Doxorubicin Chemomyectomy Is Enhanced When Performed Two Days following Bupivacaine Injections: The Effect Coincidences with the Peak of Muscle Satellite Cell Division

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**Purpose.** Doxorubicin is effective in permanently removing muscle after direct injection into the eyelid for treatment of blepharospasm and hemifacial spasm. However, patients often require two or more injection series before full abatement of their spasms is achieved. Local anesthetics cause muscle necrosis, followed by regeneration, a process that requires activation and division of muscle satellite cells. This study examined whether the muscle toxicity of doxorubicin could be amplified by injection of doxorubicin into the eyelid of rabbits 2 days after a local anesthetic injury, perhaps exploiting the toxic effects of doxorubicin on satellite cells at the peak time of their division after injury.

**Methods.** Rabbit eyelids received two series of injections of bupivacaine and hyaluronidase spaced 18 hours apart. Two days later, the eyelids were injected with either 0.5 or 1 mg doxorubicin. Animals were monitored daily for onset and duration of skin injury. After 1 month, the eyelids were assessed for muscle loss using histologic and morphometric techniques.

**Results.** Injection of doxorubicin during the peak of satellite cell activation and division 2 days after injury significantly increased muscle loss over doxorubicin alone. This treatment did not result in increased skin injury compared with doxorubicin alone.

**Conclusions.** Permanent muscle loss was increased when doxorubicin was injected at the peak of satellite cell division 2 days after injury of the muscle with bupivacaine in rabbit eyelid, taking advantage of the antimototic effects of doxorubicin on satellite cell division during the period of active regeneration. When local anesthetic injection immediately preceded the doxorubicin injection, increased myotoxicity was not seen. The injection of doxorubicin into muscle 2 days after a previous injury maximizes muscle loss. The increased muscle loss provided by this double treatment may decrease the number of injection visits required by blepharospasm and hemifacial spasm patients during their course of treatment, thus reducing the number of patients with side effects, which increases with repeated exposures of the eyelid to doxorubicin. (Invest Ophthal Mol Vis Sci. 1998;39:203-206)
spasm. Although a few patients were treated successfully with a single dose of doxorubicin per eyelid, most patients required up to three separate injection series with a cumulative dose of approximately 3 mg per eyelid for complete abatement of their muscle spasms. Each repeated exposure to doxorubicin increases the risk of skin injury. A major patient concern is the local skin reaction to the doxorubicin injection in the subjacent muscle, which consists of redness, sensitivity to touch, and development of skin ulcers. We have been developing strategies to increase the muscle loss caused by the initial doxorubicin injections, thereby reducing the need for subsequent treatment. The two main goals of any approach to increase muscle injury for the treatment of muscle spasm diseases are to avoid exacerbating the skin side effects and to make the treatment protocol easier for the patients by reducing the need for subsequent injections.

Doxorubicin is a potent skeletal muscle toxin and a potent antimitotic drug. Previous animal studies of doxorubicin-induced myotoxicity in the orbicularis oculi indicate that muscle loss is extremely rapid, with myofiber death occurring within 24 hours after doxorubicin injection. Thus the initial exposure to doxorubicin is crucial for determining how extensive the muscle loss will be. The maximum dose for a single doxorubicin administration is limited by its side effects, in particular skin injury; for patients the threshold toxic dose for a single injection is 1.5 mg per eyelid. In most cases, this dose is insufficient for total abatement of their muscle spasms. These patients require two or three subsequent injection series, with the concomitant increased risk for skin injury. To amplify muscle loss by exploiting the antimitotic effects of doxorubicin, the drug was injected into rabbit eyelid at the peak of satellite cell division after injury to the orbicularis oculi muscle.

