**Materials and Methods**

***Nanoconjugate Synthesis***

The detailed synthesis of the nanoconjugate (WGA-HRP-AuNP-proTHP) has been published elsewhere 1. Briefly, to link theophylline to the AuNP via an ester bond, a hydroxymethyl group was added to theophylline creating 7-(hydroxymethyl) theophylline or “proTHP”, using the Mannich reaction 1-2. The reaction product was analyzed by proton nuclear magnetic resonance (1H NMR) to verify the formation of proTHP. Gold chloride (HAuCl4) was reacted with tri-sodium citrate to synthesize AuNPs. Mercaptosuccinic acid (MSA) was added as the capping ligand for the AuNPs. Then proTHP was attached to the MSA-capped AuNPs via a biodegradable ester bond to enable *in vivo* drug release 3. WGA-HRP was coupled to the MSA-capped AuNPs via an amide bond 4. Each step of the synthesis was verified by UV-*vis* spectroscopy, transmission electron microscopy, dynamic light scattering, and thermogravimetric analysis (TGA). Due to the instability of the synthesized proTHP, proTHP was stored in argon to prevent hydrolysis of the hydroxymethyl group by moisture in room air. In addition, to limit variation due to the biodegradable nature of the ester bond linking the AuNP to 7-(hydroxymethyl) theophylline, the final solutions were only used up to 30 days after synthesis.

A variation of the nanoconjugate synthesized with hydroxyethyl theophylline (Sigma-Aldrich) in place of the 7-(hydroxymethyl) theophylline was also produced. The hydroxyethyl theophylline variation was tested to determine if the ester bond between theophylline and AuNPs could be further stabilized.

***C2 Hemisection Surgery***

All animal studies were approved by the Wayne State University School of Medicine Institutional Animal Care and Use Committee. In addition, all experiments were carried out in accordance with the Code of Ethics of the World Medical Association for experiments involving animals. Adult male Sprague Dawley rats were used in these studies (N=123, see Table 1). The rats were injected with atropine sulfate (0.04 mg/kg, im) 10 min prior to anesthesia induction to reduce mucus secretions during the subsequent aseptic survival surgery. The initial dose of anesthesia was a mixture of ketamine (70 mg/kg, ip) and xylazine (7 mg/kg, ip). After anesthesia induction, bupivacaine (2 mg/kg, sc), a local anesthetic, was injected into the dorsal neck. The neck was then shaved and prepared for aseptic surgery. A left C2Hx surgical procedure was carried out as previously described 5. The dorsal neck muscles were closed using 4–0 absorbable sutures (Vicryl) followed by wound clips (Reflex® 9 mm) for the skin.

***Electromyography (EMG) Analysis***

In all rats, immediately after C2Hx, paralysis of the ipsilateral hemidiaphragm was confirmed by EMG analysis. The previous study by Moreno and colleagues 5 correlated the loss of EMG activity in the ipsilateral hemidiaphragm to the lesion extent damaging the ipsilateral lateral and ventral funiculi of the cervical spinal cord where the descending respiratory drive is located, which was used here to ensure a complete hemisection 5. The abdominal surface of the diaphragm was exposed by a 6–8 cm horizontal incision made approximately 0.5 cm caudal and parallel to the costal margin. Bipolar platinum wire electrodes (Grass F-E2) were placed in each side of the diaphragm. Signals were amplified (20,000×) and band pass-filtered (30 Hz–3 kHz) by Grass amplifiers (model P511 AC, Astro-Med, Inc., West Warwick, RI) and raw EMG signals were recorded by a Cambridge Electronic Design (CED, Cambridge, England) data acquisition system integrated with CED Spike 2® software. Recordings were taken from three areas of the left hemidiaphragm: anterior (sternal), lateral (costal) and posterior (crural). C2Hx rats that showed a complete absence of activity in all three areas and could produce an augmented breath were included in the study. An augmented breath is a confirmation that the CPP is intact and functional 5-6.

To observe the course of effect of theophylline following nanoconjugate injection subsequent EMGs were sampled from day 2 post injection up to and including day 14. Each rat underwent at minimum 1 EMG and no more than 3 separate EMGs with a minimum of 1 day between surgeries. The days selected for EMG were determined based on the time course for each rat and how many surgeries had already occurred; days 2 through 7 each had a minimum of 7 rats used for data on each day. Subsequent EMG recordings were performed under ketamine/xylazine anesthesia while spontaneously breathing.

***Nanoconjugate Administration***

Following EMG confirmation of hemidiaphragm paralysis immediately after the C2Hx, intradiaphragmatic injections were administered. Groups received injections based on their weight of one of the following solutions; i) nanoconjugate (WGA-HRP-AuNP-proTHP) 0.0005 mg/kg N=3, ii) nanoconjugate 0.0008 mg/kg N=4, iii) nanoconjugate 0.0025 mg/kg N=4, iv) nanoconjugate 0.005 mg/kg N=7, v) nanoconjugate 0.0075 mg/kg N=7, vi) nanoconjugate 0.01 mg/kg N=6, vii) nanoconjugate 0.03 mg/kg N=19, viii) nanoconjugate 0.07 mg/kg N=25, ix) nanoconjugate 0.12 mg/kg N=23, x) WGA-HRP-AuNP N=6, xi) AuNP-proTHP 0.07 mg/kg N=8. Investigation of the hydroxyethyl theophylline nanoconjugate consisted of 2 groups; 0.002 mg/kg N=5, and 0.3 mg/kg N=6; summary in Table 1. Injections (10µl maximum volume per injection site) were spread equally from the posterior to anterior areas of the diaphragm using a Hamilton syringe. The needle was inserted parallel to the muscle fibers such that the tip of the needle was visualized by eye before administration of the injections, the needle was held in place for 5–10 seconds before it was slowly withdrawn from the muscle to prevent leakage7.

