelial layers, mean thickness of submucosal fibrosis, and mean degree of collagen deposition were significantly higher in group A than in groups B, C, and D (all variables; $p < 0.05$). However, the mean degrees of HSP70- and TUNEL-positive-deposition were significantly lower in group A than in groups B, C, and D (all variables; $p < 0.05$). The density grade of inflammatory cell infiltration was not significantly different among the four groups ($p = 0.726$, Kruskal-Wallis test). The mean tissue-hyperplasia area and number of epithelial layers were significantly higher in group C than in groups B and D (all variables; $p < 0.05$). The mean degree of HSP70-positive-deposition was significantly lower in group C than in groups B and D (all variables; $p < 0.05$) (**Figure 8**). The histological findings are summarized in **Table 1**, and examples are shown in **Figures 9** and **10**.

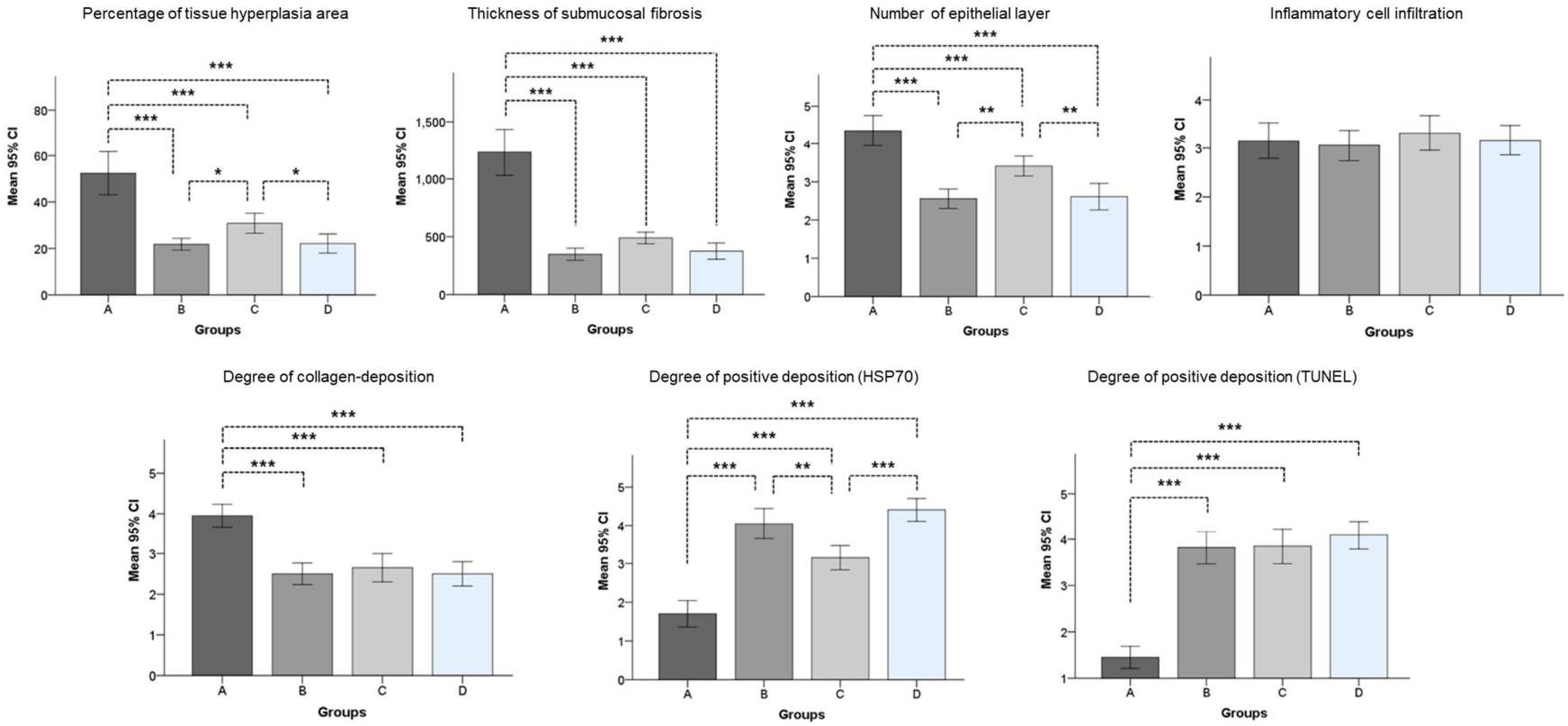


Figure 8. Histopathological findings for the stented rat esophagus at 4 and 8 weeks after stent placement in groups A-D

* $p<0.05$, ** $p<0.01$, *** $p<0.001$. CI: confidence interval.

