Effect of a Local Delivery of Triiodothyronine (T3) Within Neuroregenerative Guide on Recovery of Erectile Function in a Rat-Model of Cavernous Nerve Injury

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ABSTRACT

Introduction. A promoting effect of thyroid hormones has been established on the maturation of central and peripheral nervous systems. However, effects on autonomic nerves have never been experimentally investigated.

Aim. To assess the effect of a local treatment combining silicone guides and local administration of Triiodothyronine (T3) on the erectile function and the histological neuroregeneration of crushed cavernous nerves (CNs) in rats.

Methods. Forty-five rats were divided into five equal groups: SHAM surgery, guide without crush, crush, crush + guide, crush + guide + T3. All surgical procedures were bilateral. CNs were crushed with microvascular bulldog clamp of 100 g/cm². A silicone guide was placed around the nerves. The guides were filled with T3 neuroregenerative solution.

Main Outcome Measures. Erectile function was assessed 10 weeks post-operatively. Intra-cavernous pressure (ICP) and mean arterial pressure (MAP) were monitored during electrical stimulation of CNs at various frequencies. The main outcome was hardness of erection defined as $\Delta$ICP/MAP. Fluorescent immunohistochemical analysis of CNs was performed to assess regeneration of nerves morphologically.

Results. Electrophysiological data showed increased recovery of erectile function in the group with guide + T3 neuroregenerative solution compared with the empty guide. Immunohistochemical analysis of cavernous nerves demonstrated in morphology that regenerated axons were straighter in nerves with guide and more regular if guides had been filled with T3.


Key Words. Nerve-Sparing; Thyroid Hormone; Prostate Cancer; Prostatectomy; Neurovascular Bundle; Cavernosal Nerve Injury

Introduction

One of the main concerns of patients diagnosed with a localized prostate cancer is their sexual life after treatment. The so called “anatomical” retropubic radical prostatectomy (RP) technique described in 1982 [1] enabled a preservation of the cavernous nerves (CNs) and thus a reduction in the prevalence of post-RP erectile dysfunction (ED). A large longitudinal cohort study has reported a 56% rate of ED after this nerve-sparing technique [2]. Despite increased visual sensitivity and improved manual dexterity with laparoscopic and robotic surgery [3], the level
of post-operative sexual function still remains unsatisfactory for patients and their surgeons.

CN reconstruction was investigated to improve the post-operative functional results. In humans, autologous sural nerve-grafts after unilateral nerve-sparing RPs did not surpass functional and pharmacological rehabilitation protocols [4]. Urologists investigate new strategies such as regenerative guides to avoid nerve grafts and to deliver locally pharmacological substances. Animal experiments first assessed the use of non absorbable guides in sectioned CN [5]. Hisaue et al. provided histological evidence of better regeneration in animal models, with bigger and more CN fibers within guided nerves [6]. This approach should be investigated on a nerve-injury model that more closely mimics the damage occurring during a nerve-sparing procedure [7].

Immunophilin ligands, phosphodiesterase type 5 (PDE-5) inhibitors, erythropoietin and new innovative strategies such as hyperbaric oxygen therapy or SuperEnzyme gene therapy have, with different pathways, shown benefits in animal models of CN injury [8–10]. Clinical human trials are currently being conducted or designed to assess immunomodulatory drugs on patients after RP [8]. The role of thyroid hormones in maturation of the central and peripheral nervous systems was established three decades ago, regarding the inhibition of myelinized and unmyelinized axonal growth at deficiency of thyroid [11]. The action of triiodothyronine (T3) on responsive cells is mediated through nuclear thyroid hormone receptors (TRs) which modulate the expression of specific genes in target cells [12]. The local administration of T3—an active form of thyroid hormones produced by 5′-deiodination of Thyroxine (T4)—was evaluated on a rat sciatic nerve injury model and enhanced, respectively, 2.5 and 1.4 times the thickness and the number of axons [13]. However, there is no data available which assesses the local administration of thyroid hormones on autonomic nerves.

