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Doxorubicin does not spread systemically following a local injection into the eyelids of rabbits

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Abstract

Purpose. An experimental treatment for benign essential blepharospasm and hemifacial spasm involves the direct injection of doxorubicin into the eyelids to permanently kill muscle. This study examined the extent of local and systemic spread of doxorubicin after localized injections of low doses into the eyelid and determined the length of time doxorubicin was retained in the eyelid after injection.

Methods. Two mg doxorubicin was injected subcutaneously into the lower eyelids of rabbits. After various time periods, the eyelids were removed and dissected into three separate specimens consisting of skin, subcutaneous connective tissue including orbicularis oculi muscle, or palpebral conjunctiva. Nearby tissues were also collected, including facial muscles and extraocular muscles. Urine, blood, kidney, spleen, heart and liver samples were collected. All tissues were prepared for HPLC determination of doxorubicin concentration.

Results. Doxorubicin was detected in all three eyelid specimens for the first 4 days after injection, although by the fourth day the level of doxorubicin was greatly reduced. On and after the seventh day, there was no detectable doxorubicin in the treated eyelid tissues. There were no detectable levels of doxorubicin in the urine or any other body tissue at any of the post-injection intervals examined. There was no long term retention in any of the eyelid tissues examined.

Conclusions. The well described array of serious systemic side effects caused by the use of high systemic doses of doxorubicin as a chemotherapeutic agent made it critical to ascertain how long doxorubicin remained within the injected eyelids, and to determine to what extent and with what time course local injections of chemically intact doxorubicin might spread systemically. The short retention of the active or unmetabolized drug at the injection site is important, since more than one set of injections has been required for satisfactory amelioration of muscle spasms in blepharospasm and hemifacial spasm patients. The lack of detectable systemic spread of the drug distant from the local site of injection as well as the lack of long term retention of the locally injected doxorubicin lends support for the safety of doxorubicin administered in this manner to blepharospasm and hemifacial spasm patients.

Keywords: high-performance liquid chromatography (HPLC); doxorubicin; eyelid; orbicularis oculi muscle; rabbit

Introduction

Doxorubicin chemomyectomy is a recently developed experimental procedure for the permanent, non-surgical treatment of muscle spasm diseases, including blepharospasm and hemifacial spasm.1,2 Doxorubicin is a potent and rapidly acting skeletal muscle toxin when injected locally.3,4 This doxorubicin induced muscle loss is permanent.3,5

Intravenously administered doxorubicin has been used for chemotherapy treatment of cancer for a number of years.6 One of the side effects of the high systemic doses used for chemotherapy is the development of dose dependent cardiac myopathy.7 The development of cardiac muscle toxicity is the major basis for the lifetime limit to doxorubicin treatment to 500 to 550 mg/m², corresponding to about 13 mg/kg or 900mg for a 70 kg patient.7,8 The maximum cumulative dose given to an individual blepharospasm patient was 16.85 mg, but this was given in multiple doses over a period of 2 years.1,2 This dose is small compared to chemotherapeutic levels used in cancer treatment, but still represents a significant exposure. The average age of onset of blepharospasm is between 50 and 70 years. These doxorubicin-treated individuals may subsequently develop cancers, and previous exposure to doxorubicin might limit the subsequent use
of doxorubicin and related chemotherapeutic agents in these individuals.

There are two types of doxorubicin toxicity: acute and chronic. Acute toxicity results from high levels of initial exposure and the resultant increase in its pharmacological activity. The acute level of toxicity limits a single intravenous dose for cancer chemotherapy to about 1 mg/kg or 70 mg. Chronic toxicity is caused by sustained effects on non-renewing cell types due to the accumulated effects of doxorubicin.\(^9\) The pharmacology and toxicology of this widely used chemotherapeutic agent have been extensively studied following high dose intravenous administration. The main site for metabolism and excretion of high systemic doses of doxorubicin is the liver.\(^10\) This study was directed at assessing both levels of toxicity, by looking at both short term and long term retention and spread of locally subcutaneously injected doxorubicin. It was important to determine the duration of exposure of the initial acute exposure of the treated eyelids to doxorubicin. Secondly, it was important to verify whether the local injection of doxorubicin resulted in exposure of the body to doxorubicin due to uptake into the blood system; of particular concern was possible exposure of the heart to doxorubicin. This type of exposure could result in effects seen with chronic exposure of doxorubicin, in particular cardiac myotoxicity. It has been shown that exposure of the heart to high doses of intravenously administered doxorubicin result in injury from the first exposure\(^11\), and the heart muscle injury is cumulative with increased exposure due to the chronic progressive toxicity of repeated exposure of the heart muscle to doxorubicin.\(^7\)

One of the less life-threatening, but still unwanted, side effects of doxorubicin use for chemotherapy has been injury to the skin, both due to extravasation injury\(^12,13\) and to repeated bolus applications of doxorubicin.\(^14\) Local subcutaneous injections of doxorubicin into the eyelid also result in skin injury.\(^1,15\) While the skin heals, subsequent injections of doxorubicin needed for complete treatment of the patients usually resulted in a greater likelihood of skin injury with each additional exposure. This study examined the eyelid skin, connective tissue plus muscle, and palpebral conjunctiva to determine how long doxorubicin remained at detectable levels in these tissues and whether doxorubicin spread to other tissues distant from the site of primary application.

