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Peptide amphiphile nanofiber hydrogel delivery of Sonic hedgehog protein to the penis and cavernous nerve, suppresses intrinsic and extrinsic apoptotic signaling mechanisms, which are an underlying cause of erectile dysfunction

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Abstract

Erectile dysfunction (ED) is a common and debilitating condition with high impact on quality of life. An underlying cause of ED is apoptosis of penile smooth muscle, which occurs with cavernous nerve injury, in prostatectomy, diabetic and aging patients. We are developing peptide amphiphile (PA) nanofiber hydrogels as an *in vivo* delivery vehicle for Sonic hedgehog protein to the penis and cavernous nerve to prevent the apoptotic response. We examine two important aspects required for clinical application of the biomaterials, if SHH PA suppresses intrinsic (caspase 9) and extrinsic (caspase 8) apoptotic mechanisms, and if suppressing one apoptotic mechanism forces apoptosis to occur via a different mechanism. We show that SHH PA suppresses both caspase 9 and 8 apoptotic mechanisms, and suppressing caspase 9 did not shift signaling to

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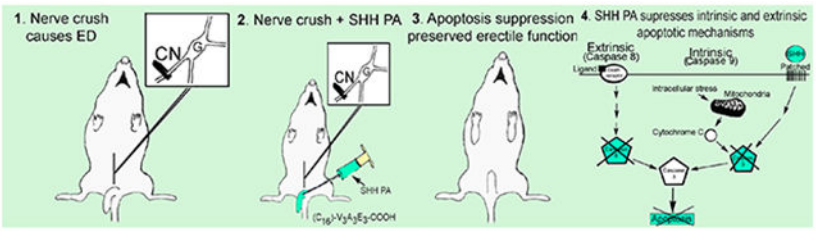
caspase 8. SHH PA has significant clinical potential as a preventative ED therapy, by management of intrinsic and extrinsic apoptotic mechanisms.

Graphical Abstract

Erectile dysfunction (ED) is a common and debilitating condition with high impact on quality of life. An underlying cause of ED is apoptosis of penile smooth muscle, which occurs with cavernous nerve injury, in prostatectomy, diabetic and aging patients. We are developing peptide amphiphile (PA) nanofiber hydrogels as an *in vivo* delivery vehicle for Sonic hedgehog protein to the penis and cavernous nerve to prevent the apoptotic response and ED. We examine two important aspects required for clinical application of the biomaterials, if SHH PA suppresses intrinsic (caspase 9) and extrinsic (caspase 8) apoptotic mechanisms, and if suppressing one apoptotic mechanism forces apoptosis to occur via a different mechanism. We show that SHH PA suppresses both caspase 9 and 8 apoptotic mechanisms, and suppressing caspase 9 did not shift signaling to caspase 8. SHH PA has significant clinical potential as a preventative ED therapy, by management of intrinsic and extrinsic apoptotic mechanisms.



Keywords

Peptide amphiphile; erectile dysfunction; intrinsic and extrinsic apoptosis; cavernous nerve injury; Sonic hedgehog; penis; peripheral nerve regeneration

Introduction

Erectile dysfunction (ED), failure to achieve or to maintain an erection, is a common and debilitating medical condition that affects more than 50% of men worldwide between the ages of 40 and 70[1], and 22% of men under 40[2-3]. Eighteen percent of US men (18 million men), and 30-64% of European males have ED (EMAS study)[4-5], and aging, diabetes, and prostate cancer treatment, including prostatectomy and radiation therapy, are risk factors that contribute significantly to ED development. ED causes physical and mental challenges for patients, and their partners, and is an early warning sign for cardiovascular disease[6], so is important to treat early, prior to disease progression. The most common therapy, oral phosphodiesterase type 5 inhibitors, is minimally effective and has inconsistent safety profiles in patients with neuropathy of the cavernous nerve (CN), including prostatectomy (up to 82% ineffective), diabetic (up to 59% ineffective) and aging ED patients[7-10]. Therefore novel therapies that are clinically durable and well tolerated are needed.

The CN (Figure 1A), a parasympathetic nerve that releases neurotransmitters essential for penile innervation, undergoes crush, tension, resection and cautery injury in up to 82%

of prostatectomy patients[7-9,11]. The CN also undergoes peripheral neuropathy in aging, and diabetic patients where the incidence of ED is as high as 75%[12-13]. CN damage and loss of innervation to the erectile tissue of the penis, initiates a cascade of architectural remodeling, starting with smooth muscle apoptosis in the corpora cavernosa[14-17] (Figure 1B). The remodeling is extensive with ~50% smooth muscle loss observed in prostatectomy and diabetic patients[18]. This is closely followed by fibrosis, with collagen increasing and a change in subtypes, making the corpora cavernosal tissue unable to respond to normal neurotransmitter signals[19-23], and ED is the result.

The sonic hedgehog (SHH) pathway plays a critical role in establishing and maintaining the smooth muscle and sinusoidal morphology of the corpora cavernosa of the penis[24]. When SHH is inhibited either in the penis, or in the pelvic ganglia (PG)/CN, smooth muscle apoptosis is increased 12-fold in the corpora cavernosa, the sinusoidal architecture is lost, and ED results[17,24-25]. SHH protein is decreased both in the penis and in the PG/CN, when the CN is injured in prostatectomy and diabetic animal models, and in ED patients[16-18,24]. Reintroduction of SHH protein to the penis at the time of CN injury, suppresses induction of smooth muscle apoptosis[17,26], hastens CN regeneration[27-28], and improves erectile function[27]. Since SHH has substantial clinical potential for development as an ED therapy, we have been developing two nanoscale self-assembling peptide amphiphile (PA) hydrogel delivery vehicles for SHH protein to the penis and CN, to quench the apoptotic response to CN injury, and to regenerate the CN more quickly[26-27]. In the first form, these biodegradable, non-invasive[29-30], extended release vehicles can be delivered by a simple injection into the penis at the time of CN injury, where they form a thin hydrogel lining *in vivo*, in the sinuses of the corpora cavernosa; this affords prolonged release of SHH protein to suppress the apoptotic response[26] (Figure 1B). PA delivery of SHH protein successfully suppressed smooth muscle apoptosis in both CN resection and CN crush injury rat models of ED[26,31]. The second distinct formulation, in which PA nanofibers are highly aligned, is formed as a linear hydrogel that is arranged on top of the CN at the time of injury (Figure 1C) to accelerate regeneration, and enhance erectile function ~60%, indicating substantial potential for clinical translation[32-33,27]. We have been optimizing SHH delivery by this methodology prior to starting clinical application[31]. However the signaling mechanism through which apoptosis occurs in response to CN injury is only recently determined[34], and to SHH inhibition, has not previously been examined in the penis. Apoptosis can be initiated by two distinct mechanisms, either via the intrinsic pathway that activates caspase-9, or the extrinsic mechanism, that activates the initiator caspase-8[34-37] (Figure 2). Patients with ED primarily utilize extrinsic caspase 8-dependent mechanisms, while ED animal models have significant intrinsic caspase-9 signaling, and a brief, early flux of caspase 8[34]. In this manuscript we investigate the mechanism of how apoptosis occurs in response to SHH inhibition, and examine if SHH treatment of the penis by PA can inhibit both intrinsic and extrinsic mechanisms. In order for clinical translation of SHH PA to occur to treat ED patients, suppression of both intrinsic and extrinsic mechanisms may be needed to effectively prevent the apoptotic response in penile smooth muscle, and thus ED.