The objective was to assess the effect of a local treatment combining silicone guides and application of T3 on the erectile function and the histological neuroregeneration of crushed CNs in rats.

**Methods**

Forty-five male Sprague-Dawley rats (Janvier, Le Genest-St-Isle, France) weighing 150–200 g were housed 7 days prior to the beginning of experiments with a 12 hours dark/light cycle. The animals had free access to standard food and water. All procedures were performed in accordance with the legislation on use of laboratory animals (National Institutes of Health publication N°85-23, revised 1996) and Animal Care Regulations in force in France as of 1988 (authorization from competent French Veterinary Services Direction—Agreement N°94-256, 3/17/2005). Five equivalent experimental groups of rats were randomly constituted. Groups are presented in Table 1.

All operative procedures were performed bilaterally. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (75 mg/kg). A midline abdominal incision was performed and CNs were exposed in all groups. Except in group 1, CNs were crushed with a microvascular bulldog clamp (100 g/mm²) applied 8 mm distally from pelvic ganglia for 30 seconds, removed 30 seconds, and then reapplied for a further 30 seconds [7]. Silicone guides (5 mm long, 300 μm diameter) were opened longitudinally and placed around CNs. No additional suture was performed to maintain guides wrapped around nerves. In group 5, the neuroregenerative solution was injected within guides. Rats from all groups had a two-lay abdominal suture.

The T3 viscous solution was prepared with a dilution of 1 mg/mL of 3,3′,5-T3 (Sigma-Aldrich, St-Quentin-Fallavier, France), added to a solution of fibrinogen (50 mg/mL) and thrombin (500 UI/mL)(Baxter). A 1/10 dilution produced a viscous solution that was conserved at 4°C for later injection within the guides, after their implantation.

The erectile function was assessed 10 weeks post-operatively by electrostimulation of CNs under general anesthesia (sodium pentobarbital, 75 mg/kg, peritoneal injection). Intracavernosal pressure (ICP) and mean arterial blood pressure (MAP) were monitored with a 24-gauge needle inserted in penile corpora cavernosa and a catheter in right internal carotid after neck incision and tracheotomy. Both catheters were connected to an amplificator (Kent®, Torrington, CT, USA) and recorded using Elphy® (CNRS, Gif-sur-Yvette, CN 1799 J Sex Med 2010;7:1798–1806

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<thead>
<tr>
<th>Table 1 Characteristics of Groups</th>
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<th>Intervention on Cavernous Nerves</th>
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<tr>
<td>Group 1 (“Exposition”)</td>
<td>9</td>
<td>Exposition</td>
<td>None</td>
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<tr>
<td>Group 2 (“Guide”)</td>
<td>9</td>
<td>Exposition</td>
<td>Guides</td>
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<td>Group 3 (“Crush”)</td>
<td>9</td>
<td>Crush</td>
<td>None</td>
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<td>Group 4 (“Crush + Guide”)</td>
<td>9</td>
<td>Crush</td>
<td>Guides</td>
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<tr>
<td>Group 5 (“Crush + Guide + T3”)</td>
<td>9</td>
<td>Crush</td>
<td>Guides + T3</td>
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France) software. A stainless steel bipolar electrode with parallel hooks (1 mm apart) was placed around the nerve, between ganglion and former site of crush injury. After a 5-minute stabilization period, each nerve was electrically stimulated at five randomized frequencies (0/2/4/7.5/10 Hertz) repeated twice every 2 minutes. Stimulation duration was 45 seconds with 1 ms pulse at 1.5 mA and 6 V. After stimulation, animals were sacrificed by intracardiac injection of Urethane (0.5 mL).