It has long been known that local anesthetics such as bupivacaine result in muscle injury when injected directly into a skeletal muscle. While bupivacaine-induced skeletal muscle injury can be quite extensive, the orbicularis oculi is somewhat more resistant to injury. Repeat injections of bupivacaine, combined with hyaluronidase to increase infiltration of the bupivacaine in the connective tissue of the eyelid, are required to obtain a substantial injury to the rabbit orbicularis oculi muscle. The peak of satellite cell activation and division after bupivacaine injury of the orbicularis oculi in rabbits is 2 days after bupivacaine injection. Thus, the orbicularis oculi muscle was treated with bupivacaine to induce local injury and regeneration, followed 2 days later by treatment with doxorubicin.
FIGURE 2 Doxorubicin chemomyectomy 2 days after preinjury. Numbers of muscle fibers from cross-sections through normal eyelids, those with doxorubicin only, and those preinjured with bupivacaine 2 days before doxorubicin treatment are shown. There was a significant increase in muscle loss in 2-day bupivacaine- and doxorubicin-treated orbicularis oculi muscle compared with doxorubicin only. (*) Significant difference from control. (**) Significant difference compared with doxorubicin only.

Theoretically, not only would the muscle be subjected to the myotoxic effects of doxorubicin, but the antimitotic capacity of doxorubicin would be maximally exploited by exposure of the activated satellite cells to the drug.

MATERIALS AND METHODS

New Zealand white rabbits were obtained from Birchwood Valley Farms and housed with Research Animal Resources at the University of Minnesota. All animal research conformed to the guidelines of the National Institutes of Health and the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Before eyelid injections were administered, the rabbits were anesthetized with an intramuscular injection of ketamine: xylazine (1:1, vol/vol) at a dose of 10 and 2 mg/kg, respectively. Proparacaine-HCl was placed in the conjunctival cul-de-sac. Both lower eyelids of seven rabbits were injected with two injections of 150 units of hyaluronidase (Wydase; Wyeth, Philadelphia, PA) in 0.5 ml of 0.75% bupivacaine-HCl in isotonic saline with 1:200,000 epinephrine (Sensocaine; Astra Pharmaceutical Products, Westborough, MA) spaced 18 hours apart. Previous studies demonstrate that the peak of satellite cell activation in the orbicularis oculi muscle is 2 days after a local anesthetic injury to the eyelid. Thus, the local anesthetic treatment was followed 2 days later with an injection of either 0.5 or 1 mg doxorubicin in 0.5 ml of sterile isotonic saline (Adriamycin; Adria, Columbus, OH). The rabbit eyelids were monitored daily for onset and duration of epithelial changes in the eyelid.

After 1 month the rabbits were euthanized with an overdose of barbiturate anesthesia, and the eyelids were removed for histologic examination. Eyelid samples from medial, central, and lateral regions of each eyelid were embedded in tragacanth gum, frozen in methylbutane chilled to a slurry on liquid nitrogen, and sectioned at 12 μm on a cryostat. The sections were processed using the alkaline myosin ATPase procedure at pH 10.5. The number of muscle fibers within the palpebral portion of the orbicularis oculi from all three areas of each eyelid was quantified using a digitizing morphometry program (Bioquant; R and M Biometrics, Nashville, TN). For each eyelid, an average muscle fiber number was determined by counting four sections from each of the three medial-to-lateral positions within the eyelid. A minimum of four eyelids (with three regions each) were examined for each experimental parameter. To prevent needless use of animals, experimental data from the bupivacaine-pretreated eyelids were compared with normal orbicularis oculi counts and 0.5 and 1 mg doxorubicin only injection data from previous studies. All onset

duration

FIGURE 3 Days to onset and total duration of skin injury. Doxorubicin only was compared with doxorubicin 2 days after bupivacaine injections. Note that 0.5 mg doxorubicin treatment resulted in a longer time to onset and a shorter total duration of skin injury than did 1 mg doxorubicin. At equivalent doses of doxorubicin, there was no significant difference between onset and duration of skin injury between eyelids treated only with doxorubicin and eyelids preinjured with bupivacaine and hyaluronidase 2 days before doxorubicin injection. Time is calculated from the day of the injection of doxorubicin.
procedures and treatment paradigms were unchanged for all experimental groups from the previous studies. Results were analyzed for statistical significance at $P < 0.001$, using an unpaired, two-tailed $t$-test. An $F$-test indicated that the variances between the control and experimental groups were not significantly different. The statistical tests were conducted using the StatMate and Prism software (Graphpad, San Diego, CA).