Methods

Animals:

Adult (P115-P120) Sprague Dawley rats (n=55) were procured from Charles River. Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health instructions. The protocol was approved and animals were cared for in accordance with the University of Illinois at Chicago Office of Animal Care and Institutional Biosafety.

Patients:

Human corpora cavernosal tissue was obtained from 3 patients who were undergoing prosthesis implant at Loyola University Medical Center, and at the University of Illinois at Chicago. Corpora cavernosal tissue was from patients who had diabetes. Exclusion criteria include men less than 18 years of age. The Institutional Review Board of Loyola University Medical Center, and the University of Illinois at Chicago, approved the protocol, and written informed consent was obtained from all patients. Diabetic corpora cavernosal tissue was used as a positive control for caspase 8-cleaved analyses since diabetic corpora cavernosa has documented apoptosis using caspase 8 dependent mechanisms[34].

Material synthesis:

Peptide amphiphiles were synthesized by conventional automated solid-phase synthesis methods using FMoc-based chemistry, as described in detail previously[26]. Peptides with the sequence VVVAAAEEE were synthesized on a glutamic acid-functionalized Wang resin. These were then capped by reaction with a palmitic acid (C16) alkyl tail. These PAs ((C16)-$V_3A_3E_3$-COOH) were cleaved from the resin, precipitated in cold ethyl ether. The raw PAs were re-dissolved in water, adjusted to pH 7 with dilute base until fully solubilized, aliquoted and lyophilized. $V_3A_3E_3$ PAs were analyzed on an Agilent 6520 Q -TOF LC/MS, to determine purity and to confirm the molecular weight of the product. Analysis confirmed > 95% purity, and identified two major peaks: 1154 g/mol, representing the expected molecular weight, and 1171 g/mol, representing the expected +17 ammonium ion adduct from the 0.1% ammonium hydroxide that was used in analysis solvents.

Affi-Gel bead delivery of SHH inhibitor and IgG control to the corpora cavernosa of the penis:

Affi-Gel beads (100-200 mesh, Life Sciences) were soaked over night at 4°C in 5E1 SHH inhibitor (325μg/mL, Hybridoma Bank, University of Iowa) or in mouse IgG (control, Sigma). The SHH inhibitor used is an antibody, so the appropriate control is the same fraction of serum as the antibody (IgG), but without the inhibitor. A midline incision was made in the outer skin layer rostral to the pubis to expose the proximal portion of the penis. Affi-Gel beads were injected directly into the corpora cavernosa using a 26-gauge needle (50μl volume), as described previously[17] (Figure 3A). SHH inhibitor (n=6) and IgG (n=4) treated rats were sacrificed after two days and penis tissue was snap frozen with liquid nitrogen and stored at −80°C.

AffiGel bead delivery of SHH inhibitor and IgG control to the PG/CN:

An anterior posterior incision of the abdomen was performed to expose the prostate and pelvic neural plexus in adult Sprague Dawley rats. The PG and CN were identified and Affi-Gel beads previously soaked overnight at 4°C with 5E1 SHH inhibitor or mouse IgG (control), were placed under the PG bilaterally, as described previously[25] (Figure 4A). Approximately 25 beads were place under each PG using the tip of a 26-gauge needle as a vehicle to move the ```` beads. Rats treated with SHH inhibitor (n=4) and IgG (control, n=3), were sacrificed after two days and penis tissue was frozen with liquid nitrogen and stored at −80°C.

Surgical procedures for CN crush and sham:

After exposing the PG/CN, microforceps (size 0.02 X 0.06mm) were used to bilaterally crush the CN for 30 seconds. This CN crush methodology is an established and accepted model in the literature[38-39] and we previously verified the intensity and reproducibility of CN crush injury in our laboratory[27]. Rats were sacrificed after 2 (n=3), and 4 (n=3) days after injury and were used as positive controls for caspase 3-cleaved and caspase 9-cleaved analyses.

CN crush with SHH or control treatment of the penis by PA for 9 days:

We performed CN injury as described above. Self-assembling PA hydrogel ($V_3A_3E_3$-COOH) containing SHH (R&D Systems) or MSA (control, Sigma) proteins were injected into the corpora cavernosa of the penis as previously described[26], and are summarized below. After CN crush, a silk tie was placed around the base of the penis. Then 50 μl of PA (20-mM) was added to 5 μl of SHH protein (1.25 μg/μl, R&D Systems, Minneapolis, MN, USA) in an eppendorf tube. Just prior to injection into the distal penis, 50 μl of $CaCl_2$ (40 mM) was added to the SHH-PA solution, which was quickly drawn into a syringe and was injected with a 26-gauge needle directly into the corpora cavernosa (Figure 5A). The final amount of SHH protein injected to cause maximal apoptosis suppression was previously optimized to be 6.25 μg per rat[31]. The total volume of PA, $CaCl_2$ and SHH protein injected was 105 μl. Once injected, the PA gelled *in vivo*, within the sinusoidal spaces within the corpora cavernosa, forming a loose gel coating the sinuses, but did not obstruct them[26]. The PA visibly gels within a minute of addition of the $CaCl_2$. This was verified previously by sacrificing the rats immediately after injection and examining PA dispersion within the sinusoids of the corpora cavernosa[26]. As the PA broke down, SHH protein was delivered and up taken into the penile smooth muscle[26]. Duration of PA within the corpora cavernosa was examined previously at 1 hour, 7 and 14 days after injection. PA was still abundant at 7 days and had broken down by 14 days after injection[26]. In order to keep SHH protein levels constant, a second SHH (n=8) or MSA/BSA (n=7) PA injection was performed at day 5, and rats were sacrificed 4 days after the second injection (9 days of treatment)[31]. An additional group, treated with only one SHH PA injection at the time of CN injury, was sacrificed after 9 days (n=4).

Affi-Gel bead delivery of caspase 9 inhibitor to the corpora cavernosa of the penis after CN injury:

Affi-Gel beads (100-200 mesh, Life Sciences) were soaked over night at 4°C in Ac-LEHD-CMK caspase 9 inhibitor (1 μg/μL, Chem-Impex International, Inc.). CN injury was performed as described above and Affi-Gel beads were injected directly into the corpora cavernosa using a 26-gauge needle (50μl volume, Figure 6A). Caspase 9 inhibitor (n=4) and CN injury only (control, n=3) treated rats were sacrificed after two days and penis tissue was snap frozen with liquid nitrogen and stored at −80°C.

CN crush with SHH PA treatment of the penis for 1 and 2 days:

CN crush was performed as described above on adult Sprague Dawley rats (n=6). At the time of CN injury surgery, SHH PA was injected into the corpora cavernosa of the penis of half of the rats, as described above (n=6, Figure 7A). Rats were sacrificed one and two days after CN injury.

Immunohistochemical analysis (IHC):

IHC was performed on frozen sections cut 10μm in thickness, assaying for 1/100 rabbit caspase 3-cleaved, 1/50 rabbit caspase 9-cleaved, and 1/100 mouse caspase 8-cleaved (active forms, Cell Signaling), as described previously[34]. Secondary antibodies were chicken anti-rabbit 594 (1/100, Molecular Probes) and goat anti-mouse 594 (1/150, Molecular Probes). IHC was also performed on sections in which the primary antibody was omitted; this was done for all secondary antibodies to ensure non-specific staining was not present.

