Ultrastructure of Mepivacaine-Induced Damage and Regeneration in Rat Extraocular Muscle

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Adult rats were given a single retrobulbar injection of 50 μl of 2.0% mepivacaine and the lateral rectus muscles were examined ultrastructurally at intervals from 15 min to 30 days post-injection. There were three purposes of the study: (1) to determine the extent of muscle fiber damage caused by the anesthetic; (2) to document the subsequent course of muscle fiber regeneration; and (3) to relate these findings to clinical data on possible adverse effects of local anesthetics on human extraocular muscle function. The lateral rectus muscle was massively damaged by exposure to the anesthetic, with membrane lesions seen as early as 15 min after the injection. Intracellular damage was followed by the phagocytic removal of the remnants of the damaged muscle fibers. The activation of satellite cells to myoblasts began during the phase of phagocytosis, and between 3 and 4 days after injection multinucleated myotubes actively forming sarcomeres appeared. Even during later stages of muscle fiber regeneration, evidence of damage was seen in muscle fibers that were not destroyed during the first 2 days post-injection. The results of this experiment show (1) that the vast majority of lateral rectus muscle fibers are rapidly broken down by the anesthetic, but that the destroyed muscle fibers are replaced by regenerating ones; and (2) that the ultrastructure of regeneration of extraocular muscle fibers differs little from the regeneration of somatic muscle fibers. The myotoxic effects of retrobulbarly applied local anesthetics in rats seem to be much greater than they are in primates. Invest Ophthalmol Vis Sci 30:1643–1651, 1989

In 1985, Rainin and Carlson reported the occurrence of postoperative diplopia and ptosis in some patients whose rectus muscles had been exposed to bupivacaine prior to cataract surgery, and they postulated that the basis for the decreased muscle function is a myotoxic effect of the injected local anesthetic on the muscle fibers. In a subsequent laboratory study, the same authors obtained massive damage to the extraocular muscle fibers of rats after a single injection of 50 μl of bupivacaine, lidocaine or mepivacaine. Histological analysis revealed that the anesthetic-induced damage to the extraocular muscle fibers was followed by virtually complete regeneration. In addition to muscle fiber destruction, the epithelial cells of the Harderian gland were severely damaged.

It has been known for almost two decades that in laboratory animals many of the local anesthetics have a myotoxic effect on striated muscle, including cardiac muscle, and skeletal muscle fibers in vivo and in vitro. In addition, some local anesthetics are also toxic for other tissue components in skeletal muscles. Although some ultrastructural studies have been conducted on bupivacaine- and lidocaine-induced damage of limb muscles and regeneration after mepivacaine-induced damage has not been studied at the electron microscopic level.

The current study was undertaken because, despite a couple of reports, the regeneration of extraocular muscles, which are highly specialized, has not been systematically studied. Mepivacaine was selected as the damaging agent on the basis of our previous study. At the histological level, mepivacaine produced a pattern of damage similar to that of lidocaine and bupivacaine, but the uniformity of the lesions was somewhat greater than that produced by the other two anesthetics. The major aim of the study was to provide a detailed description of the degeneration and early regeneration of extraocular muscle fibers in the rat.

Materials and Methods

Twenty-eight adult F-455 rats (a Wistar strain) were anesthetized with ether and given a single injection of 50 μl of 2% Carbocaine (Mepivacaine hydrochloride, Winthrop-Breon, New York, NY) into the
At intervals of 15 and 30 min, 2, 6 and 24 hr, and 1, 2, 3, 4, 7, 14 and 30 days after injection, lateral rectus muscles were prepared for analysis. Under heavy anesthesia, the extraocular muscles on both the control and experimental eyes were fixed in situ for 15 min by infusing 0.1 M phosphate-buffered Karnovsky fixative into the retrobulbar cavity. In order to prevent the build-up of fluid pressure, vents were created to allow fixative to drain. The lateral rectus muscles were dissected out, cut transversely into pieces and immersion-fixed for 2 hr in the same fixative. The tissue was washed briefly in a phosphate-buffered 4% dextrose solution, postfixed for 1 h in 1% OsO4, dehydrated through an acetone series and embedded in Epon 812. Sections were cut on a Reichert Ultracut microtome. Ultrathin sections were stained 30 min in a saturated solution of uranyl acetate, 5 min in Reynolds's lead citrate, and examined with a Philips 400 microscope operating at 60 kV.

