Verapamil Substantially Increases the Chemomyectomy Effect of Doxorubicin Injected Into Rabbit or Monkey Eyelid

Linda Kirschen McLoon, Michael Ekern, and Jonathan Wirtschafter

Local doxorubicin injections have been used clinically to treat blepharospasm, hemifacial spasm, and other related disorders permanently and nonsurgically. Doxorubicin is an effective myotoxic agent for the removal of the orbicularis oculi muscle in the eyelid after local injection. Injections of this drug alone resulted in removal of up to 70% of the muscle fibers from the treated eyelids in monkeys. The authors attempted to optimize the conditions for doxorubicin myotoxicity of the orbicularis oculi. Doxorubicin was injected shortly after local verapamil injection in rabbits and a monkey in an attempt to maximize the muscle injury in the eyelid. Verapamil (dose, 0.5 mg or 1.6 mg in the rabbits), injected with a range of doses of doxorubicin, caused substantially increased muscle loss in the eyelid compared with doxorubicin alone. In the monkey, verapamil (dose, 0.25 mg) injection was followed by an injection of 1 mg of doxorubicin. Verapamil cotreatment resulted in increased muscle loss over that caused by doxorubicin alone in both rabbits and the monkey. Injection of verapamil alone also caused muscle loss, and this was quantified. The muscle loss with doxorubicin and verapamil injections included muscle in the preseptal portion of the muscle and even in the pretarsal muscle (which previously was difficult to destroy). This technique clinically might be used to decrease the dose of doxorubicin injected and/or decrease the total number of injections necessary but still retain a clinically effective treatment for blepharospasm and hemifacial spasm. The reduction in the dose of doxorubicin also may decrease the risk of skin injury from doxorubicin chemomyectomy in these patients. Invest Ophthalmol Vis Sci 33:3228-3234, 1992

Injection of doxorubicin into the eyelid may be an effective and economic permanent nonsurgical treatment for blepharospasm, hemifacial spasm, and other related facial dystonias. In studies using cynomolgus monkeys, doxorubicin treatment caused a loss of up to 70% in the number of muscle fibers. In the first clinical trial of this novel treatment protocol, 8 of 20 patients had major long-term improvement, which has now continued for up to 1 yr.

The major drawback with the clinical use of doxorubicin chemomyectomy is the potential for eyelid skin injury as a result of the drug's toxicity. In both animal and patient studies, skin ulceration has occurred at the doses most effective for muscle removal. Repeated injections have been necessary in these patients to achieve maximal relief of muscle spasms. Each injection series is followed by inflammation and redness for 1-2 mo, and repeated injections increase the risk of ulcer formation. It may be possible to reduce the dose of doxorubicin and the number of injections needed by using a drug that would amplify the toxic effects of doxorubicin.

One drug that has been effective in potentiating the toxic effects of doxorubicin in cancer cells is verapamil. Verapamil is a calcium-channel antagonist that inhibits the calcium ion influx through slow channels into muscle cells. Several hypotheses have been advanced to explain its ability to enhance the toxic effect of doxorubicin and other anthracyclines in cancer cells. Verapamil has been shown to increase doxorubicin accumulation in treated cells in a number of ways, including decreasing doxorubicin efflux from the cells, altering cellular localization of doxorubicin in the cells from the cytoplasm to the nucleus, and enhancing disruption of DNA. There is conflicting evidence whether changes in intracellular calcium concentration are involved, with evidence both for and against the role of calcium. Verapamil
also can bind to the P-glycoprotein expressed by the multidrug resistance gene product in doxorubicin-resistant cells. This binding apparently prevents doxorubicin binding and efflux,13-15 thus raising the intracellular doxorubicin level.

We injected verapamil and doxorubicin into rabbit or monkey eyelids to determine whether there was an increase in the toxic effect of the doxorubicin as a result of verapamil pretreatment of the muscle. A successful result might permit us to decrease the number of injections and/or the dose of doxorubicin needed to provide relief of muscle spasms in a number of muscle spasm disorders, including blepharospasm and hemifacial spasm patients.