***Post Procedure Care***

Incisions were cleaned and the rats were given yohimbine (2mg/kg, ip) to reverse the xylazine, and buprenorphine (0.01mg/kg, sc) in 10mL saline for pain management. The rats were recovered on a heating blanket in a supine position to limit pressure on the diaphragm. Once rats could ambulate, they were returned to clean soft litter-lined cages with food and water provided ad libitum on the floor of the cage. The rats were also given cereal (Fruit-Loops®) as an enticement to eat. For the following 48-hours post-operative period, injections of buprenorphine (0.01mg/kg, sc) were given every 8-12 hours for pain management. Rats survived for 3, 7, or 14 days’ post injection depending on the timeline for each experimental group.

***Immunohistochemical Visualization of THP Nanoconjugate***

72 hours after intradiaphragmatic injection of the nanoconjugate 0.12mg/kg (N=3) into the left hemidiaphragm, rats were euthanized and underwent transcardial perfusion using heparinized saline followed by 4% formaldehyde (Fisher, F-79) 7. The cervical spinal cord (C3-C6) and medulla were removed and post fixed in 4% formaldehyde for 24 hours followed by 72 hours in 30% sucrose for cryoprotection. Tissues sections 50 microns thick were cut transversely on the cryostat and collected in PBS. Tissue sections containing the phrenic nuclei and rVRGs underwent the immunohistochemistry protocol as described by Minic *et al.* 7; sections were washed three times in the immuno-buffer (PBS with 0.3% Triton) and then blocked using 10% normal horse serum (Invitrogen) in the immuno-buffer. Sections were incubated in primary antibody goat anti-WGA (1:200, AS-2024, Vector Laboratories), diluted in 10% normal horse serum immuno-buffer solution for 72 h, and then washed in PBS. Sections were then incubated in the biotinylated secondary antibody, donkey anti-goat overnight (1:400, Jackson Immuno Research Laboratories). Sections were then incubated in streptavidin-tagged-Cy3 for 4 h and then mounted wet on the slides and coversliped. Tissue sections were examined on a Zeiss Axioimager.M2 fluorescent microscope. Images were captured using the Zen 2 pro Blue edition program. Off-line processing was used to acquire high-power views of the phrenic nuclei and the rVRGs.

***Phrenic Nerve Recordings***

Bilateral phrenic nerve recordings were sampled at day 3, 7, and 14 under standardized conditions. The rats were injected with atropine sulfate (0.06mg/kg, im) 10 minutes prior to anesthesia induction to reduce mucus secretions during the procedure. The anesthesia was a mixture of ketamine (70mg/kg, ip) and xylazine (7mg/kg, ip), a supplement was provided as needed. Phrenic nerves were accessed from the ventral surface of the neck and cut distally to eliminate afferent activity. Each nerve was positioned on platinum bipolar electrodes and coated with mineral oil to prevent fluid interference with the signal and to reduce electrical noise. Before acquiring nerve activity data, the following standardizing conditions were performed: (1) animals were bilaterally vagotomized; (2) animals were paralyzed using pancuronium bromide (0.5 mg/kg, iv); and (3) animals were placed on a ventilator set at 3–5mmHg above the apnea threshold 7. The procedure for determining the apnea threshold has been described previously 8. The unprocessed recording was amplified 5000 times using Grass P511 amplifiers (Grass Technologies), filtered at 0.3–1 kHz. The recordings were digitized, rectified, and integrated in 0.1 s intervals using the Cambridge Electronic Design data acquisition system and the Spike 2 software.

***Statistical Analysis***

As described by Minic *et al.* 7, EMG analysis was intended to only analyze the *incidence* of recovery not to determine the *amount* of recovery. Treatment groups were divided into a “Yes” or “No” category based on the following criteria described by Minic *et al.* 7; Recovery of the left hemidiaphragm was considered positive/Yes in each rat only if the following conditions were met: (1) the EMG activity was detected in at least two of the three diaphragm areas (posterior, lateral, or anterior); and (2) if the activity persisted for the duration of the study. In each treatment group, rats were then divided into those having recovery or not, and the Fisher exact test was performed to determine whether the proportion of rats with an incidence of recovery in the treated groups was statistically different from the ratio of control animals with an incidence of recovery. The Fisher’s exact test was followed by the Bonferonni adjustment to correct for the number of comparisons tested. A α level of 0.016 was considered statistically significant (SigmaPlot 13.0). Groups that demonstrated a significantly higher incidence of recovery during the initial screening process, to determine optimal drug dose, were further investigated using phrenic nerve recordings to quantify recovery observed.

Terminal bilateral phrenic nerve recordings were used to estimate the increase in phrenic nerve output after nanoconjugate injections as described by Minic *et al.* 7. The areas under 10 consecutive integrated waveforms (INT) in the right phrenic nerve (RPN) and left phrenic nerve (LPN) activity were averaged and expressed as mean ±SE. Activity of the LPN was used as an index of the recovered respiratory activity in the THP nanoconjugate-treated rats. Both maximum (MAX) LPN amplitude activities as well as the areas under the curves (AUC) were expressed as the percentage of the RPN whose signal was set to be 100%. Then the average % INT and the average % MAX for each group was averaged and subjected to statistical examination using SigmaPlot 13.0. All groups underwent One-way ANOVA followed by Holm-Sidak pairwise comparison to determine the effect of the injected solution. A α level of 0.05 was considered statistically significant.

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