Western:

Western analysis was performed as described previously[18,34], assaying for rabbit caspase 9-cleaved, mouse caspase 8-cleaved (Cell Signaling) and mouse β-ACTIN (protein loading reference, Sigma). Secondary antibodies were HRP conjugated donkey anti-rabbit (1/35,000, Sigma-Aldrich), and chicken anti-mouse (1/15,000, Santa Cruz).

Apoptosis:

TUNEL assay was performed using the Apoptag kit (Millipore) on frozen penis tissue, cut 10μM in thickness, that was post fixed in acetone at 4°C for 15 minutes as described previously[26].

Results

SHH inhibition in the penis:

Affi-Gel beads containing 5E1 SHH inhibitor (antibody inhibitor, n=6) or mouse IgG (control for antibody fraction, n=4) were injected into adult (P120) Sprague Dawley rat penis (Figure 3A), which were sacrificed after two days, and TUNEL assay and the active form of caspase 3, caspase 9 and caspase 8 (caspase 3-cleaved, caspase 9-cleaved, and caspase 8-cleaved) were assayed by IHC analysis. TUNEL assay identified apoptosis in response to SHH inhibition in the corpora cavernosa (Figure 3B). Apoptosis was confirmed by abundant caspase 3-cleaved protein in the corpora cavernosa with SHH inhibition (Figure

3C), that was not identified in the IgG control (Figure 3C). Caspase 3-cleaved protein was abundant in the positive control (corpora cavernosa two days after CN injury, Figure 3C), but did not show staining when the primary antibody was omitted (negative control), indicating the absence of artifact from the secondary antibody (Figure 3C, bottom left).

In order to determine the signaling pathway by which SHH inhibition induces apoptosis in the penis, caspase 9-cleaved IHC was performed, which showed increased caspase 9-cleaved in the SHH inhibited corpora cavernosa, but not in the IgG treated control penis (Figure 3C). Caspase 9-cleaved was abundant in the positive control (corpora cavernosa 4 days after CN injury, Figure 3C).

Caspase 8-cleaved was not identified in SHH inhibited or IgG control treated penis, however staining was abundant in human diabetic tissue which was used as a positive control (Figure 3C). The human diabetic penis did not show staining in the absence of primary antibody, indicating the absence of secondary artifact (Figure 3C, bottom right).

SHH inhibition in the PG/CN:

Affi-Gel beads containing 5E1 SHH inhibitor or mouse IgG (control), were placed under the PG, to deliver SHH inhibitor or control IgG to the neurons that innervate the penis (Figure 4A). This mimics decreased SHH protein that occurs in the PG/CN with CN injury. TUNEL assay and caspase 3-cleaved, caspase 9-cleaved and caspase 8-cleaved (active forms) were assayed by IHC and western analysis. TUNEL assay identified apoptosis in the corpora cavernosa of the penis in response to SHH inhibition in the PG/CN (n=4). A minimal apoptotic response was observed with IgG control treatment (n=3, Figure 4B)[25]. Apoptosis was confirmed by abundant caspase 3-cleaved protein in the corpora cavernosa, that was not present in the IgG treated controls (Figure 4C). Caspase 3 staining was verified with a positive control (corpora cavernosa two days after CN crush, Figure 4C).

IHC analysis for caspase 9-cleaved was performed to determine if parallel mechanisms of apoptotic signaling occur with SHH inhbition in the PG/CN and in the penis. Caspase 9-cleaved increased in the corpora cavernosa in response to SHH inhibition in the PG/CN; IgG treated controls did not show increased caspase 9-cleaved (Figure 4C). Caspase 9-cleaved was abundant in positive controls (corpora cavernosa 4 days after CN injury). Negative controls in which the primary antibody was ommitted did not show staining (Figure 4C, bottom left). Western analysis confirmed abundant caspase 9-cleaved in the corpora cavernosa with SHH inhibition in the PG/CN (Figure 4D).

Caspase 8-cleaved protein was barely detectable in corpora cavernosa after SHH inhibition in the PG/CN, and was not identified in IgG treated controls (Figure 4C). Corpora cavernosal tissue from diabetic patients (positive control), showed abundant caspase 8-cleaved protein, that was absent when primary antibody was omitted (Figure 4C, bottom right). Western analysis confirmed low abundance caspase 8-cleaved in the penis, with SHH inhibition in the PG/CN (Figure 4D).

SHH PA treatment of the penis prevents caspase dependent apoptotic signaling after CN injury:

When the CN is injured in the Sprague Dawley rat, apoptosis is quickly induced in the penis, primarily via a caspase 9 dependent mechanism; a small flux of caspase 8 signaling was detected in day one and two after CN injury[34]. We examined if SHH protein treatment of the corpora cavernosa by PA (Figure 5A) for 9 days can suppress caspase 9 induction after CN injury. Caspase 9-cleaved was abundant in the corpora cavernosa 9 days after CN injury (n=7, Figure 5B). SHH PA injection into the penis at the time of CN injury, suppressed caspase 9-cleaved in the corpora cavernosa (n=4, Figure 5B). A second SHH PA injection at day 5 maintained elevated SHH for longer, and further suppressed caspase 9-cleaved at 9 days after CN injury (n=8, Figure 5B). SHH suppression of caspase 9 after CN injury did not shift the apoptotic response to the extrinsic caspase 8 dependent pathway, since IHC analysis for caspase 8-cleaved did not identify staining after CN injury and SHH PA treatment (Figure 5C).

Caspase 9 inhibition in the penis two days after CN injury:

Caspase 9 inhibitor was given to adult Sprague Dawley rats via Affi-Gel beads injected into the corpora cavernosa of the penis at the time of CN injury (Figure 6A), and rats were sacrificed after two days (n=4). We verified that caspase 9 was inhibited by performing IHC analysis for caspase 9-cleaved, which was identified away from the Affi-Gel beads, but not where the caspase 9 inhibitor was delivered (Figure 6B). A positive control (corpora cavernosa 2 days after CN crush) confirmed caspase 9-cleaved staining after CN injury (Figure 6B). TUNEL assay identified decreased apoptosis in the region of the Affi-Gel beads after CN injury and caspase 9-inhibition (Figure 6C, n=3). Caspase 3-cleaved protein also decreased in the vicinity of the beads, indicating that the caspase 9-inhibitor was suppressing the apoptotic response to CN injury (Figure 6D). A positive control (corpora cavernosa 2 days after CN injury) showed abundant caspase 3-cleaved that was absent when the primary antibody was omitted (Figure 6D). Caspase 8-cleaved protein was not increased with caspase 9-inhibition (Figure 6E), indicating that the extrinsic apoptotic pathway was not induced with suppression of intrinsic apoptotic mechanisms. Positive controls confirmed caspase 8-cleaved staining (diabetic human corpora cavernosa, rat corpora cavernosa 2 days after CN injury), that was not present when the primary antibody was omitted (negative control, Figure 6E).

SHH PA suppresses caspase 8 apoptotic signaling mechanisms:

IHC analysis for caspase 8-cleaved was performed to examine if SHH PA treatment of the penis for 1 and 2 days after CN injury (Figure 7A) can suppress extrinsic caspase 8 dependent apoptotic mechanisms. Caspase 8-cleaved protein was detected at low abundance in the rat corpora cavernosa, 1 and 2 days after CN injury (Figure 7B). SHH PA treatment of the corpora cavernosa suppressed caspase 8 signaling after CN injury (Figure 7B). Positive controls (diabetic human corpora cavernosa), confirmed caspase 8-cleaved staining (Figure 7), that was not observed when the primary antibody was omitted (negative control, Figure 4C). All experimental results are summarized in Table 1.