Materials and Methods

Thirty-three New Zealand white rabbits were obtained from Birchwood Laboratories through the University of Minnesota Research Animal Resources. One cynomolgus monkey was obtained through the University of Minnesota Research Animal Resources. All research conformed to the guidelines of the ARVO Resolution on the Use of Animals in Research.

Before the injection of the drugs into the eyelids, the rabbits were anesthetized with intramuscular injections of a 1:1 mixture of ketamine HCl (concentration, 100 mg/ml; dose, 10 mg/kg) and xylazine (concentration, 20 mg/ml; dose, 2 mg/kg). The monkey was anesthetized with an intramuscular injection of ketamine HCl (dose, 10 mg/kg). Proparacaine HCl was placed in the conjunctival cul-de-sac to reduce blinking. For the rabbits, two injections of a solution of verapamil HCl (5 mg/ml or 16 mg/ml in sterile isotonic saline; total dose, 0.5 mg [17 rabbits] or 1.6 mg [16 rabbits]) were administered to include the entire lateral-to-medial extent of the right upper and left lower lids. Higher doses of verapamil are lethal to rabbits. This was followed immediately by injection of doxorubicin in sterile isotonic saline at one of the following doses: 2 mg (six rabbits), 1 mg (ten rabbits), 0.5 mg (ten rabbits), or 0.1 mg (six rabbits). The concentration of the doxorubicin solution was altered to keep the total volume injected into the eyelids constant. The eyelids of five rabbits were injected with 0.5 mg of the drug (2 mg/ml in isotonic saline) only. The eyelids of two rabbits each were injected with either 0.5 or 1.6 mg verapamil HCl only to serve as controls. For the monkey, two injections of verapamil HCl (5 mg/ml) in isotonic saline were placed into the left lower and right upper eyelids (total dose, 0.25 mg in each lid). This was immediately followed by injection of doxorubicin (5 mg/ml) in sterile isotonic saline (total dose, 1 mg in each lid). These two lids were compared with data published previously based on the injection of four monkeys.

One to three months later, the animals were anesthetized deeply as described. Portions of the treated and contralateral control eyelids were taken for histologic processing. Part of the medial, central, and lateral parts of the treated eyelids and a portion of the central region of the control lids were taken. The samples were embedded in tragacanth gum and immediately frozen in 2-methylbutane that had been chilled in liquid nitrogen. The tissue was sectioned at 12 μm (using a cryostat) and processed for alkaline myosin adenosine triphosphatase histochemical analysis by standard techniques at pH 10.5.16 The muscle loss was quantified using a Bioquant image analysis system (R and M Biometrics) to determine (1) the number of muscle fibers in each eyelid section, (2) the muscle fiber cross-sectional areas for each fiber in the pretarsal and preseptal portions of the muscle, and (3) the total muscle area (number of muscle fibers × cross-sectional areas for each fiber) for each section.

Our previous work showed that there were differences in the regional toxicity of doxorubicin.3 We also determined the number of fibers in the pretarsal portion of the muscle (from the eyelid margin to the end of the tarsal glands) and the preseptal portion (from the tarsal glands to the end of the conjunctiva).17 A total of 5 sections were counted for each portion of the lid, making a total of 15 sections per eyelid. The standard error of the mean was determined for each set of measured parameters. The results were statistically analyzed using a paired Student’s t-test. Unless otherwise stated, we used P < 0.01 as statistically significant.

Results

Light microscopic examination of the treated muscle in the rabbit eyelids showed there was substantial muscle loss when verapamil injections preceded the doxorubicin administration (Fig. 1). There was an obvious difference in the amount of muscle loss caused by the different doses of doxorubicin; an increase in the amount of muscle loss was found as the dose of doxorubicin was raised from 0.1 mg (Fig. 1D) to 2 mg (Fig. 1A) compared with the normal muscle (Fig. 1E).

