Comparison of Angiogenic Activities of Three Neuropeptides, Substance P, Secretoneurin, and Neuropeptide Y Using Myocardial Infarction

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Abstract

BACKGROUND: The interplay between neurogenesis and angiogenesis is crucial during the development mediated by neuro-angiogenic morphogens. In particular, the angiogenic activity of neuropeptides and their role in tissue regeneration have long been investigated for better understanding of their biological mechanisms and further applications. However, there have been few studies for direct comparison of angiogenic activities of neuropeptides for *in vitro* and *in vivo* models. In this study, we report that direct comparison of the angiogenic activities of neuropeptide Y, secretoneurin, and substance P (SP) immobilized on hydrogels in *in vitro* and *in vivo* experiments.

METHODS: A hyaluronic acid-based hydrogel is prepared by utilizing acrylated hyaluronic acid and thiolated peptides as a crosslinker and angiogenic factors, respectively. Angiogenic activities of three neuropeptides are evaluated not only by *in vitro* angiogenic and gene expression assays, but also by an *in vivo* chronic myocardial infarction model.

RESULTS: The comparison of *in vitro* angiogenic activities of three peptides demonstrates that the SP-immobilized hydrogel shows a higher degree of cell network formation and angiogenic-specific genes than those of the other peptides and the control case. In addition, a three-dimensional angiogenic assay illustrates that more sprouting is observable in the SP group. Evaluation of regenerative activity in the chronic myocardial infarction model reveals that all three peptide-immobilized hydrogels induce increased cardiac function as well as structural regeneration. Among all the cases, the SP group provided the highest regenerative activity both *in vitro* and *in vivo*.

CONCLUSION: In our comparison study, the SP-immobilized hydrogel shows the highest angiogenic activity and tissue regeneration among the test groups. This result suggests that nerve regeneration factors help angiogenesis in damaged tissues, which also highlights the importance of the neuro-angiogenic peptides as an element of tissue regeneration.

Keywords Neuro-angiogenic peptide · Hyaluronic acid-based hydrogel · Angiogenesis · Chronic heart failure

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1 Introduction

Angiogenesis in tissue regeneration has long been explored due to the importance of repairing damaged tissue, which requires the formation of a proper vascular network supplying oxygen and nutrients [1]. Therapeutic manipulation of such angiogenesis provides unique opportunities for facilitating the cure of morbidity and mortality in heart disease [2]. Thus, the use of angiogenic factors, including growth factors and cytokines, is of great interest for promoting the regeneration of vascular structures [3, 4]. In

particular, neuropeptides secreted by the sensory and autonomic nerves are known to play an important role in growth factor and cytokine release, which regulates immune surveillance, cardiovascular homeostasis, and, importantly, angiogenesis [5]. This suggests that the finding of effective neuropeptides with angiogenic potential is critical to develop the precise manipulation of angiogenesis for tissue regeneration.

To date, a number of neuropeptides including neuropeptide Y (NPY) [6], substance P (SP) [7], calcitonin gene-related peptide (CGRP) [8], vasoactive intestinal peptide (VIP) [9], secretoneurin (SN) [10], and catestatin [11] are emerging as important regulators enabling researchers to mediate the vascular system through angiogenesis and vascular remodeling. For instance, SN has been shown to stimulate vascular endothelial growth factor (VEGF) signaling for angiogenesis by promoting the migration and incorporation of endothelial progenitor cells [12]. Unlike VEGF-induced angiogenesis, NPY promotes vascular sprouting from existing blood vessels, resulting in the formation of microvessels in tree-like structures rather than in meshed structure, which is analogous to those induced by fibroblast growth factor (FGF)-2 [13]. Another neuropeptide, SP, has the capability of inducing angiogenesis by enhancing the proliferation of endothelial cells and the recruitment of cells with angiogenic potential [14]. These neuropeptides have potential uses as therapeutic agents in cardiovascularization, which could improve the cardiac function in heart disease.