Eight muscles, one control and seven muscles sampled at 15 and 30 min, 2, 6 and 24 hr after injection, were treated with Ruthenium red as a test for membrane damage. The Ruthenium red solution employed was prepared in the manner described by Mykelbust, but contained 4% dextrose. After initial in situ fixation, as described above, the entire lateral rectus muscle was fixed for 2 hr in Karnovsky's solution and washed in phosphate buffer. It was then postfixed in a 1:1 mixture of 1% OsO4/Ruthenium red solution. After postfixing and rinsing, the muscle was cut transversely into pieces and then processed for embedding as described above. The muscle fibers had to be intact during the period of exposure to Ruthenium red or artifactual leakage of Ruthenium red into the muscle fibers occurred.

Results

Control Lateral Rectus Muscle

After retrobulbar injections of physiological saline, the muscles appeared normal (Fig. 1). Ruthenium red
Fig. 4. Transverse section of the global layer 15 min after mepivacaine injection. Myofibers show an accumulation of Ruthenium red in the SR-cisternae (arrows). C = capillaries (×5000).

Fig. 5. Ruthenium red-stained tissue, 30 min after mepivacaine injection. Phagocytes (P) begin to surround flocculent material (*) in the intercellular space, and parts of the contractile material in the myofibers (M) are damaged (×3000).

did not enter the muscle fibers. Only if the needle directly penetrated a muscle did transient minor edema occur.

Degeneration and Regeneration Pattern

The vascular bed, nerve endings and satellite cells appeared normal at all time periods. The vast majority of muscle fibers were affected by the anesthetic. Counts are impractical in an electron microscopic study, but histological preparations from our former study showed the complete degeneration and regeneration of from 95–100% of the muscle fibers in the rectus muscles.

15 min Lesions: The affected myofibers had a ruptured sarcolemma, as well as ruptured and swollen T-tubules and sarcoplasmic reticulum. The extracellularly applied stain, Ruthenium red, penetrated into the junctional parts of the sarcoplasmic reticulum (Fig. 2). Ruthenium red tended to accumulate on the outer mitochondrial membrane, but it did not penetrate into the interior. At this time, the nuclei remained morphologically unaffected (Fig. 2). Most of the myofibers, in both the global and orbital layers, had a normal sarcomere length (2.3 μm). Neighboring myofibrils typically drifted apart and were not always in register. The resulting space between the myofibrils appeared empty (Fig. 2). Some of the thinnest myofibers in the orbital layer lacked contractile material and were empty except for mitochondria and nuclei (Fig. 3). At low magnification the thickest myofibers in the global layer appeared normal, but even in these, the presence of Ruthenium red in cisternae of the sarcoplasmic reticulum attested to damage of the sarcolemma and membranes of the sarcoplasmic reticulum (Fig. 4).

30 min Lesions: The number of mononucleated cells in the affected muscles increased, and unidentified flocculent material was abundant in the intercellular space. Phagocytes had already begun to surround the flocculent material (Fig. 5). Many of the thinnest myofibers had no contractile material in cross-section and the completely empty myofibers were outlined by their persistent basal laminae (Fig. 6). Accumulations of Ruthenium red were obvious in the junctional cisternae of the sarcoplasmic reticulum (Fig. 7). Outer mitochondrial membranes were still continuous. Among the muscle fibers in which complete cytoplasmic dissolution had not occurred, the

Fig. 6. Thirty minutes after mepivacaine injection, an empty basal lamina (arrowheads) outlines the remains of a muscle fiber. An intact satellite cell (S) persists within the basal lamina (×7800).

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distinct banding pattern of sarcomeres began to fade away, leaving the Z-band hardly visible.

2 hr Lesions: Damage to the sarcolemma progressed to the point where myofibers had only rudiments of the sarcolemma, leaving the continuous basal lamina as the only border to the intercellular space. Many myofibers appeared partially empty, containing pyknotic nuclei, mitochondria and scattered bundles of myofilaments (Fig. 8). The outer mitochondrial membranes were permeable to Ruthenium red.