Two rats from each group underwent a transcardial perfusion of phosphate-buffered saline followed by paraformaldehyde (PAF) (4% paraformaldehyde in 0.1 M phosphate-buffered saline). Perfusion fixed tissues before dissection and sample prelevement. Prostatic lobe with CN was post-fixed in the same fixative, followed by 24 hours in sucrose (30% in phosphate-buffered saline). Serial transverse and longitudinal sections of 10 µm thickness were realized on a cryostat and were mounted on slides. For two rats in each group (no statistical analysis performed), the thickness of the external neural sheath was measured with Lucia Software® (Laboratory Imaging, Prague, Czech Republic) on transversal sections in optical microscopy. Sections were pre-incubated with normal goat serum for 30 minutes. Double fluorescent staining was performed with anti-neurofilament 200 (1/25, mouse, Sigma-Aldrich®) and anti-laminin (1/50, rabbit, Sigma-Aldrich®, St. Louis, MO, USA) and analyzed with confocal laser scanning microscopy.

Statistical Analysis

The difference between ICP in the flaccid state, i.e., before electrostimulation, and ICP during the plateau phase of the erectile response was calculated for each stimulation. The rigidity of erections was assessed by the ΔICP (mm Hg)/MAP (mm Hg) × 100 rate during plateau phase as main outcome. For each frequency of each group, elicited results were averaged and expressed as mean ± standard error of the mean. Data comparisons were performed with two-way analysis of variance (ANOVA) statistical test followed by Bonferroni’s post-test. P values < 0.05 were considered significant.

Results

Eighty-seven (97%) nerves were stimulated. In group 2, one rat died from anesthetic overdose during electrostimulation, and another had a traumatic section of one nerve.

Functional results are presented in Figure 1.

No statistical difference was observed between curves from the “Exposition” group and the “Guide without Crush” group (P > 0.05, N = 25, two-way ANOVA, Figure 1A), suggesting that surgical implantation of guides around the nerves did not modify the erectile function more than nerve exposition.

Groups “Crush” and “Exposition” had significantly different curves (P < 0.0001, n = 18, two-way ANOVA, Figure 1B), confirming the occurrence of an erectile dysfunction after the experimental crush of CNs. At 10 Hertz, the ΔICP/MAP rates were, respectively, 13.2 ± 2.4% and 32.7 ± 1.7% (P < 0.001, N = 18, Bonferroni).

Curves from groups “Crush + Guide” and “Crush” were significantly different (P < 0.001, N = 18, two-way ANOVA, Figure 1C), with ΔICP/MAP rates of 25.5 ± 3.9% and 13.2 ± 2.4%, respectively, at 10 Hertz (P < 0.01, N = 18, Bonferroni). Guides enabled a better recovery of erectile function than spontaneous nerve regeneration.

The difference between curves from groups “Crush + Guide” and “Crush + Guide + T3” was also statistically significant (P < 0.01, N = 18, two-way ANOVA, Figure 1D). At 10 Hertz, the ΔICP/MAP rates were, respectively, 25.5 ± 3.9% and 35.5 ± 3.2% (P < 0.05, N = 18, Bonferroni). The addition of T3 within guides provided an additional benefit in erectile function recovery.

A general survey of functional results is presented in Figure 2.

Histological longitudinal nerves sections are presented in Figure 3.

At ×4 magnifying analysis of longitudinal sections, the course of CNs appeared sinusous in the “Crush group”, compared with the straight nerves from the “Exposition” group. In groups where a guide had been implanted, nerves kept their rectilinear course, suggesting a link between the mechanical properties of the physical support and neuroregeneration.

At ×20 magnifying observation of longitudinal and transversal sections, remodeling of the nervous architecture was predominant after crushes. Pycnotic vesicles were noted often in the sections from the “Crush” group. Inflammatory infiltration was observed within neural tissues,
whose resistance to cryosection was low and decreased by numerous and wide interaxonal spaces. In groups “Crush + Guide” and “Crush + Guide + T3”, regular organization of surrounding nuclei, lack of remodeling and inflammation were signs of successful regeneration. Compared with the retracted crushed nerves from the “Sham” group, guided nerves appeared as fully functional regenerated nerves, with a normal 150 µm diameter, a regular structure, and some resistance at section. Mean thickness of neural sheaths was inferior to 1 µm in the “Crush” group, 13.5 µm in rats from group “Exposition”, and, respectively, 29 and 27.5 µm in group “Crush + Guide” and “Crush + Guide + T3”.