Delivering bioactive molecules such as small molecule drugs, growth factors, or peptides is another challenging issue for effective tissue regeneration. Hydrogels have been used as an ideal delivery vehicle for controlling the release of bioactive molecules. The encapsulation of growth factors such as VEGF or FGF in the polymer network could increase the angiogenesis in the damaged tissues such as the heart or hind limb [15, 16]. The controlled release of smaller molecules such as bioactive peptides by encapsulation has limitations because of the larger polymer network pores, which facilitate the initial burst of bioactive peptides. The immobilization of peptides is an alternative strategy for the stimuli-sensitive controlled release of small molecule peptides. Ac-SDKP peptide is immobilized in a hydrogel, and the application of this hydrogel increases the regenerative activity in the infarcted hearts. In our previous study, we show that hydrogels with growth factors or bioactive peptides increase the regeneration potential of infarcted hearts by enhancing the angiogenic activity of the damaged tissues [17].

Here, we report the angiogenic activity of three neuro-angiogenic peptides, NPY, SN, and SP, immobilized on hydrogels by employing *in vitro* and *in vivo* myocardial infarction (MI) models. We find that these peptides have

different angiogenic activities and distinct roles in angiogenesis. Since these peptides are extensively used for angiogenic modulations, direct comparisons of their angiogenic activities and regeneration potentials will be beneficial for future researches in the field of neurogenesis and angiogenesis.

2 Materials and methods

2.1 Materials

Sodium hyaluronate (molecular weight: 200 kDa) was purchased from Lifecore Biomedical (Chaska, MN, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, adipic acid dihydrazide, and triethanolamine (TEA) were acquired from Sigma-Aldrich (St. Louis, MO, USA). 1-Hydroxybenzotriazole hydrate was purchased from Fluka Chemical (Buchs, Switzerland). N-acryloxysuccinimide was purchased from Polyscience (Warrington, PA, USA). RGD (GCGYGRGDSPG), NPY (SKPDNPGEDAPAEDMARYYSLRHYINLITRQRYC), SN (TNEIVVEEQYTPQS LATLESVFQELGKLTGPNNQC), SP (RPKPQQFFGLMC) and a matrix metalloprotease (MMP)-sensitive peptide (GCRDGPQGIWGQDRCG) were synthesized by Anygen (Gwangju, Korea). Human umbilical vein endothelial cells (HUVECs), human mesenchymal stem cells (hMSCs), Endothelial Growth Medium (EGM-2) BulletKit and mesenchymal stem cell growth medium (MSCGM) were purchased from Lonza (Walkersville, MD, USA). Trizol were purchased from Invitrogen-Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS), penicillin/streptomycin, trypsin, Dulbecco's modified Eagle's medium (DMEM), and phosphate-buffered saline (PBS; pH 7.4) were purchased from Gibco BRL (Grand Island, NY, USA). A LIVE/DEAD Viability/Cytotoxicity Assay Kit was purchased from Molecular Probes (Eugene, OR, USA). The PrimeScript first-strand cDNA synthesis kit and PrimeScript RT reagent kit were purchased from Takara Bio Inc (Shiga, Japan).

2.2 Preparation of hyaluronic acid (HA)-based hydrogel with immobilized neuro-angiogenic peptide

The HA was first acrylated as shown in previous studies [18], and the acrylated HA (200 kDa, 3 wt%) was dissolved in 0.3 M TEA buffer to add MMP-sensitive peptide with an equimolar ratio of 80% acryl and thiol groups. RGD and neuro-angiogenic peptides (NPY, SN, and SP) were immobilized on the MMP-HA at a molar ratio of 20% of the acrylic groups by dissolving them into TEA buffer. The reaction mixture was incubated at 37 °C for gelation.

These HA-based hydrogels were used for the three-dimensional (3D) hMSC cultures of the *in vitro* studies and for the *in vivo* MI model.