Discussion

The PA hydrogels that we are developing for clinical application as a preventative therapy for ED, and as a potentially reversible strategy for architectural remodeling of the penis, offer significant potential for management and prevention of intrinsic and extrinsic apoptotic mechanisms. These self-assembling hydrogels were first developed as a scaffold to deliver cells *in vivo*[29-30], and have been adapted in other capacities for bone regeneration[40], blood vessel repair[41], and spinal cord injury treatment[42-43]. We have been developing the PA's to prevent apoptosis of penile smooth muscle that occurs in response to loss of innervation in prostatectomy, diabetic and aging patients, and to promote CN regeneration after injury. In this study we examine two important aspects of this process, which are required for clinical application of the biomaterials, if SHH PA treatment of the penis, suppresses both the intrinsic and extrinsic apoptotic signaling mechanisms required to prevent the architectural remodeling of the penis and thus ED development, and if suppressing one apoptotic mechanism forces apoptosis to occur via a different mechanism, or truly suppresses the apoptotic response. It is essential when developing nanoparticle based therapies, that we examine the mechanism of how they function *in vivo*, the extended release of delivery, durability of the physiological changes, and if impacting one signaling mechanism effects other pathways in unexpected ways that could impact clinical management of disease.

Caspases are a group of protease enzymes that are essential for apoptotic signaling to occur[37,34]. The intrinsic pathway is activated by intracellular signals and is dependent on release of cytochrome c from the mitochondria[37], while the extrinsic pathway is initiated by death receptors[35]. Both pathways activate executioner caspases, which cleave proteins and induce the apoptotic response[37]. Apoptosis occurs in the penis in response to loss of innervation from the CN. The SHH pathway plays a critical role in this process. SHH protein decreases in the penis, when the CN is injured, causing substantial smooth muscle apoptosis and ED[17,24]. SHH protein also decreases in the PG/CN when it is injured, initiating the down stream remodeling events in the corpora cavernosa[28]. SHH inhibition in the penis or in the PG/CN, in the absence of CN injury, induce the same remodeling response in the penis as does loss of innervation, causing abundant smooth muscle apoptosis, altering the sinusoidal architecture of the corpora cavernosa, and ED develops[17,24-25,44], indicating the critical role of the SHH pathway in maintaining penile architecture. In this study we examined the mechanism by which apoptosis takes place in the penis with SHH inhibition, in order to identify points of intervention to suppress the apoptotic response. We show that apoptosis takes place in the rat penis in response to SHH inhibition via an intrinsic, caspase 9 dependent mechanism. This is the same mechanism utilized by the rat with CN injury[34], and reiterates the importance of the SHH pathway for ED management.

SHH inhibition in the PG/CN also utilizes primarily a caspase 9 dependent mechanism of apoptosis induction, similar to what was observed when SHH was inhibited directly in the corpora cavernosa. However, low abundance caspase 8 (extrinsic signaling) was also observed in the penis after two days of SHH inhibition in the PG/CN. This parallels what happens in the CN when it is injured. There is an initial low response of caspase 8 signaling in the penis the first two days after CN injury (Figure 7B). At the same time there is a robust caspase 9 induction from 1-7 days after CN injury[34]. It may be that at longer time points

after SHH inhibition in the PG/CN (3-7 days) that there is a shift to only caspase 9 signaling, as occurs with CN injury, however this is difficult to evaluate since the Affi-Gel delivery vehicle has limitations of inhibitor loading and delivery to the PG/CN. It is unclear why the neuronal apoptotic mechanism includes low level caspase 8 signaling, even if only for a couple of days as part of the initial response, and the down stream penile smooth muscle does not. However therapy design to suppress apoptosis with CN injury, such as occurs with prostatectomy, diabetic, and aging patients, must take this into account in order for effective clinical treatment of ED.

SHH treatment at the time of CN injury suppresses caspase 9 induction, which occurs first in a broad layer under the tunica, and after a couple of days in between the sinuses of the corpora cavernosa, in response to CN injury[34]. We did not see a shift to caspase 8 induction with SHH PA treatment, indicating that when we give SHH PA to the penis we are truly suppressing the apoptotic response, rather than shifting it to another signaling mechanism. This is an important consideration when designing effective therapies to suppress morphological remodeling in prostatectomy, diabetic and aging patients. We confirmed this finding when the penis was treated with a caspase 9-inhibitor at the time of CN injury. While caspase 9 signaling was decreased, as was apoptosis and caspase 3, caspase 8 was not induced.

We have shown previously, that the most commonly used animal model of ED, the CN injured rat, mimics the morphological remodeling that occurs with CN injury in prostatectomy, diabetic, and aging ED patients, with first smooth muscle apoptosis being induced and then increased collagen, making the corpora cavernosa unable to respond to normal neurotransmitter signaling mechanisms as the CN regenerates. However, the mechanism of how apoptosis takes place in response to CN injury is not identical in ED patients and animal models[34]. Apoptosis occurs in ED patients primarily via caspase 8, and in rat models via caspase 9. There are addendums to this with diabetic ED patients also utilizing low-level caspase 9 apoptotic signaling, and rats having an early flux of caspase 8 immediately after CN injury. This discrepancy does not invalidate the animal model, however it raises an important point to be aware of in that what ever therapies are developed to suppress CN injury induced apoptosis, they need to be effective against both the intrinsic and extrinsic apoptotic mechanisms.

In this study we show that SHH PA treatment of the penis is effective in suppressing both caspase 9 and caspase 8 apoptotic signaling mechanisms, and therefore should be effective for therapy development in ED patients and our animal models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Grant Sponsor:

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Statement of Significance:

In this study we examine two important aspects of our biomaterials that are required for clinical translation; if Sonic hedgehog delivered by peptide amphiphile to the penis, suppresses both the intrinsic and extrinsic apoptotic signaling mechanisms required to prevent the architectural remodeling of the penis and erectile dysfunction, and if suppressing one apoptotic mechanism forces apoptosis to occur via a different mechanism, or truly suppresses the apoptotic response.

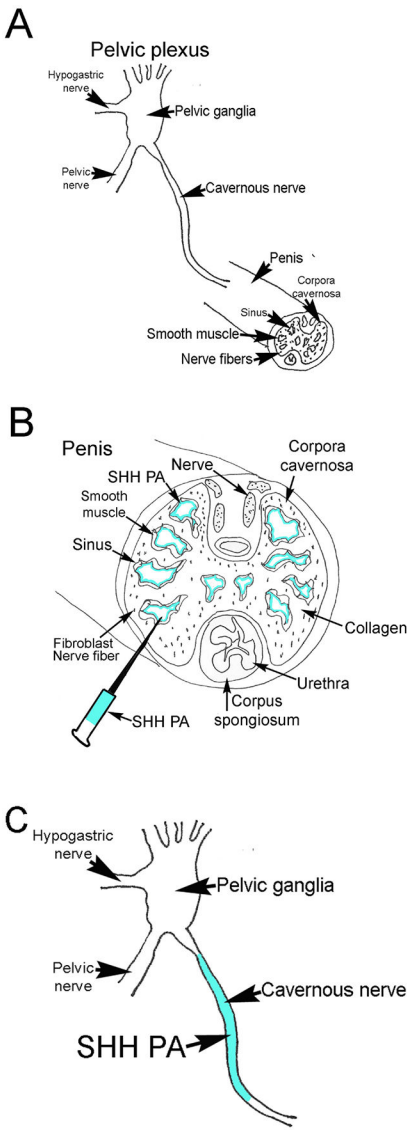

Figure 1:

(A) Diagram of innervation between the pelvic ganglia and penis. (B) Diagram of the penis showing SHH PA delivered to the sinusoidal spaces, to prevent smooth muscle apoptosis, penile remodeling, and ED. (C) Diagram of SHH PA delivery to the CN.