Table 1. Histological findings after local heat treatment using gold nanoparticle (AuNP)-coated stent in rat esophagus

	Group A	Group B	Group C	Group D	†*p*-value	**p*-value (A vs. B)	**p*-value (A vs. C)	**p*-value (A vs. D)	**p*-value (B vs. C)	**p*-value (B vs. D)	**p*-value (C vs. D)
HSP70 positive deposition (Grade)	1.70±0.73	4.05±0.90	3.15±0.67	4.41±0.67	< 0.001	< 0.001	< 0.001	< 0.001	0.001	0.671	< 0.001
TUNEL positive deposition (Grade)	1.45±0.51	3.82±0.80	3.85±0.81	4.09±0.68	< 0.001	< 0.001	< 0.001	< 0.001	1.000	1.000	1.000
Collagen deposition (Grade)	3.95±0.60	2.50±0.60	2.65±0.75	2.50±0.67	< 0.001	< 0.001	< 0.001	< 0.001	1.000	1.000	1.000
Tissue hyperplasia area (%)	52.43±8.88	21.80±2.74	30.84±5.18	22.14±4.44	< 0.001	< 0.001	< 0.001	< 0.001	0.027	1.000	0.036
Thickness of submucosal fibrosis (mm)	1,232±419	348±104	489±103	375±141	< 0.001	< 0.001	< 0.001	< 0.001	0.432	1.000	0.870
Number of epithelial layers (Number)	4.35±0.79	2.56±0.51	3.41±0.51	2.61±0.70	< 0.001	< 0.001	< 0.001	< 0.001	0.001	1.000	0.002
Inflammatory cell infiltration (Grade)	3.16±0.76	3.06±0.64	3.32±0.75	3.17±0.62	0.726	1.000	1.000	1.000	1.000	1.000	1.000

Note. Data values are a mean ± standard deviations. †Kruskal–Wallis, *Mann–Whitney U test