2.3 Cell preparation and culture

HUVECs were cultured in endothelial basal medium-2 with supplementation of the provided kit, and hMSCs were cultured in mesenchymal stem cell growth medium. All cells were maintained in the incubator at 37 °C with 5% CO₂ and humidification. The culture medium was exchanged every 2–3 days. Five passage HUVECs and CB-MSCs were used for experiments.

2.4 *In vitro* 3D cultures in HA hydrogels and evaluation of cell morphology

The hMSCs were cultured in a T-75 cell culture plate and then trypsinized for the dissociation of the adherent cells. The suspended cells were mixed with a peptide-immobilized HA hydrogel solution (1×10^6 cells/100 µL gel). Four different peptide groups were used: (A) RGD, (B) NPY, (C) SN, and (D) SP, respectively. The volume of the 3D construct was equally adjusted to 25 mL ($n = 4$ for each group). The mixture samples were crosslinked for 20 min at 37 °C, and the resulting cell-containing hydrogels were cultured in EGM-2 and DMEM at a 1:1 ratio for 14 days (5%, CO₂, 37 °C). Cellular morphology in the HA-based hydrogels was analyzed by light micrography at 7 and 14 days.

2.5 Measurement of cell roundness using image analysis program

To measure the degree of cell morphology change, an index value for the cell roundness was estimated. The roundness of cells was calculated by employing the formula $4 \times \pi \times (\text{area/perimeter}^2)$ [19]. This equation enabled us to measure the perimeter of the cell area. When a cell expands and becomes wider or longer, the circle exponent approaches 0, and when the cell remains circular, the circle exponent approaches 1.

2.6 Evaluation of angiogenic gene expression

HA hydrogels containing hMSCs were cultured for 14 days and then homogenized in TRIzol reagent according to the manufacturer's instructions. Purified RNA samples were then reverse-transcribed to cDNA with the PrimeScript RT reagent kit. Their cDNAs were subjected to real-time polymerase chain reaction (PCR) (SYBR Premix Ex Taq; Takara) to evaluate the expression of von Willebrand factor (vWF, forward: CCAGCTTCTGAAGAGCACCT; reverse:

GTACAGCACCATTCCCTCCT) and endoglin (CD105, forward: TGCAGAAAGAGTCGGTTGTG; reverse: TCTCAGTGCCATTTTGCTTG) genes as angiogenic differentiation markers. As an internal control, the expression of β -actin gene (forward: ATGGCAATGAGCGGTTC; reverse: GGATGCAGGACTCCAT) was tested. Gene expression level was estimated as fold changes by comparing the cycle threshold values for the β -actin gene and the mRNAs of interest.

2.7 3D angiogenesis assay with endothelial cell spheroids

The HUVEC spheroids was prepared as previously described [20]. In short, HUVECs were suspended and aggregated to form cellular spheroids (500 cells/spheroid) within the culture medium by the hanging drop method. Spheroids were embedded into collagen gels containing culture medium with neuro-angiogenic peptide (10 µL/100 mL). After 16 h of incubation, the HUVEC spheroids were stained with calcein AM. Fluorescent images from five to seven spheroids per experimental group were captured via confocal microscopy (LSM 700, Zeiss, Germany), and the lengths and total number of sprouts were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8 Animal experimental groups

The procedures of animal experiment were approved by the institutional animal care and use committee of Korea University College of Medicine (Protocol Number: KUIACUC-2015-165). All institutional and national guidelines for the care and use of laboratory animals were followed. Rats of chronic MI model were used for the *in vivo* experiments, and they were grouped into six ($n = 3$ –6 for each group): (A) sham operation, (B) chronic myocardial infarction, (C) hydrogel alone as the control group, (D) neuropeptide Y/hydrogel, (E) secretoneurin/hydrogel, (F) substance P/hydrogel. The thoracotomy of all animal groups was performed by three times, which produces surgical stress.