This muscle loss was quantified for each of the doses of verapamil and doxorubicin. When only doxorubicin was injected into rabbit eyelid, there was a significant muscle loss in the treated orbicularis oculi of 30% of the muscle fibers (Figs. 2, 3). In every instance, the injection of verapamil 20 min before the administration of doxorubicin resulted in a significant increase in muscle injury. When an injection of verapamil (dose, 0.5 mg) preceded the doxorubicin, a significant decrease in muscle fiber number was seen.
Fig. 1. Photomicrographs of the pretarsal region of the orbicularis oculi muscle in rabbits after injections of 1.6 verapamil with the following doses of doxorubicin: (A) 2 mg doxorubicin injection; (B) 1 mg doxorubicin injection; (C) 0.5 mg doxorubicin injection; (D) 0.1 mg doxorubicin injection; and (E) uninjected control. Arrowheads indicate type 2 muscle fibers. White arrows indicate type 1 muscle fibers. Bar is 50 μm.
at all doxorubicin doses compared with injection of doxorubicin alone (Figs. 2, 3). Although there was no statistical difference between the muscle fiber number at any of the doxorubicin doses used after 0.5-mg verapamil injections, there was a dose-related decrease in total muscle fiber number with increasing doxorubicin doses. The percent decrease in muscle fiber number compared with control was 98% after both 1- and 2-mg doxorubicin injections, 83% after 0.5 mg, and 70% after 0.1 mg. After injections of 1.6 mg of verapamil and graded doses of doxorubicin, there was again a highly significant decrease in muscle fiber number compared with doxorubicin alone (Figs. 2, 3). Both the 2- and 1-mg doxorubicin injections produced a significant reduction of muscle fiber number compared with doses of 0.5 and 0.1 mg. The percent decrease in muscle fiber number compared with control was 94% after a dose of 2 mg of doxorubicin, 84% after 1 mg, 79% after 0.5 mg, and 75% after 0.1 mg.

Differential counts were made of the muscle loss in the pretarsal and preseptal regions of the treated orbicularis oculi muscle of the rabbits (Fig. 4). In every instance, the muscle loss included both the pretarsal and preseptal regions of the lid. This contrasted with the results after injection of doxorubicin alone; significant muscle loss was found only in the preseptal region.

We examined the muscle loss in samples from medial, central, and lateral portions of the treated eyelids in individual rabbits (Fig. 5). We observed that there tended to be differences in muscle fiber number in these three areas in the same rabbit eyelid, presumably subjected to the same dose of doxorubicin across the entire extent of the eyelid. The region of greatest muscle loss after doxorubicin injection varied from animal to animal, but usually it was greatest in the center of each eyelid. This is most likely a reflection of the slightly different placement of the verapamil and doxorubicin injections.

In the monkey eyelid, the verapamil and doxorubicin injection protocol resulted in an increased loss of muscle fiber number compared with doxorubicin alone (Fig. 6). An injection of 2 mg of doxorubicin alone caused a 70% loss in the number of muscle fibers compared with a 79% loss when verapamil and 1 mg of doxorubicin were injected sequentially into the eyelid. These differences were significantly different from control values and from each other.

Injections of either 0.5 mg or 1.6 mg verapamil
Regional Muscle Loss in the Eyelid After Doxorubicin and Verapamil injections

Fig. 5. Graph of the distribution of muscle fiber number in one rabbit eyelid treated with 0.5 mg verapamil and 2 mg doxorubicin, counted in lateral, central, and medial portions of the eyelid. The total muscle fiber number is the sum of the pretarsal and preseptal muscle fiber number.

alone into the rabbit eyelid also resulted in a significant decrease in muscle fiber number compared with untreated muscle. This represented 10% and 52%, respectively, of the number of muscle fibers in the control orbicularis oculi (Fig. 2).

There was no difference in the appearance of skin ulceration between animals injected with doxorubicin alone and those injected with both verapamil and doxorubicin. No lids ulcerated on the conjunctival surface, although an occasional small patch of epithelial cell proliferation was seen.