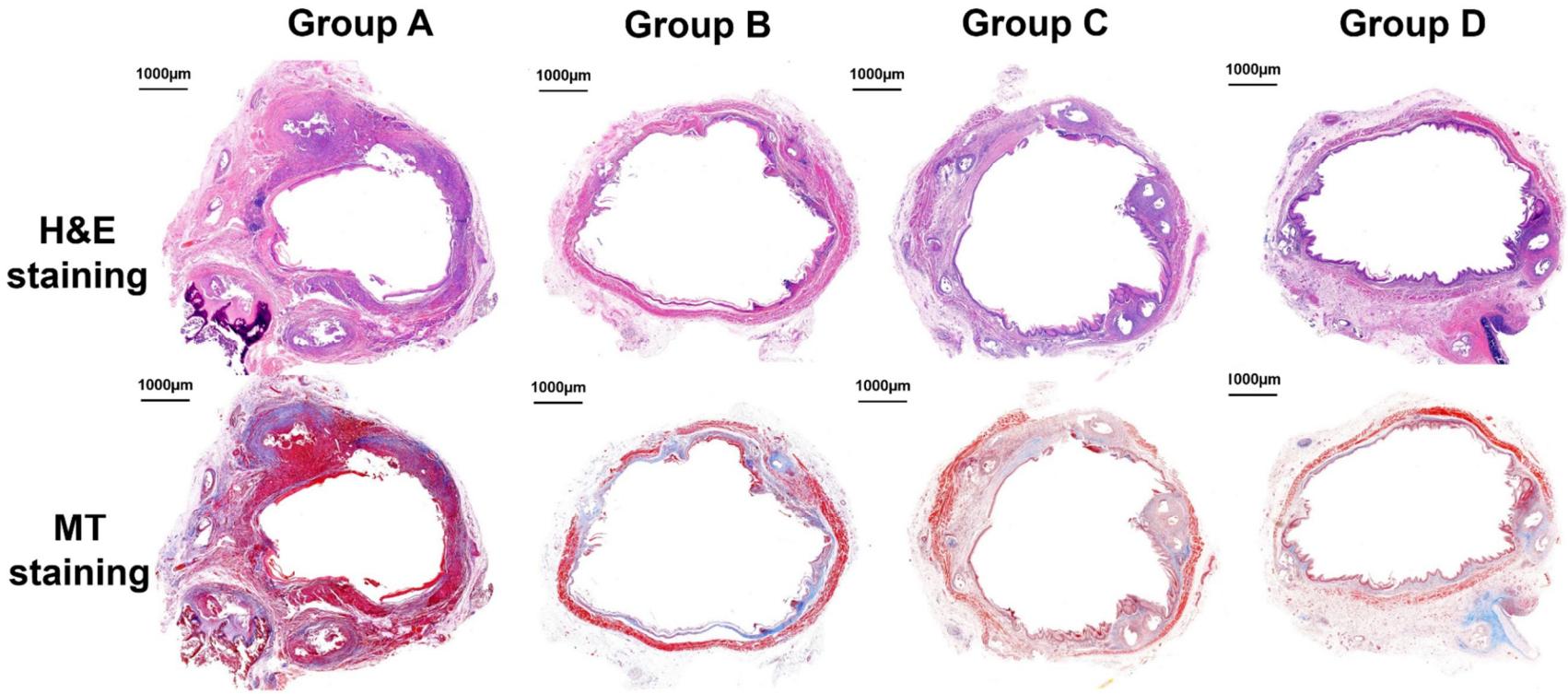


Figure 9. Representative microscopic images of histological sections obtained at 4 weeks (groups A and B) and 8 weeks (groups C and D) after stent placement. Hematoxylin and eosin and Masson's trichrome stained sections are shown (magnification, ×1.25).