6 hr Lesions: Supercontraction and the accompanying tearing of neighboring sarcomeres was frequently seen in muscle fibers that still retained the basic organization of the sarcomeres (Fig. 9). Phagocytes were more abundant in the injected areas, but none were seen yet inside the basal laminae of the muscle fibers. Activation of some satellite cells had begun, as evidenced by a general enlargement and a paler than normal staining reaction (Fig. 10). Rough endoplasmic reticulum was visible in these cells. Other satellite cells retained an inactive appearance. There was no obvious correlation between the state of satellite cell activation and the degree of degeneration of the associated muscle fiber. Dormant-appearing satellite cells could be seen within basal laminae of totally destroyed myofibers or, as in Figure 10, active satellite cells could be associated with muscle fibers showing minimal signs of damage. At low magnification some of the largest myofibers still appeared normal, but at higher magnification one could detect disruption of the sarcolemma and some of the nuclear membranes.

12 and 24 hr Lesions: For the first time, phagocytes were seen within the damaged myofibers. A spectrum of stages of degenerating myofibers could still be seen. This ranged from completely empty basal laminae to thicker muscle fibers, which showed obvious signs of damage but contained most cytoplasmic constituents. The breaking up of sarcomeres at the Z-bands and anomalies of the mitochondrial cristae were common (Fig. 11). Even the seemingly less damaged muscle fibers were readily distinguished in both semithin and ultrathin sections by a pale staining reaction. Very few nuclei remained in the necrotic myofibers.
Fig. 10. Six hours after mepivacaine injection myofibers show a range of degeneration from severe (M1) to mild (M2). An activated satellite cell (S) is associated with M2. C = capillary (x3600).

2 day Lesions: For the majority of muscle fibers, destruction of the remaining cytoplasm was virtually complete. Although satellite cells were spread out along the inside of the old basal laminae, most of their growth occurred by thickening. Regenerating muscle fibers were surrounded by a convoluted basal lamina (Fig. 12), and areas of doubling and tripling of the basal lamina material were seen. The original basal laminae of small muscle fibers were filled with one or two myoblasts in transverse section (Fig. 12), whereas larger muscle fibers were replaced by a ring of myoblastic cells with an empty or phagocyte-filled center. Severely damaged, but surviving muscle fibers were still encountered.

3 day Lesions: The regenerating muscle was still in a principally myoblastic phase. Phagocytes were still common, and they were often found inside the old basal laminae along with myoblasts (Figs. 13, 14). Although many of the regenerating muscle cells contained small bundles of myofilaments, the membrane systems of these cells were disorganized and rudimentary, and no couplings between T-tubules and sarcoplasmic reticulum were observed. Nerve endings formed synaptic contacts with regenerating muscle fibers.

4 day Lesions: The fusion of myoblasts into myotubes and most sarcomerogenesis took place between the third and fourth day. Although their morphology was irregular, the myofibrils, as well as the membrane systems, had taken shape by day 4 (Fig. 15). The sarcomeres possessed a full set of bands. The myotubes contained only a few myofibrils, and the myofibrils were not always aligned. The interior couplings sometimes strayed so that they were found at other band levels than the normal A-I junction, and at times double sets of couplings were found side by side. In one muscle, exposed to Ruthenium red, the Ruthenium red had accumulated in the T-tubules and not in the junctional parts of the sarcoplasmic reticulum. Within the myotubes, nucleoli, containing activated nucleoli, formed central chains.

7 day Lesions: Myotubes were fully formed, but most retained the central nucleus (Fig. 16). Occasional myoblasts were still found. Phagocytes were still abundant in the interstitial spaces.

14 and 30 day Lesions: The regenerating muscle fibers were mature in appearance, but some central nuclei persisted. The others had moved to a peripheral position (Fig. 17).
Damage to Surviving Muscle Fibers

Although the great majority of muscle fibers exposed to mepivacaine underwent a sequence of rapid degeneration and regeneration, others remained histologically intact. However, ultrastructural analysis revealed that many of these fibers sustained damage as well.

Commonly encountered signs of late-appearing damage to muscle fibers were the following: central nucleation (Fig. 18), swelling of the sarcoplasmic reticulum, swelling and destruction of the interior of mitochondria (Fig. 18), and the occasional appearance of rough endoplasmic reticulum. The convolution and/or doubling of the basal lamina was frequent (Figs. 18, 19). At relatively late intervals after a single injection of anesthetic, surviving, but damaged, muscle fibers were invaded by phagocytic cells. Even at 2 to 4 weeks after injection surviving myofibers showed persisting evidence of damage, and early stages of muscle regeneration could be seen within the persisting basal laminae of muscle fibers (Fig. 20).