2.9 Myocardial infarction model

Sprague–Dawley rats, 6 week male, were anesthetized with enflurane and then kept under anesthesia with 2% isoflurane and 0.3 L/min oxygen after tracheal intubation. Ventilation was performed at a rate of 76 cycles/min with a tidal volume of 2.5 mL. In a left-sided thoracotomy, partial ligation of a left anterior descending (LAD) artery was performed with a 7-0 polypropylene suture at 2–3 mm distance from its origin. The re-expansion of the lungs was

carried out, and the chest, muscle, and skin were closed with a 3-0 silk suture. Baytril was intramuscularly injected to prevent infection at a dose of 5 mg/kg on the day after surgery. The animals were maintained with ventilation of 0.3 L/min oxygen until awoken from anesthesia. The sham animals were treated with the same procedure, except that the suture was passed under the LAD artery. Thoracotomy was carried out at four weeks after the infarction, followed by injection of each hydrogel composite (total volume: 50 μ L) into the epicardium of the border zone of the infarcted area by utilizing a 100- μ L Hamilton syringe and a 26-G curved needle. Evaluation of heart function was performed by the use of a Millar catheter at four weeks after the injection. All the animals were then sacrificed, and their hearts were histologically analyzed.

2.10 Heart functional analysis using pressure–volume (PV) catheter

To evaluate the pressure–volume relationship of the heart, rats were similarly anesthetized. A micro-tip (2F) PV catheter apical stab was inserted into the left ventricle and connected to a pressure–conductance system (MPVS-300, Millar Instruments, Inc., Houston, TX, USA). The pressure and volume were continuously measured in real time using a software (Chart 5 v5.4.2, AD Instruments, Sydney, Australia) on a computer. After stabilization, the signal from the left ventricle pressure–volume loop was used as a base line and following parameters were characterized using special cardiac pressure–volume analysis software (PVAN v3.5; Millar Instruments, Inc.): stroke volume, ejection fraction, cardiac output, and arterial elastance.

2.11 Statistical analysis

All data are reported as the mean $\pm$ SD. Differences between the experimental groups were analyzed by Origin Pro 8 (OriginLab Co., Northampton, MA, USA) and Student's *t* test of Excel, and $p < 0.05$ was considered statistically significant.

3 Results

3.1 Preparation of HA-based hydrogels with immobilized neuro-angiogenic peptides

To explore the angiogenic activity of neurogenic peptides, we utilize HA-based hydrogels by immobilizing NPY, SN, or SP on them through a Michael-type addition reaction. The MMP-sensitive peptides are copolymerized as degradable crosslinkers, which are responsible for the stimulus-responsive release of the neurogenic peptides.

Since the cells secreted metalloproteases, the hydrogels are slowly degraded, allowing for the incorporation of the peptides into the cells during *in vitro* and *in vivo* experiments. Three kinds of hydrogels, incorporating NPY, SN, or SP, are employed to estimate their angiogenic activity and further regeneration potential in *in vitro* experiments and an *in vivo* MI model (Fig. 1).

3.2 Evaluation of cell morphology of hMSCs in HA-based hydrogel

The effects of the neuropeptides on the morphology of hMSCs are evaluated by culturing the cells in the HA hydrogel scaffolds that contain either NPY, SN, SP, or RGD. Seven days later, the morphology of the hMSCs begins to differentiate for each group. In contrast to the RGD group's cells that mostly remain in round shape, each neuropeptide group displays changes in the cell morphology. The hMSCs in the NPY hydrogel begin to stretch out randomly, and such change is more obvious for the SN and SP groups that are characterized by star-like spreading and the formation of small islands connecting with each other, respectively. By the 14th day, the morphology of the hMSCs is dramatically changed, proving that the neuropeptides immobilized on the HA hydrogels act as angiogenic activators for cell network formation. The hMSCs in the NPY hydrogel form progressed networks among the cells, and those in the SN hydrogel show cell aggregation and extensive tubular structures (Fig. 2A). It is also interesting to note that the SP hydrogel induces the formation of clustered hMSC islands in a spheroid shape, where they are interconnected with each other by long branches. To quantify the change in the hMSCs' morphology, their micrographs are analyzed by roundness index using image analysis software on the 7th day. The roundness indices of the RGD group and the NPY group are 0.62 ± 0.1 and 0.24 ± 0.05 , respectively. The indices of the SN group and the SP group are close to zero, indicating that the circumferences of the hMSCs are different from that of a circle (Fig. 2B).