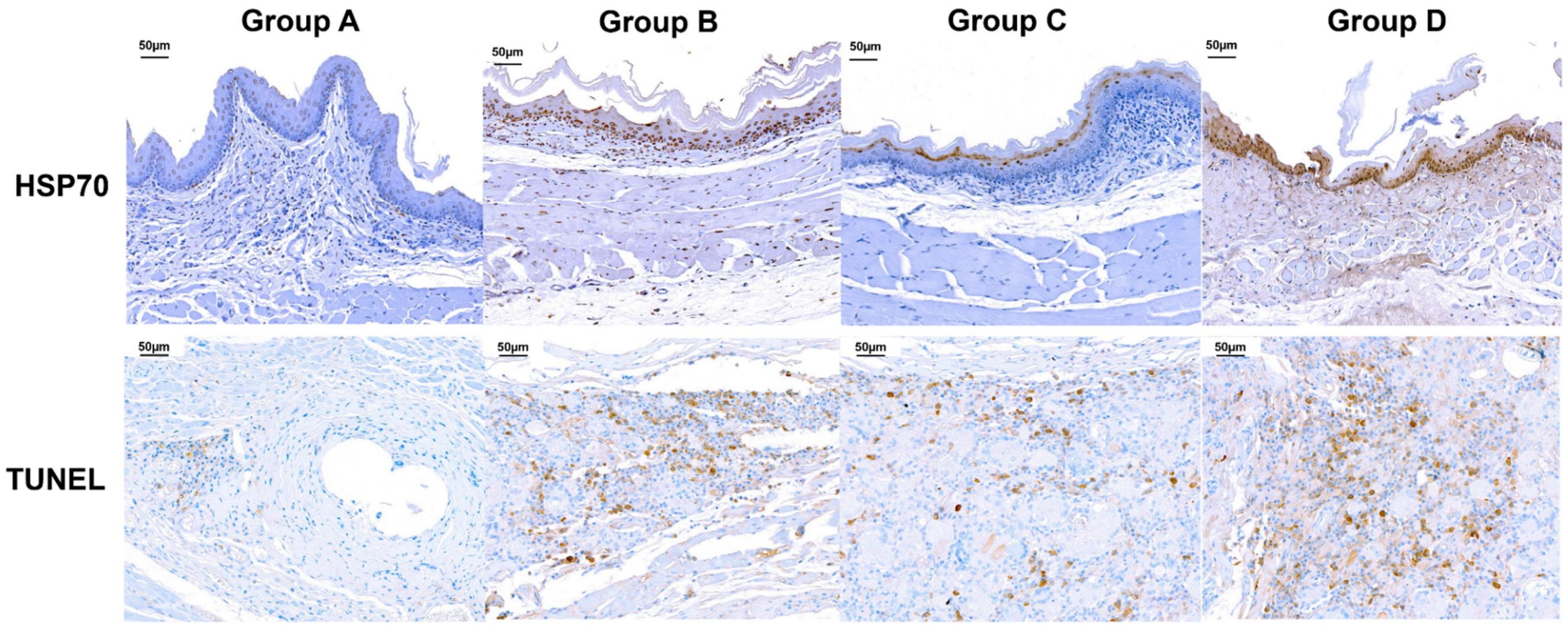


Figure 10. Representative microscopic images of immunohistochemistry sections obtained at 4 weeks (groups A and B) and 8 weeks (groups C and D) after stent placement. HSP70 and TUNEL expression were significantly increased in the heated groups compared to the control group (magnification, ×20).

DISCUSSION

Our present results have demonstrated that PTT via an AuNP-coated SEMS under NIR laser irradiation will successfully treat tissue hyperplasia after stent placement in the rat esophagus. Tissue hyperplasia was significantly decreased in the PTT groups compared to a control group. PTT-induced cell apoptosis was significantly elevated in the heated esophageal mucosa, and markers of cellular proliferation were significantly decreased after PTT when compared to the control animals. Increases in HSP expression and thermal induced-apoptosis are well-characterized features of the heat shock response, and previous studies have reported that HSP70 is an indicator of heat stress in different species (22, 23).

Our study groups were evaluated for the rebound effect. The group C rats treated once at 4 weeks after stent placement had a significant increase in tissue hyperplasia at 8 weeks compared to the group D animals treated twice at 4 and 8 weeks after stent placement. The area of tissue hyperplasia and number of epithelial layers in group C were found to have gradually increased compared with the group D rats. Our results confirmed that the PTT was more effective when administered every 4 weeks and indicated therefore that periodic PTT seems to be necessary to treat stent-induced tissue hyperplasia after stent placement. Further studies with a long-term follow-up are required to confirm our present findings.

Although PTT has been previously reported to successfully treat tissue hyperplasia, there is no consensus regarding the optimal temperature for local heating for the treatment of stent-induced tissue hyperplasia (11, 13-15, 21). Several studies have reported that at 43°C to 65°C can help inhibit tissue hyperplasia but that an increase to 70°C induced immediate tissue burn (11, 13-14). In our current study, stent-induced tissue hyperplasia in the rat esophagus was effectively treated with AuNP coated SEMS-mediated local heating at 65 °C, which may effectively burn the granulation tissues generated around the stent. Taken together, our present results demonstrated that local heat treatment at 65°C was optimal for successful PTT.

Our previous studies have reported that an AuNP-coated SEMS can be easily fabricated using simple synthesis steps and is rapidly heated to therapeutic temperatures within a few seconds of NIR laser irradiation (11, 21). The AuNP-coated SEMS used in our current experiments thus rapidly reached a high temperature, which increased in proportion to the NIR power. Hence, the PT properties could be easily controlled by adjusting the NIR irradiation levels. These properties can be attributed to the anisotropic structural characteristics of AuNP, consistent with our prior results (11, 25). Our previous studies involved local heat treatment via the AuNP-coated stent under NIR irradiation at one week after stent placement to prevent stent-induced tissue hyperplasia. In our current study, local heat treatment was performed at 4 weeks after tissue hyperplasia had already occurred. Restenosis caused by stent-induced tissue hyperplasia occurs as an excessive proliferative response within 4 weeks to the mechanical injury caused by stent placement (10, 11, 26). Taken together with previous evidence, our current findings support the notion that local heat treatment via the NIR irradiation of an AuNP-coated stent is an effective therapeutic option for the prevention as well as the treatment tissue hyperplasia after stent placement.

Advances in stent technologies have resulted in a decrease in complications and a prolonged stent patency period. Although a stent has been commonly used as a minimally invasive method to treat malignant and benign esophageal strictures, tissue hyperplasia through the mesh or around the edges of esophageal stents has been reported after the placement of up to 60% of bare stents and 13% of covered stents (27-29). Hence, permanent stent placement in patients with a relatively long life expectancy has not yet become widespread due to the likelihood of late adverse events, including the development of new strictures caused by stent-induced tissue hyperplasia, stent migration, and esophageal ulceration (30, 31). We believe however that our therapeutic strategy using AuNP-coated SEMS could be applied also to an uncovered SEMS to treat tissue hyperplasia and may prolong

stent patency by reducing stent-induced tissue hyperplasia, thus improving the patients' quality of life.