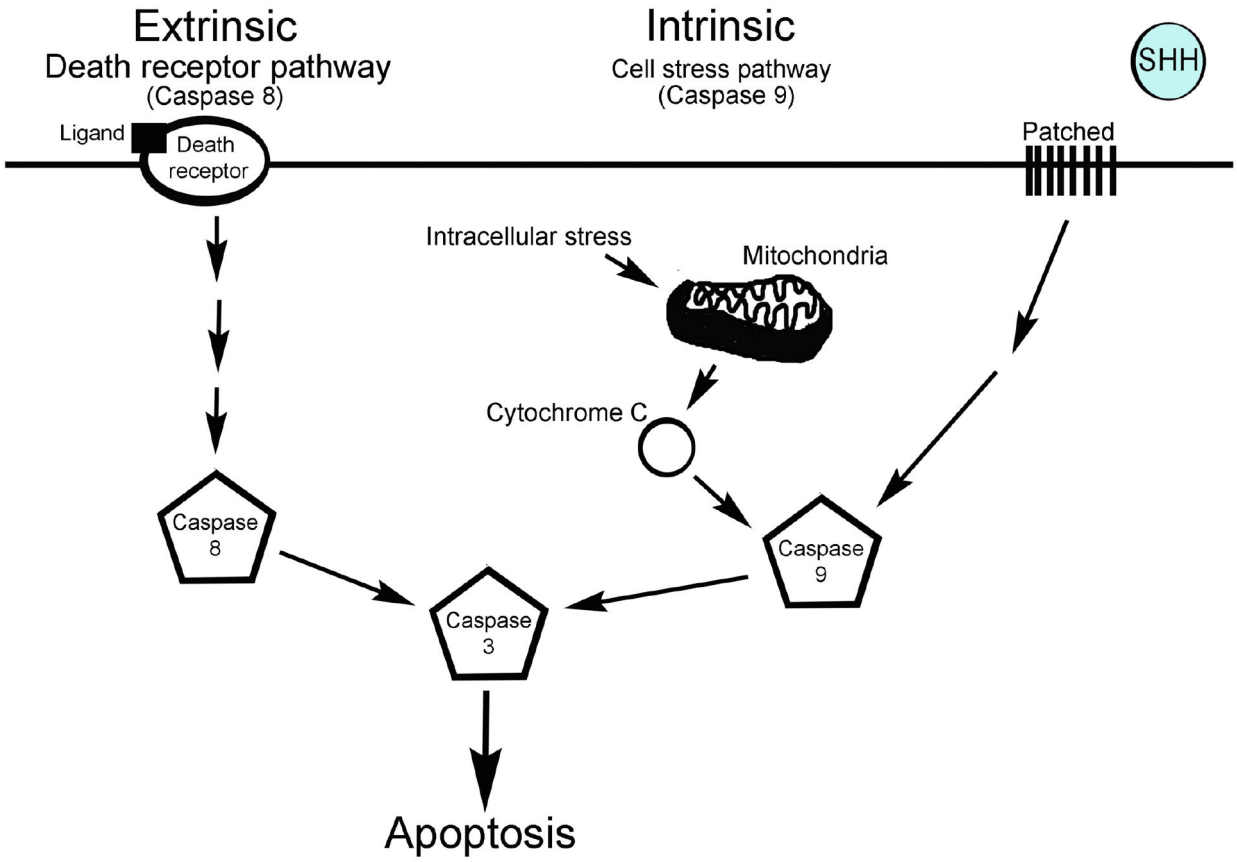

Figure 2:
Diagram of intrinsic and extrinsic apoptotic mechanisms.

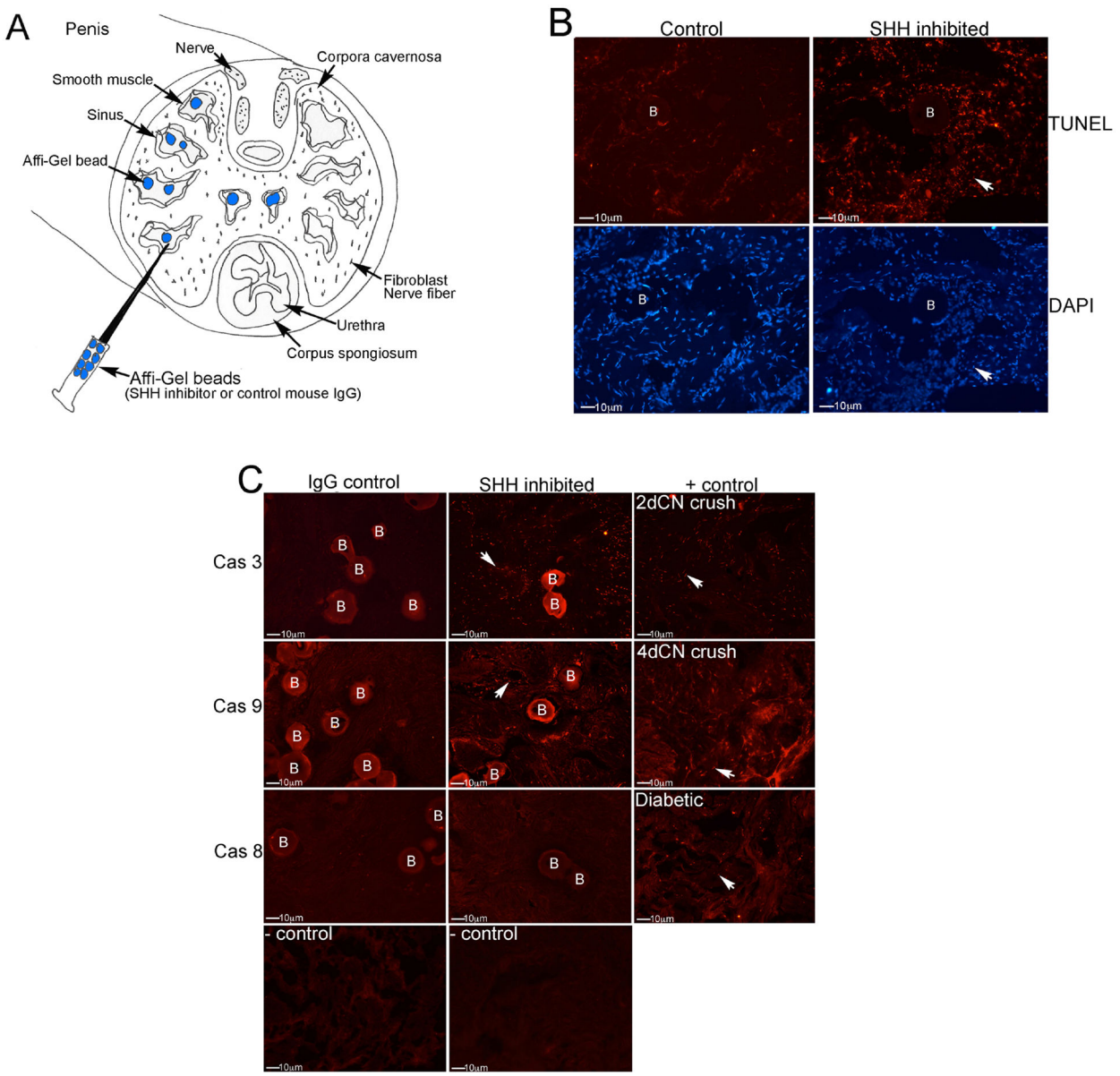

Figure 3:

Affi-Gel beads containing 5E1 SHH inhibitor (n=6) or control mouse IgG (n=4) were injected into adult Sprague Dawley rat penis; rats were sacrificed after two days, and TUNEL assay and caspase 3-cleaved, caspase 9-cleaved and caspase 8-cleaved were assayed by IHC analysis. (A) Diagram of the penis showing Affi-Gel bead delivery of ShH inhibitör or control mouse IgG, to the penis. (B) TUNEL showed abundant apoptosis in response to SHH inhibition, but not with control treatment. DAPI staining identified all cells. (C) Caspase 3-cleaved was identified in the corpora cavernosa after two days of SHH inhibition but not in the control. Caspase 3-cleaved was observed in the positive control (2day CN injured penis), but not when the primary antibody was omitted (bottom left). Caspase 9-cleaved was abundant in the SHH inhibited corpora cavernosa, but not in the control. Caspase 9-cleaved was abundant in 4day CN crushed penis (positive control). Caspase 8-

cleaved was not identified in SHH inhibited or control penis, however staining was abundant in human diabetic tissue (positive control). Human diabetic penis did not show staining in the absence of primary antibody (bottom right). 200X magnification. Arrows indicate staining. B=Affi-Gel beads.

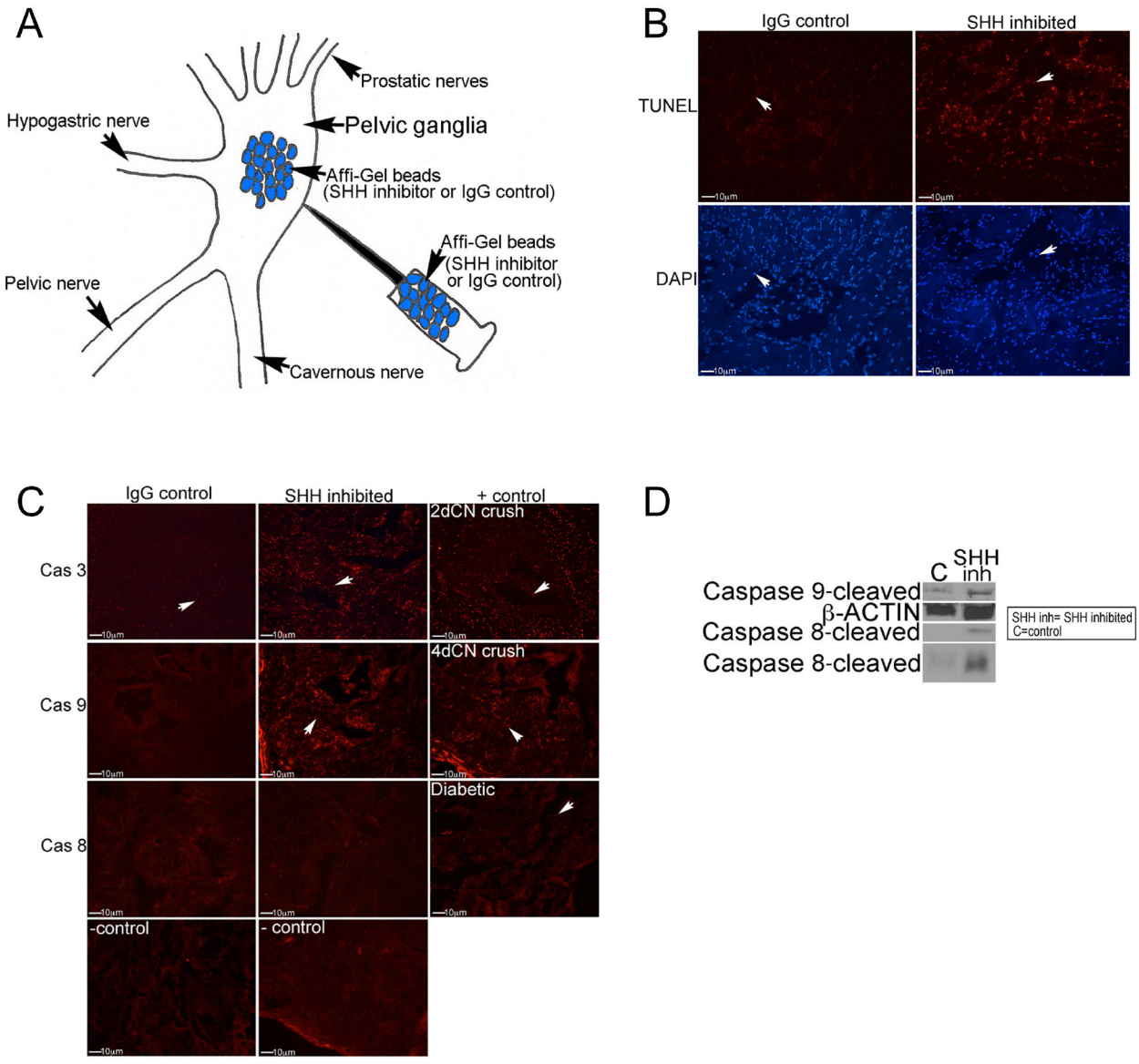

Figure 4:

Affi-Gel beads containing 5E1 SHH inhibitor or control mouse IgG, were placed under the PG, to deliver inhibitor to the neurons that innervate the penis, and TUNEL and caspase 3-cleaved, caspase 9-cleaved and caspase 8-cleaved were assayed by IHC and western analysis. (A) Diagram of Affi-Gel beads containing either SHH inhibitör, or control Mouse IgG, under the PG. (B) TUNEL assay of the penis in response to SHH inhibition in the PG/CN (n=4), showed increased apoptosis with SHH inhibition. Minimal staining was observed with IgG control treatment (n=3). DAPI staining identified all cells. (C) Apoptosis was confirmed by identification of abundant caspase 3-cleaved protein in the corpora cavernosa after two days of SHH inhibition in the PG/CN, but not with IgG treated controls. Penis tissue from 2d CN crushed rats, which was used as a positive control, showed abundant caspase 3 staining. Caspase 9-cleaved was increased in the corpora cavernosa of the penis in response to SHH inhibition in the PG/CN, but not with control IgG treatment.

Caspase 9-cleaved was abundant in penis tissue from 4day CN crushed rats, which were used as a positive control, and staining was not identified in the absence of primary antibody (bottom left). Caspase 8-cleaved protein was barely detectable in SHH inhibited penis, while IgG treated controls did not show caspase 8-cleaved staining. Corpora cavernosal tissue from diabetic patients, which was used as a positive control, showed abundant caspase 8-cleaved protein, that was absent when primary antibody was omitted (bottom right). 200X magnification. Arrows indicate staining. (D) Western analysis confirmed abundant caspase 9-cleaved induction with SHH inhibition in the PG/CN, and low abundance caspase 8-cleaved.