Immunofluorescent transverse sections are presented in Figure 4.

The immunofluorescent labeling of axons (anti-neurofilament, in red) and neural sheaths (anti-laminin, in green) provided characteristics of the nerve structure of groups. On longitudinal sections, nerves within guides had straight labeled axons, organized in beams and surrounded by neural sheaths. On transverse sections, the labeling was more intense and numerous in the “Exposition” and “Guide” groups than in the “Crush” group. The label intensity was maximal in group “Crush + Guide + T3”, suggesting a promoting effect of the T3 on neuroregeneration. In one case (observed in the “crush + Guide + T3” group) a regenerated labeled axonal beam was visible beyond the longitudinal slit of the guide, exteriorly (Figure 4D): in this case, despite a late migration and a failure to guide fibers durably, there was a promotion of early neuroregeneration.

**Discussion**

The devising of CN reconstruction was initiated on rat models, in which a nerve graft after CN section provided a 90% recovery of the erectile function [14]. In humans, the surgical technique was found to be more elaborated regarding the scattering of nerve fibers in the cellulo-adipous peri-prostatic tissue [15]. In recent randomized prospective cohort with 2 years follow-up, autologous unilateral sural nerve grafts after unilateral nerve-sparing RP did not improve patients’
potency rate more than standard post-operative rehabilitation protocols [4].

Our study demonstrates that nerve guides provided a 62% recovery of erectile function, defined as the difference between groups “Crush + guide” and “Crush” compared with the difference between groups “Exposition” and “Crush” at a 10-Hertz stimulation frequency. In accordance with previous studies, we note that CN guides enhanced recovery of sexual function, with accurate methodology: (i) taking into account a post-operative erectile dysfunction after exposition of CNs in rats [7], CNs were exposed in the control group, thus isolating nerve trauma from other confusing factors such as surgical and anesthetic stress, cicatrization or adherent processes; (ii) an experimental nerve crush, more likely to closely mimic the “nerve-sparing” surgical damage (electrocoagulation, stretch, or crush of the CNs) was performed, rather than nerve resection; and (iii) the nerve stimulation protocol included low and high frequencies among a spectrum of five, thus reproducing the stimuli of physiological erections with more accuracy than a maximal-single-frequency procedure. Results presented as half-sinusoid curves represented responses to all frequencies occurring during physiological erections.

Our histological data indicate a reduction of irregular sprouting and preservation of the functional architecture in regenerated nerves by using guides. It validates the concept of a mechanical support for CN regeneration on a crush-injury model, thus foreseeing its application after a nerve-sparing surgical procedure. However, in

Figure 3 HES longitudinal sections of cavernous nerves (×20 and ×4) (A) Group “Exposition”. (B) Group “Crush”. (C) Group “Crush + Guide”. (D) Group “Crush + Guide + T3”.

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humans, the difficulty to identify the CN fibers, the use of surgical clips, and the lowering of the bladder during the tightening of the urethrovesical anastomosis points makes it technically difficult to put guides in place.

These technical issues should justify further research to adapt the “physical support” concept to a per-operative situation. Precise anatomical research may help standardize the surgical technique to place the supports. For example, Tak-enaka described the course of CNs in adults immunohistochemically and electrophysiologically, suggesting that it had a separate location from neurovascular bundles at the bladder–prostate junction [15]. It now appears that surgeons who used to perform nerve grafts were missing these recent anatomical findings. The immunohistochemical analysis with a three-dimensional reconstruction has provided a precise cartography of the pelvic innervation in the fetus [16]. It led to a computer-assisted anatomical dissection that is applicable on adult male cad-
avers to supply some landmarks to devise a neuroregenerative-guiding procedure [17].

Another concern when using guides in humans is their tolerance and eventual pro-inflammatory properties obstructing neuroregeneration. Barakat-Walter showed an enhancer effect of T3 on sciatic nerve regeneration, and she compared the results of a local application of T3 within silicone vs. bioresorbable guides, and reported equivalent results [18]. However, used in humans, biodegradable guides evade a removal procedure and provide an obvious safety benefit in the case of guide migration.