Discussion

Doxorubicin injections into the orbicularis oculi of rabbits and monkeys caused a significant decrease in muscle fiber number. When doxorubicin was injected after an injection of verapamil in both rabbits and the monkey eyelids, there was a substantial increase in muscle toxicity compared with that seen with doxorubicin administration alone. Both the 0.5- and 1.6-mg doses of verapamil were effective in reducing muscle fiber number; the higher dose of verapamil was more effective when injected with 1 or 2 mg of doxorubicin. The loss of up to 98% of the muscle fibers after a single injection of 1–2 mg of doxorubicin and 1.6 mg of verapamil represents a substantial amplification of muscle loss compared with doxorubicin alone. Although the increase in muscle loss was less pronounced in the monkey, the dose of verapamil was only 0.25 mg (compared with 0.5 and 1.6 mg in the rabbits). The higher dose of verapamil was more effective in killing muscle fibers in the treated rabbits, and presumably, a higher dose of verapamil would cause a greater loss of muscle fibers in the monkey also. The dose of doxorubicin was only 1 mg in the verapamil-treated monkey eyelids (compared with 2 mg for the eyelids treated with doxorubicin alone). Thus the verapamil again increased the amount of muscle fiber loss over that seen with the higher dose of doxorubicin injected alone.

The myotoxic effect of doxorubicin appeared to be greater in the monkey than in the rabbit orbicularis oculi muscle. There are several possible explanations for this apparent difference. The first is that we injected a slightly lower dose of doxorubicin into the eyelids of the rabbits. The rabbit eyelid is substantially larger than that of the primate, in both the canthal-to-canthal dimension and in the distance from the eyelid margin to the conjunctival cul-de-sac. This would lower the actual concentration of doxorubicin at any given location in the eyelid. The rabbit eyelid also is thicker than the primate eyelid, with a great deal of dense connective tissue. This could decrease the accessibility of the muscle cells to the injected doxorubicin. From tissue culture studies, it was evident that the toxicity of doxorubicin was directly proportional to the intracellular concentration of the drug.

The mechanism for the enhancement of doxorubicin myotoxicity by verapamil is not understood. There is ample evidence that verapamil can cause increased intracellular accumulation of doxorubicin, probably by preventing cellular efflux of the drug and altering its intracellular localization. This increased intracellular doxorubicin accumulation might increase its muscle cell toxicity. The myotoxic effects of doxorubicin have been well character-
ized.\textsuperscript{21,22} Its toxic effects are, in part, the result of disruption of subcellular organelles in the muscle cells, particularly the contractile proteins.\textsuperscript{23,24} As early as 15 min after doxorubicin injection into the eyelid, disruption of the myofibrillar organization is apparent in the treated orbicularis oculi muscle fibers.\textsuperscript{25}

Injection of doxorubicin alone resulted in a significant loss only in the preseptal region. Injections of verapamil and doxorubicin caused muscle loss in both the pretarsal and preseptal regions of the orbicularis oculi muscle. It is possible that the vasodilator function of verapamil helped increase the spread of the doxorubicin in the injected eyelid, resulting in greater availability of doxorubicin to the muscle. In other studies, agents known to increase the tissue infiltration of drugs, such as hyaluronidase or collagenase, increased the doxorubicin toxicity when the eyelids were pretreated with these drugs.\textsuperscript{26} Presumably increased extracellular space permits expanded spread of the doxorubicin in the treated eyelid.

There is less experimental evidence to explain the apparent permanent myotoxic effect of verapamil alone on the orbicularis oculi muscle. Verapamil is a calcium-channel blocker used to treat supraventricular tachycardia. It has been shown in tissue culture studies to be cytotoxic in concentrations greater than 6.6 \textmu mol/L.\textsuperscript{27} It inhibits tumor growth when used alone.\textsuperscript{28} Specific myotoxic effects have not been described during its systemic use. It would appear that direct injection of verapamil into the orbicularis oculi muscle is sufficient to injure some muscle fibers permanently.

The ability to selectively destroy most of the orbicularis oculi muscle fibers after a single set of doxorubicin and verapamil injections would be a substantial improvement over the current protocol in use in the clinical trial. The patients who have had maximal improvement of their muscle spasms have all required three sets of doxorubicin injections spaced at least 3 mo apart.\textsuperscript{4} Each subsequent injection series resulted in a greater chance of skin ulceration, particularly as the cumulative dose reached 4 mg in one eyelid. The long-term retention of doxorubicin and subsequent skin reactions in cases of extravasation in the skin of the hand or arm has been documented.\textsuperscript{29} By using verapamil to amplify the myotoxic effects of the doxorubicin, we hope we can reduce the total number of injections needed for relief of spasm in patients with blepharospasm and hemifacial spasm.