3.3 Angiogenesis-related gene expression of hMSCs in neuropeptides immobilized on hydrogels

To determine the angiogenic potential of the neuropeptides, we characterize the gene expression profiles of hMSCs cultured in HA hydrogels on which NPY, SN, SP, or RGD are immobilized (Fig. 3). The mRNA levels of angiogenesis-specific genes including vWF, CD31, CD105, and alpha smooth muscle actin (α -SMA) are measured by utilizing real-time PCR. The results show that the SP group tends to upregulate all four mRNA levels of vWF, CD31, CD105, and α -SMA by 1.7-, 1.5-, 1.5-, and 2-fold,

Fig. 1 Schematic diagram illustrating neuro-angiogenic peptide immobilized on a hyaluronic acid-based hydrogel.
* MI: myocardial infarction

Fig. 2 Evaluation of cell morphology of hMSCs in HA-based hydrogels with immobilized neuro-angiogenic peptides. **A** Cellular morphology was observed at days 7 and 14. **B** The cell roundness index was measured using an image program on a microscope image

captured at 7 days. Scale bars: 200 μm , * $p < 0.05$, ** RGD: Arg-Gly-Asp peptide, NPY: neuropeptide Y, SN: secretoneurin, SP: substance P

respectively. For both the SN and NPY groups, the mRNA levels of CD105 (a specific marker to represent the activated endothelial cells) and α -SMA (a marker of mature vessels) are similarly increased by 1.5- and 2-fold, respectively, but there are negligible changes in CD31 (a marker of endothelial cells). These results suggest that SP-immobilized hydrogels provide the highest angiogenic potential among the three neuropeptides and the RGD group, as represented by the increased expression levels of angiogenic and vascularization-related genes of the co-cultured hMSCs.

3.4 3D angiogenic assay of spheroids in collagen gels treated with neuropeptides

The angiogenic potentials of neuropeptides are also estimated by conducting 3D endothelial cell sprouting assays, which closely mimics the actual environment *in vivo* (Fig. 4A). Unlike HA-hydrogel based experiments immobilized with the peptides, HUVEC spheroids are cultured in collagen gels containing soluble NPY, SN, SP, or RGD, and the numbers and lengths of their branches are measured. Among the three neuropeptides, the SP group shows

Fig. 3 Gene expression of hMSCs in various HA-based hydrogels by real-time PCR at 14 days. The mRNA expression levels of von Willebrand factor (vWF), CD31, CD105, and smooth muscle actin (α -SMA) were higher in the SN and SP groups than in the control and NPY groups. * $p < 0.05$

Fig. 4 Angiogenesis by three neuropeptides on HUVEC spheroids in collagen gels. **A** Calcein AM-stained fluorescent image after 16 h of neuro-angiogenic peptide treatment (10 μ L/100 mL). **B** The average

length of branches and average number of branches were higher in the SN and SP groups than in the RGD group. Scale bars: 200 μ m. * CON: control, NPY: neuropeptide Y, SN: secretoneurin, SP: substance P

the highest angiogenic potential in comparison to the other neuropeptides and the RGD control, which is highlighted by the longer length and higher number of branches of the spheroids. It is worthwhile to note that all of the

neuropeptides induce significantly longer branches than those of the RGD control (Fig. 4B), but exhibit slight changes in the number of the branches with no statistical significance (Fig. 4C).