The other main result of the study could support a neuroprotective strategy of post-RP ED prevention. Considering the 51% additional benefit on erectile function at 10 Hz ($P < 0.01$) in the group “Crush + Guide + T3” compared with the group “Crush + Guide”, we confirmed that there was a local effect of the active thyroid hormones. Histological results showed that nerve architecture was mainly modified with guides alone, which suggests that thyroid hormones have molecular and functional regenerative pathways. But contrary to sensori-motor nerves, the local effect of T3 on CNs—an autonomic nerve—cannot be explained by its stimulating action on Schwann cells. The effect of local T3 is supported by the presence of thyroid hormone nuclear receptors [12] that would regulate the gene expression of neurons in the autonomic pelvic ganglia, which in turn may enable a lasting and stimulating effect on nerve regeneration [18].

The choice of T3 was indicated as it is an active form of thyroid hormone, produced by 5'-deionidation of the T4. This reaction is catalyzed by type-2-deiodinase (D2). The lesion of a peripheral nerve induces an increase in D2 activity in the external sheaths in the hours following the lesion, and thus would enhance deiodination of T4 into T3 [19]. The implication of thyroid hormones in the maturation of central nervous system led to the experimental use of T3 in the promotion of neuroregeneration in peripheral nervous system [12]. In our model, the local delivery of T3 within a viscous gel could enable a high and long-lasting concentration of thyroid hormone and was associated with a better histological and functional neuroregeneration. For the first time, experimental data suggest a beneficial role of thyroid hormones in the neuroregeneration of the autonomic nervous system, supporting similarities in its neuropharmacology with the central and the neuro-sensitive peripheral nervous system. Receptors of T3 may be solicited in the vegetative neuroregeneration after a mechanical stress. The duration of neural response to such a stress is only few days in the peripheral nervous system [20], and its prolongation with a long-lasting T3 delivery could lengthen the sprouting period of injured nerve.

Regarding the surgical stress as a sum of some hormonal, oxidative, anesthetic, psychological, and mechanical components, the monitoring of post-operative thyroid function would be informative. Indeed, a 6-day long hypothyroidism period was described in human after cardiovascular surgeries [21]. Despite the assessment of varied types of drugs such as immunophilin ligands [22], neurotrophic factors [6] or PDE-5 inhibitor [23], the balance of thyroid function has never been investigated as a potential factor of recovery; whereas, thyroid function was found decades ago to regulate the nervous system maturation [11]. Thus, a short general supplementation in thyroid hormones in the early peri-operative days could be assessed on an animal model.

Previous studies have reported some beneficial effects of neurotrophic growth factors on a similar nerve lesion or resection model. However, we are cautiously monitoring the local use of growth factors in the context of prostate cancer because of the risk of cancer resurgence [24]. Thyroid hormones were found to be safer in this context as their concentration did not influence the growth of benign, malignant, and metastatic prostatic cells on an in vitro cell culture model [25].

In the main in vivo research data on RP-ED prevention during the past decade, it mainly seems that only single strategies have been assessed [26]. The opportunity thus exists to design a combination of efficient therapies to treat RP-ED. Indeed, the RP-ED is the result of nerve trauma and of cavernosal fibrosis occurring in the early post-operative neurapraxic period [27,28]. The use of a mechanical (guide) or pharmacological (immunophilin ligand) neuroprotection could be combined with the administration of a vasculo-endothelial agent. This overall concept of a multi-modal strategy to prevent RP-ED should be developed. It could also integrate and evaluate the place of a thyroid balance in the post-operative period.

**Conclusions**

The study demonstrated, with morphological evidence, a partial but prevalent enhancement of erectile function recovery following use of neuroregenerative guides on injured CNs in rat

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**References**

models. The local addition of T3 within guides revealed an increased benefit and widened the spectrum of strategies to combine to prevent post-RP ED. To restore both cavernosal oxygenation and neuro-hormonal regenerative conditions, combinations of interventions should be developed in a multimodal scheme of RP-ED prevention.

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