RESULTS

Injury of the orbicularis oculi muscle with bupivacaine before doxorubicin administration resulted in a substantial increase in muscle loss compared with doxorubicin alone$^7$ (Fig. 1). As is usual with injections into the eyelid, muscle loss was somewhat variable, but always significant, when examined from the medial to lateral extent of the lid. Often there were areas where all of the muscle was killed. The muscle loss in all treated eyelids was quantified (Fig. 2). When either 0.5 or 1 mg doxorubicin was injected 2 days after a bupivacaine-induced muscle injury, muscle loss was significantly increased over that seen with doxorubicin alone (Fig. 2). By injuring the orbicularis oculi muscle in advance of doxorubicin exposure, muscle loss was increased by 55% at the 1-mg doxorubicin dose and 63% at the 0.5-mg doxorubicin dose.

The onset and the duration of eyelid skin injury in the rabbits that received the local anesthetic preinjury 2 days before doxorubicin injections were similar to that seen with doxorubicin alone$^5$ (Fig. 3). Onset of skin injury always occurs sooner with the greater dose of doxorubicin, and the duration is always longer after a 1-mg dose of doxorubicin compared with a 0.5-mg dose of doxorubicin. This trend persists when doxorubicin is injected at the peak of satellite cell division 2 days after bupivacaine injury.

DISCUSSION

Local anesthetics are known to injure skeletal muscle.$^4$ Skeletal muscle responds to this injury by activation of satellite cells within the muscle. The satellite cells are myogenic precursors, and their division results in efficient regeneration of the injured muscle.$^5$ Satellite cell activation can take from 24 to 96 hours after a muscle injury. Previous studies indicated that the peak of satellite cell activation in bupivacaine-injured orbicularis oculi in rabbits is 2 days after injection,$^6$ although after injections of local anesthetic only the muscle completely regenerates.$^3$ Bupivacaine alone is not toxic to skin.$^5,6$ When doxorubicin was injected into the rabbit eyelid 2 days after the local anesthetic injury, at the peak of satellite cell mitosis, there was a significant increase in muscle loss compared with that seen after doxorubicin alone. This is in marked contrast to doxorubicin injected immediately after a bupivacaine injury in rabbit eyelid, where the bupivacaine injection did not enhance the doxorubicin-induced muscle loss.$^9$ This amplification in muscle loss when doxorubicin is injected into a regenerating muscle bed could be the result of one or two separate processes. Increased muscle loss could be due to increased effectiveness in the acute toxicity of the doxorubicin, caused by free oxygen radicals or calcium changes, on already compromised muscle cells directly and/or could be the result of the antimitotic effect of doxorubicin on the satellite cells that were activated and dividing in response to the first injury.$^6$ Doxorubicin can specifically repress the myogenic transcription factor MyoD,$^{10}$ which would prevent myoblast fusion and retard muscle cell differentiation. Preliminary in vitro studies indicated that even at concentrations below toxic levels, doxorubicin still inhibited mitosis in satellite cell cultures from rabbit muscle (McLoon, unpublished results). Additional work is needed to distinguish between these two mechanisms.

The injection of doxorubicin into muscle at the peak of satellite cell activation and division is an effective strategy for maximizing muscle loss with a single doxorubicin administration. This treatment did not result in greater skin injury compared with doxorubicin alone. This method should allow for a more effective treatment of patients with muscle spasm disease, since a single injection series may be sufficient to treat their disease. We are continuing to explore multiple techniques to enhance the safety and efficacy of chemomyectomy. For example, we have recently found that direct intrallesional injection of corticotropin releasing factor can inhibit the undesirable skin changes without decreasing the muscle loss.$^7$ The local anti-inflammatory effect of the corticotropin releasing factor peptide is not dependent on increased systemic production of corticosteroids. The injection of doxorubicin during the peak of satellite cell mitosis 2 days after bupivacaine-induced muscle injury might be used in combination with corticotropin releasing factor to allow for maximal muscle injury and minimal inflammation.

References