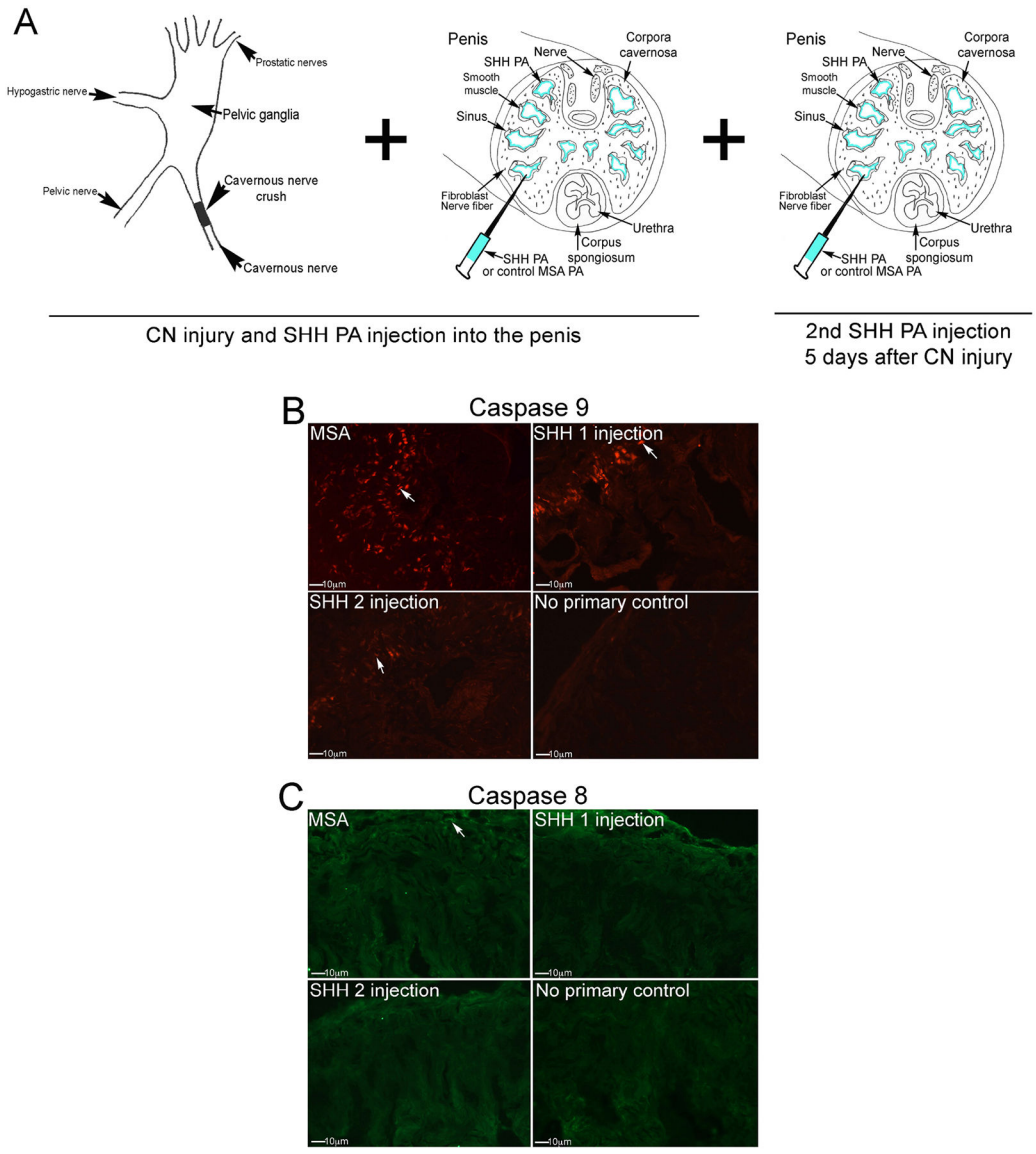

Figure 5:

We examined if SHH protein treatment of the corpora cavernosa by PA versus MSA treated controls, can suppress caspase 9 induction after CN injury. (A) Diagram of CN crush and the penis with SHH or MSA control treatment by PA. (B) Caspase 9-cleaved was abundant in the corpora cavernosa 9 days after CN injury (n=7) and in the MSA treated controls (n=7). SHH PA injection into the penis at the time of CN injury, suppressed caspase 9-cleaved in the corpora cavernosa (n=4). A second SHH PA injection at day 5 maintained elevated SHH for longer, and further suppressed caspase 9-cleaved at 9 days after CN injury (n=8). (C) SHH suppression of caspase 9 after CN injury did not shift the apoptotic response to the extrinsic caspase 8 dependent pathway, since IHC analysis for caspase 8 cleaved was not identified after CN injury and SHH PA treatment. 200X magnification. Arrows indicate staining.