3.5 Comparison of regenerative activity of neuropeptide-immobilized hydrogels in myocardial infarction animal model

The regenerative activities of neuropeptide-immobilized hydrogels are compared by injecting them into the infarcted area in a chronic MI model. In chronic MI, heart tissue undergoes extensive remodeling that leads to the larger size of the heart and its decreased contractility. This suggests that the comparison of the remodeled heart size is crucial to determine the therapeutic effects of the hydrogels. The heart size of the chronic MI group is larger than that of the control group due to hypertrophy, and the ischemic portion of its myocardium shows changes in color (Fig. 5A). We observe that the effects of NYP- or SN-immobilized hydrogels on heart sizes are negligible in comparison with those of the MI group, which implies that the therapeutic effects of the NYP and SN hydrogels are limited in the extensively remodeled chronic MI. In contrast, treatment with the SP hydrogel results in a significant decrease in the heart size when compared to the MI group and a similar size to the healthy control hearts. Our experimental results

demonstrate that the therapeutic activity of SP is the highest among the three neuro-angiogenic peptides.

Histological analysis reveals that hydrogels containing SP could regenerate the heart muscle from the fibrous tissue formed at an infarct site (Fig. 5B). Hematoxylin and eosin staining of histologic sections indicate that the SP group induces thickening of the left ventricular wall in comparison to the NYP and SN groups. In addition, Masson's trichrome staining of the histologic section clearly shows that the fibrous tissue at the infarct site is less in the SP group than in the other peptide groups (Fig. 5B). Based on both analyses, we propose that SP treatment as a hydrogel embedded form can regenerate myocardium after MI. In parallel, we identify the formation of mature blood vessels on the histological sections by immunostaining with α -SMA antibody (Fig. 6). The results show that the SP-treated group develops more blood vessels than the other groups, suggesting that SP is a potent candidate for angiogenesis.

Functional analysis of the heart using a PV catheter enables us to characterize the accurate pressure–volume loop, which provides diverse parameters related to heart functions (Fig. 7). Treatment with all three types of neuropeptide-immobilized hydrogels results in increased stroke

Fig. 5 The morphologies of the heart. **A** Comparison of heart size after the remodeling process with different compositions of hydrogel. **B** The morphologies of the left ventricle with staining by hematoxylin and eosin and Masson's trichrome (MT). Note that the arrows indicate

the border area (square box) between normal and ischemic tissue. Scale bars: top low is 2000 μm; middle and bottom lows are 400 μm. * MT: Sham: control, CMI: chronic myocardial infarction, Gel: hydrogel alone, NPY: neuropeptide Y, SN: secretoneurin, SP: substance P

Fig. 6 Representative immunostaining of vascular smooth muscle cells (VSMCs). Using immunofluorescence, VSMCs were stained for alpha smooth muscle actin (α -SMA). Scale bars: 400 μ m. * Sham:

control, MI: myocardial infarction, Gel: hydrogel only, NPY: neuropeptide Y, SN: secretoneurin, SP: substance P, DAPI: 4',6-diamidino-2-phenylindole

Fig. 7 Functional analysis of the heart. Pressure and volume in the left ventricle were measured by catheter, and functional indices were calculated. * SV: stroke volume, EF: ejection fraction, CO: cardiac output, Ea: arterioles elastance

volume compared to the MI or RGD gel group. However, there are no significant differences among the neuropeptide groups (NYP, SN, and SP). The value of the ejection fraction is the highest for the SP group among the neuropeptide

groups, and its value recovers up to 70% of that of the control group. Cardiac outputs from the neuropeptide-treated groups improves up to 64.5% compared to those of the MI group, with no differences between the treated groups.

Evaluation of arterial elastance indicates that the three neuropeptide groups yield the lowest values, even compared to that of the control group. Considering the various functional indices, we conclude that SP hydrogel treatment in the chronic MI model provides the highest regenerative activity among three neuropeptide hydrogels.