**Materials and methods**

Adult New Zealand white rabbits were purchased from Birchwood Valley Farm and housed with Research Animal Resources at the University of Minnesota. All research conformed to the guidelines from the National Institutes of Health on the proper use of animals in research.

Rabbits were anesthetized with an intramuscular injection of ketamine:xylazine, 1:1 (10 mg/kg:2 mg/kg, respectively). All rabbits weighed between 1.8 and 2.1 kg at the time of injection. The lower eyelids of the rabbits received an injection of 2 mg doxorubicin in sterile isotonic saline (Adriamycin, Adria Labs.) in a volume of 1 ml. Euthanasia was performed by administration of an overdose of barbiturate anesthesia intravenously after various time periods, including 2, 4, 24 hours and 4, 7, 180 and 360 days. Tissue samples were removed from the eyelids, which were divided into three samples: skin, a combined sample which contained subcutaneous connective tissue and orbicularis oculi muscle, and palpebral conjunctiva. By 24 hours, muscle could not be clearly identified and presumably all samples contained mainly connective tissue. Previous work indicated that local injections of 2 mg doxorubicin rapidly destroys the muscle, particularly in the preseptal and orbital regions.\(^4\) Additionally, samples of adjacent tissues, neck fat and other facial muscles, were collected at the time of sacrifice at each of the above listed post-injection intervals. The following tissues were removed from each doxorubicin injected rabbit at each of the above listed post-injection intervals for HPLC analysis: heart, blood, liver, spleen, and kidney. Urine was collected at the time of sacrifice by direct needle insertion into the bladder. The tissue was frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until analysis. Each time point represents a minimum of 4 animals, and each tissue sample was prepared for HPLC analysis and run in triplicate.

The doxorubicin concentration in each of the tissue samples was determined using a Gilson high pressure chromatograph equipped with a C18 column (SynChrom Inc.).\(^16\) All reagents were HPLC grade. Tissue samples were weighed and were diluted 200 \(\mu\text{l}\) PBS/mg tissue or per ml for urine and blood. Samples were homogenized for 5 repetitions of 15 seconds in a sonicator. Aliquots of homogenate were removed, transferred to a clean microtube and diluted 1:4 in methanol. Aliquots of 12 mM phosphoric acid were added, and the precipitated proteins were removed by centrifugation at 8000g for 10 minutes. The supernatant was filtered through a 0.2 \(\mu\text{m}\) syringe filter. Samples of the filtrate were injected onto the C18 column. A linear gradient of 25 mM ammonium dihydrogen phosphate/30 mM
phosphoric acid:acetonitrile (85:15,v:v) to 25 mM ammonium dihydrogen phosphate/30 mM phosphoric acid:acetonitrile (1:1,v:v) was run through the column at a flow rate of 1.5 mL/min, with the final concentration held for 5 minutes. Column effluent was monitored at excitation and emission wavelengths at 475 nm and 580 nm, respectively. A standard curve of doxorubicin concentration was prepared. The lowest detectable concentration of doxorubicin was 0.0448 mg/ml.

Results
The local concentration of doxorubicin was determined after direct injection into the eyelid. Doxorubicin was present in the injected eyelids in detectable amounts for the first week after administration. After two hours, doxorubicin was detected in the lid skin, combined muscle and connective tissue samples, and palpebral conjunctiva. By 4 hours, these levels were significantly reduced in skin and combined connective tissue/muscle samples but remained relatively stable in the palpebral conjunctival tissues examined. The levels were relatively unchanged after 24 hours (Figure 1). After 48 hours, there were detectable but very low levels of doxorubicin in the eyelid skin and combined connective tissue/muscle samples, but the level in palpebral conjunctiva was unchanged. After 4 days (96 hours), the level of doxorubicin was significantly reduced in all tissues examined. By 7 days and thereafter, no doxorubicin was detected in any of these eyelid tissue samples.

Doxorubicin was not detected in any of the other adjacent regions of the face examined. Doxorubicin treated eyelids were examined 6 months and 1 year after local injection. No doxorubicin was detected within the eyelid by HPLC analysis after these time periods.

Doxorubicin was also never detected in any of the other tissues examined, including the heart, liver, spleen, kidney urine and blood.