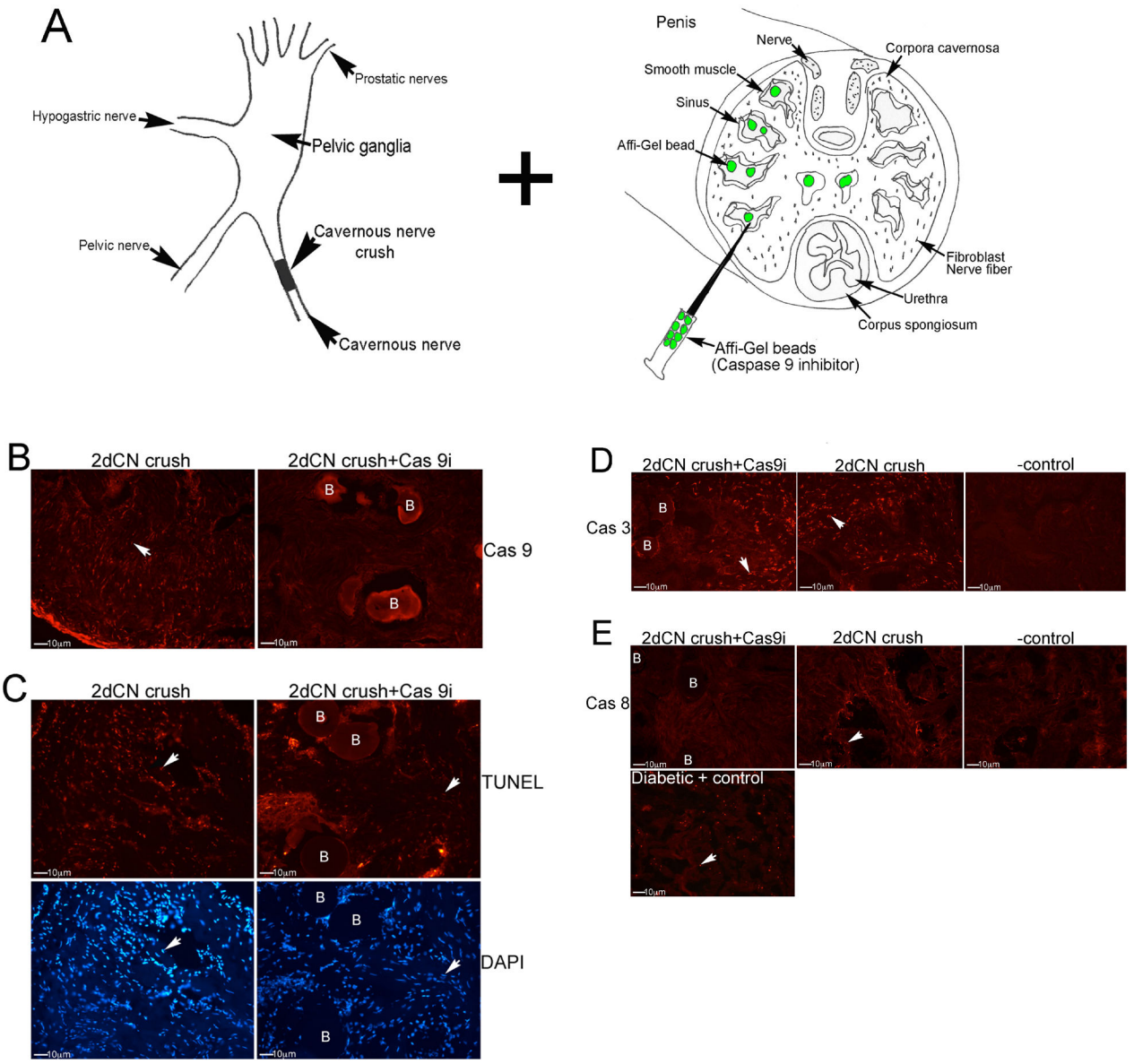

Figure 6:

(A) Caspase 9 inhibitor was given to adult Sprague Dawley rats via Affi-Gel beads injected into the corpora cavernosa of the penis at the time of CN injury to suppress the apoptotic response, and rats were sacrificed after two days (n=4). CN crush only rats were performed for comparison (n=3). (B) We verified that caspase 9 was inhibited by performing IHC analysis for caspase 9-cleaved, which showed staining away from the Affi-Gel bead delivery vehicle, but reduced staining in the region of the beads. (C) TUNEL staining identified decreased apoptosis in the region of the Affi-Gel beads with caspase 9 inhibition. DAPI staining identified all cells. (D) After two days there was reduced caspase 3-cleaved protein in the vicinity of the beads, indicating that the caspase 9-inhibitor was suppressing the apoptotic response to CN injury in the region around the beads. Two day CN crushed rat penis, which was used as a positive control, showed abundant caspase 3-cleaved that was absent when the primary antibody was omitted. (E) Caspase 8-cleaved protein was not

increased in the region of the Affi-gel beads when caspase 9 was inhibited, indicating that the extrinsic apoptotic pathway was not induced with suppression of intrinsic apoptotic mechanisms. Caspase 8-cleaved was identified in diabetic human corpora cavernosa, which was used as a positive control, but not when primary antibody was omitted. 200X magnification. Arrows indicate staining. B=Affi-gel beads.

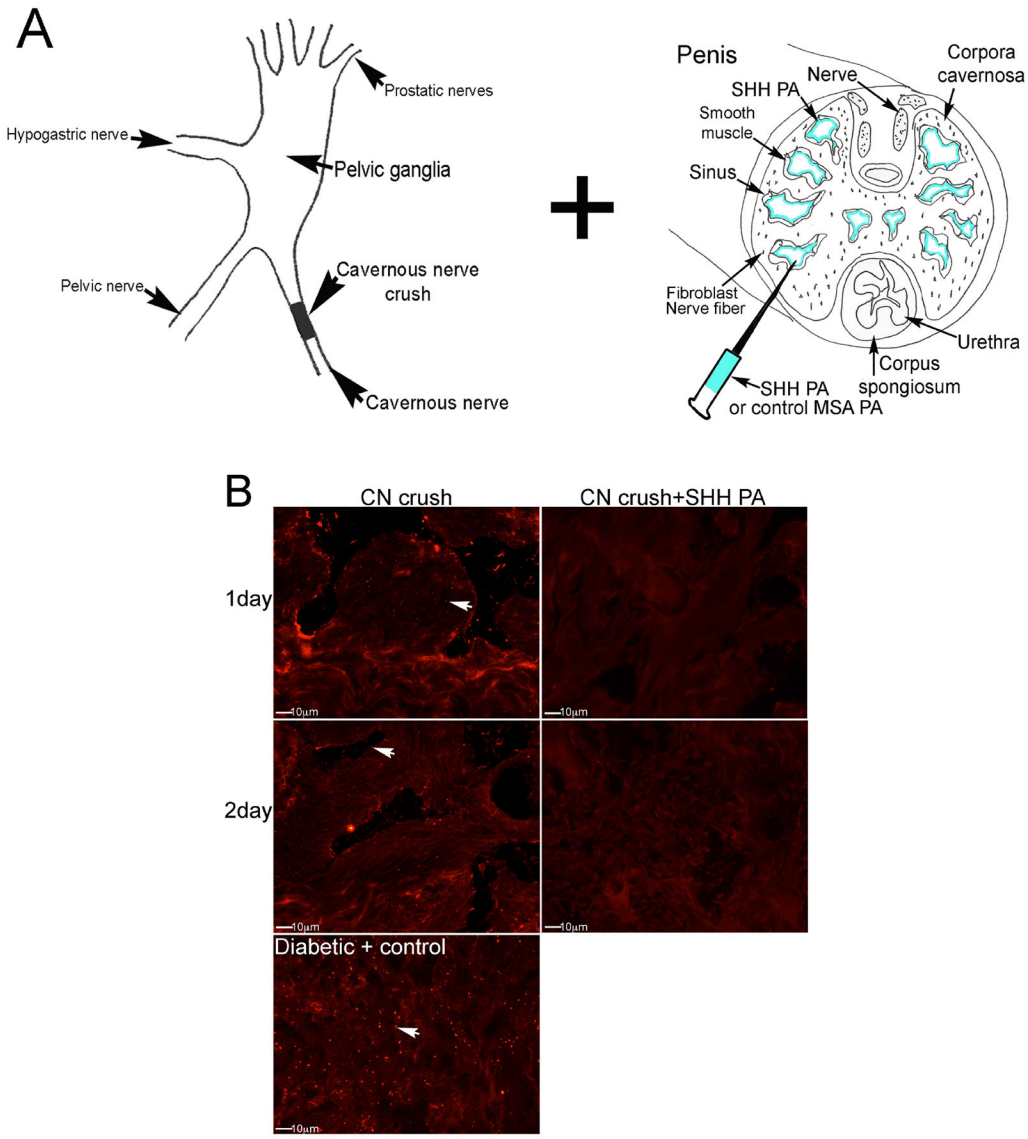

Figure 7:

IHC analysis for caspase 8-cleaved was performed to examine if SHH PA treatment of the penis at the time of CN injury can suppress extrinsic caspase 8-dependent apoptotic mechanisms (A). (B) Caspase 8 signaling has been identified at low abundance at 1 (n=3) and 2 days (n=3) after CN crush injury in the rat penis. SHH PA treatment of the corpora cavernosa suppressed caspase 8 signaling at one (n=3) and two (n=3) days after CN injury. Diabetic human corpora cavernosa (n=3), which was used as a positive control, showed abundant caspase 8-cleaved protein, that was not observed when the primary antibody was omitted (Figure 4C). 200X magnification. Arrows indicate staining.

Table 1

Experimental summary

Experiment	Caspase 3-cleaved	Caspase 9-cleaved	Caspase 8-cleaved
SHH inhibition of penis (2d)	x	x	-
SHH inhibition of PG/CN (2d	x	x	x
SHH PA tx of penis (4-9d)	-	-	
Caspase 9 inhibition in penis (2d)	-	-	-
SHH PA tx of penis (1-2d)	-	-	-