There were some limitations to our study of note. First, our findings may not reflect all of the pathological mechanisms occurring in humans following a stent placement. Second, it is necessary to determine the optimal timing for local heating after stent placement to treat stent-induced tissue hyperplasia. Third, we did not evaluate the depth penetration of the AuNP-coated SEMS in the rat esophageal model. Although additional studies will be required to further validate our current data, our study supports the premise that PTT via an AuNP-coated stent can successfully treated stent-induced tissue hyperplasia.

In conclusion, An AuNP-coated SEMS appears to be an effective approach for the local treatment of stent-induced tissue hyperplasia in the rat esophagus. An AuNP-coated stent-mediated local PTT protocol could be used for not only to treat tissue hyperplasia but also tumor ingrowth or overgrowth though the stent meshes. Although further preclinical studies are needed to investigate its efficacy and safety, this therapeutic strategy shows considerable promise for the treatment of tissue hyperplasia after stent placement.

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국문요약

연구 필요성: 자가 확장형 금속 스텐트 삽입술(Self-expandable metallic stent; SEMS)은 주로 절제 불가능한 악성 식도암 또는 양성 식도암 협착증을 치료하는데 사용되어왔다. 그러나, 스텐트 삽입술 후 스텐트 내부 또는 위, 아래에서 조직 과증식(tissue hyperplasia)으로 인해 스텐트가 폐색되는 문제가 계속해서 대두되고 있으며, 이를 해결하기 위해 조직 과증식을 억제하는 약물을 방출하는 스텐트(Antiproliferative drug-eluting SEMS) 또는 생분해성 스텐트 등 다양한 스텐트를 개발학하고 있으나 현재까지 충분한 임상적 결과를 얻기 힘든 실정이다.
연구 목표: 국소열 치료술(Local heat treatment)은 세포 증식 및 면역 반응 등을 조절하는데 이용되고 있고 있으며, 최근에는 금 나노 입자 (Gold nanoparticle; AuNP)와 자성 나노 입자 (Magnetic nanopaticles)등은 높은 효율과 정확도로 국소열 치료술 연구에 주로 사용되고 있다. 본 연구에서는 금속 스텐트에 금 나노 입자를 코팅하고 스텐트 삽입술 후 발생되는 식도의 조직 과증식을 국소열 치료술을 통해 이러한 치료 효과를 입증하고자 한다.
연구 방법: 총 40 마리의 백서(Rat)에 금 나노 입자를 코팅한 스텐트를 삽입하고, 총 4 그룹(Group A, B, C, D)으로 분류하여 Group A 는 스텐트 삽입 후 4 주 후에 희생하여 조직을 적출한다. Group B 는 스텐트 삽입 후 4 주 후에 국소열 치료술을 시행하고 희생하여 조직을 적출한다. Group C 는 스텐트 삽입 후 4 주 후에 국소열 치료술을 시행하고 8 주 후에 희생하여 조직을 적출한다. Group D 는 스텐트 삽입 후 4 주와 8 주 후에 각각 국소열 치료술을 시행하고 희생하여 조직을 적출한다. 적출한 조직을 병리학적 검사를 시행하여 조직 과증식의 여부를 확인한다.
연구 결과: 비 피폭형 금속 스텐트는 2 단계의 합성 과정을 통해 성공적으로 금 나노 입자가 코팅되었음. 스텐트 삽입술 및 국소열 치료술은 모든 백서에서 성공적이었으나 2 마리의 백서에서 stent migration 이 발생되었고 5 마리가 합병증으로 폐사하였음. 스텐트 삽입으로 유발된 조직 과증식은 A 군에 비해 B, C, D 군에서 통계적으로 유의하게 낮았음 ($p < 0.05$). HSP70 과 TUNEL 발현 정도는 A 군에 비해 B, C, D 군에서 통계적으로 유의하게 낮았음 ($p < 0.05$). 조직 과증식 관련 변수는 C 군에 비해 B 와 D 군에서 통계적으로 유의하게 낮았음 ($p < 0.05$).
연구 결론: 백서 식도 모델에서 비 피복형 스텐트 삽입 후 발생되는 조직 과증식을 국소열 치료술을 이용하여 효과적으로 치료함을 확인하였음.
(**중심 단어**: 자가 확장형 금속 스텐트, 국소 열 치료, 금 나노 입자 스텐트, 조직 과증식)