Discussion
Due to the known, and potentially serious, side effects caused by the intravenous administration of doxorubicin, it was important to ascertain the extent and timing of doxorubicin that might spread systemically after local injection into the subcutaneous and muscle tissue of the eyelid. It was also important, faced with the need in some patients for two or three repeat injection series of doxorubicin in each eyelid for the complete treatment of the blepharospasm and hemifacial spasm patients, to determine how long doxorubicin remained in the injected eyelids. Doxorubicin apparently is either removed within days from the injection site or is completely metabolized within the eyelid after local injection, as it was greatly reduced by 4 days and no longer detectable in the various eyelid tissues 7 days after local injection of 2 mg doxorubicin into the eyelid. The detection of doxorubicin within all the palpebral structures in short time periods after injection, including the skin and palpebral conjunctiva, was expected as a natural consequence of the injection procedure used throughout the eyelid. No doxorubicin was detected in nearby orbital structures, facial muscles or orbital fat. No doxorubicin was detected in other body tissues, including blood, heart, liver, spleen, kidney or urine. While it may be possible that doxorubicin was present systemically that was below the level of detection of the method employed, doxorubicin toxicity is known to be directly related to its local concentration. This is in contrast to botulinum toxin injections. Some evidence of systemic spread is EMG evidence of subclinical, but
detectable muscle jitter in the distant limb muscles after a local injection in the face or neck muscles.\textsuperscript{17} Other evidence for systemic spread of botulinum toxin is the development of serum antibodies.\textsuperscript{18} Neither of these responses are applicable to doxorubicin administration.

Extravasation of doxorubicin locally during intravenous administration causes tissue destruction.\textsuperscript{12,13,19} This has been postulated to be the result of the persistence of doxorubicin for at least one month in the dermis at an extravasation site in the hand.\textsuperscript{20} The removal of most of the doxorubicin within the eyelid after just a few hours may in part explain why the severity of skin injury is less pronounced than after extravasation at intravenous therapy sites where the drug enters subcutaneous tissues not associated with muscle as is the case in the treated eyelids. Even after repeated injections of doxorubicin in human eyelids, up to a maximum dose for all treated eyelids in one patient of 12.8 mg, only one patient out of 29, required out-patient surgical closure of a small ulcer.\textsuperscript{21} This is in marked contrast to intradermal injections of 0.5 mg doxorubicin in a mouse skin lesion model, where single injections resulted in pronounced skin lesions.\textsuperscript{19} In blepharospasm and hemifacial spasm patients, the maximum cumulative dose in a single eyelid was 5 mg, yet the development of skin ulcers was less severe than after extravasation exposure.\textsuperscript{1,2} It is possible that the specific uptake of doxorubicin into the muscle cells themselves,\textsuperscript{22} and their subsequent rapid necrosis and removal, protects the skin and palpebral conjunctiva from the long term injury seen after extravasation, especially as seen in the dorsum of the hand.\textsuperscript{20} One of the main causes of doxorubicin induced skin toxicity may not be its acute toxicity on the epithelial cells themselves, but rather due to a long term inhibition of turnover in the epithelial stem cells (McLoon, unpublished observations).\textsuperscript{14}

The clearance behavior of doxorubicin after high dose systemic administration is complex, and studies have shown that the kinetics of doxorubicin clearance varies greatly from patient to patient.\textsuperscript{23,24} In such studies, intravenous concentrations of doxorubicin showed a precipitous fall within the first two minutes after intravenous administration and an initial half-life for elimination varying between 30 minutes to 3 hours.\textsuperscript{10,25,26} The half-life for terminal elimination and an initial half-life for elimination varying between 50 minutes to 3 hours.\textsuperscript{10,25,26} The half-life for terminal elimination of high dose doxorubicin treatment in plasma varied from 18 to 50 hours.\textsuperscript{10} The lack of detectable doxorubicin in any body tissue and the great reduction of detectable doxorubicin within the eyelid after four days in the present study is well within the range of these previous studies. It would appear that the mode of doxorubicin administration, whether intravenous infusion or local injection, results in a similar timetable for its elimination. This study in rabbits may indicate that localized administration of doxorubicin in the human eyelid constitutes a minimal, if any, additive risk of cardiomyopathy for persons who may later require systemic chemotherapy with this agent.

In other studies the majority of circulating doxorubicin after a high systemic dose was excreted in the urine and was not broken down into metabolites.\textsuperscript{25} However, excretion into the urine does not represent the major pathway for doxorubicin elimination, even after high systemic exposure.\textsuperscript{27} Liver is the major site of high-dose doxorubicin removal from the plasma, primarily by excretion in the bile.\textsuperscript{10} Doxorubicin was never detected in the liver after localized injections into the eyelid. It should also be pointed out that patients who received doxorubicin injections into their eyelids for treatment of blepharospasm or hemifacial spasm had liver enzyme studies, and these were always normal.\textsuperscript{2,21} Thus, the total exposure of the systemic organs to doxorubicin after localized eyelid injections is so small that it is undetectable, presumably because it is broken down completely within the injection site.

The present study indicates that local injection of doxorubicin does not result in significant long-term retention of the drug within the eyelid. Doxorubicin is not detected at the injection site one week after treatment. Doxorubicin was never detected in any other body tissue at any other time. This gives added assurance to patients of the safety of direct injection of low levels of doxorubicin for the treatment of muscle spasm diseases.

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