4 Discussion

Neurogenesis and angiogenesis are tightly related to the formation of neural and vascular networks during development and regeneration [21, 22]. Research on the interplay of key molecules between these biological pathways has focused on the controlled modulation of angiogenesis [23]. In particular, neuroactive peptides such as substance P, secretoneurin, and neuropeptide Y have been extensively studied in the field of regenerative medicine where they play a role in enhancing angiogenesis during regeneration. Although molecular basis of the three neuropeptides remains to be elucidated, we find that they tend to increase angiogenesis for myocardial infarction model. Furthermore, when substance P is immobilized on a bioactive peptide [7], secretoneurin is delivered using nanoparticles [24], or NPY [25] is transferred into the hind limb ischemic model, the surrounding tissue is regenerated. In light of our experimental results we confirm that neurogenic factors have angiogenic effects.

Common methods to evaluate the angiogenic potentials of these peptides are either administering them into endothelial cell culture medium as a soluble form for *in vitro* trials or injecting them into the site of animal infarction models for *in vivo* experiments [26, 27]. The immobilization of bioactive peptides in biodegradable scaffolds is an example of advanced treatments in tissue engineering and drug delivery research areas. However, the angiogenic potential of neuropeptides immobilized in biomimetic hydrogels remains to be elucidated for *in vitro* and *in vivo* applications. Moreover, few studies have focused on comparisons of the angiogenic activity of hydrogel-immobilized neuropeptides in infarcted tissues. In our research, we examine the angiogenic activities of three well-known neuropeptides by immobilizing them to the biomimetic hydrogels *in vitro* and *in vivo*. There is a caveat in precisely evaluating the angiogenic activities that are dependent on the release profile of the neuropeptides from hydrogels. Although we indirectly estimate the release profile based on our previous works [28], there are experimental limitations to correlate the actual release profile in this study.

We demonstrate that NPY-, SN-, or SP-immobilized hydrogels induce different aggregation patterns of hMSCs in *in vitro* co-culture experiments. For example, cells in NPY hydrogels show cell spreading, whereas cells in SP hydrogels

form spherical 3D structures. This reflects the fact that each peptide has a distinct role in the heart [29]. Gene expression profiles also show the differential angiogenic activities of the three peptides. Differentiation of mesenchymal cells to endothelial cells shows that cells in the SN and SP groups increase the expression of endothelial cell-specific genes by 50%. Specifically, CD105 and α -SMA are expressed at the highest level in the SP group, which is correlated with the *in vivo* experiments displaying mature vascular structures.

For sub-acute or acute infarction models, the simple injection of hydrogels allows for the regeneration of tissues. However, the chronic myocardial infarction model is challenging for regeneration, mainly due to the irreversible remodeling of left ventricles and hypertrophic phenotypes [30]. Interestingly, our study demonstrates that all three neuropeptides showed increased myocardial tissue regeneration in the border area and functional recovery in the chronic infarction model. Specifically, SP shows the highest regeneration potential among the three peptides by improving the cardiac output, ejection fraction, and stroke volume. Limited regeneration potential of peptide immobilized hydrogels in chronic MI model needs further approaches such as combination therapy of peptides and growth factors. Although we focus on the angiogenic activities of the peptides, the modulation of inflammation could be involved in heart regeneration, considering the fact that the regeneration process in chronic myocardial infarction is highly complex [31].

Neurogenesis and angiogenesis are two important events during development, which is also fundamental during the regeneration of damaged tissue at which the developmental process is recapitulated [32–34]. Here, we hypothesize that three well-known neuroactive peptides have angiogenic as well as neurogenic activities and their processes involved during development and regeneration are distinct. To evaluate our hypothesis, we performed direct comparison of the angiogenic activities of the peptides in *in vitro* and *in vivo* myocardial infarction (MI) models. For the effective therapeutic treatment in tissue regeneration, we also employed HA-hydrogel to immobilize the peptides, allowing their controlled release according to their function *in vivo*. Based on the experimental setup, our results support that neuropeptides and their immobilization into biomimetic hydrogels are promising approaches for regeneration in a chronic infarction model as well as other tissue defects requiring angiogenesis.

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Compliance with ethical standards

Conflict of interest The authors have no financial conflicts of interest.

Ethical statement The animal experiment procedures were approved by the institutional animal care and use committee of Korea University College of Medicine (KUIACUC-2015-165).

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