Discussion

The study confirms the earlier histological findings of Carlson and Rainin that a single retrobulbar injection of a local anesthetic, in this case 2.0% mepivacaine, causes the degeneration of the vast majority of the muscle fibers of the lateral rectus muscle in the rat. Of the muscle fibers that don't break down com-
Fig. 17. Fourteen-day regenerating muscle fibers. In some, the nuclei (arrows) have migrated to the periphery, whereas in others, the nuclei (N) remain central. C = capillary (X3600).

Fig. 18. Four days after injection. Transverse section of a surviving myofiber with a central nucleus (N), mitochondrial damage (arrows), and a double basal lamina (arrowheads) (X6400).

Fig. 19. Transverse section of two surviving myofibers 7 days after injection, showing drug-related abnormalities of mitochondrial cristae (arrows), double basal laminae (arrowheads), and a central nucleus (N) (X12,000).

Fig. 20. Thirty days after injection. The highly convoluted basal lamina (arrowheads) of a regenerating fiber contains one single myoblast (M). C = capillary (X12,000).

From the purely ultrastructural standpoint, our data point to early membrane damage as the earliest site of damage to muscle fibers exposed to mepivacaine. On the basis of both ultrastructure and the penetration of Ruthenium red into various components of the muscle fibers, we propose that mepivacaine disrupts the sarcolemma (and T-tubules) and membranes of the sarcoplasmic reticulum as early as a few minutes after exposure to the anesthetic, and causes damage to the nuclear and mitochondrial membranes a few hours later. Wakayama and Shibuya examined extensor digitorum longus muscle fibers of the rat 15 min after an injection of 0.5% bupivacaine by freeze-fracture, as well as conven-
tional electron microscopy, and found decreases in the density of caveolae and non-array particles. After exposing rat lumbrical muscles to bupivacaine for short periods (5 to 180 min) in vitro, Hall-Craggs reported both membrane damage and supercontraction within the myofibrils, as was seen in the current study.

Other authors, using biochemical methods, have noted early disturbances in the intracellular distribution of Ca++.Opinions among these authors vary concerning the relative importance of extracellular vs. intracellular Ca++ as the source of the increased Ca++ to which the contractile elements are exposed. The morphological findings in this study are compatible with either or both intra- and extracellular sources of Ca++.

Karpati and Carpenter reported rapid increases in local Ca++ following the micro-puncture of muscle fibers with a needle. Regardless of the source, the high local concentrations of Ca++ around the myofibrils could explain the early supercontraction seen in several studies (refs. 26, 28, this study). It has been suggested that Ca++-activated proteases or cathepsins may account for early removal of the Z-bands (and loss of a-actinin) but intracellular enzyme activity may not suffice to account for the removal of all of the contractile proteins. Whatever the mechanisms of early damage, the affected muscle fibers are soon attacked by large numbers of phagocytic cells, which remove essentially all remnants of the original muscle fibers.

Despite the devastating effects of local anesthetics on muscle fibers, their associated satellite cells remain relatively unscathed, despite being exposed to the same local concentrations of the anesthetics. These cells proliferate and serve as the source of the newly regenerating muscle fibers. Parallel results were obtained in vitro, where local anesthetics disrupted myotubes, but not myoblasts. In addition to satellite cells, motor nerve terminals are spared from the major destructive effects of local anesthetics. This results in the early restoration of neuromuscular continuity during the regenerative process.

Once the regeneration of new muscle fibers began, the ultrastructure of the process did not differ to any significant degree from the regeneration of somatic muscle fibers already reported in rats. Because there was no obvious ischemic necrosis in the lateral rectus muscle, the process of regeneration was synchronous among the early degenerating muscle fibers.

The evidence of damage to the surviving muscle fibers (mainly to mitochondria and the sarcoplasmic reticulum) and the late invasion of some of these muscle fibers by macrophages has not been previously reported. Although quantitatively a small part of the overall picture of breakdown and regeneration, this finding suggests a chronic effect of the anesthetic, as well. Whether this effect is primary or secondary could not be determined from this descriptive study.

Although there is no question that a single retrobulbar dose (50 μl) of commonly used local anesthetics (bupivacaine, lidocaine and mepivacaine) produces a massive toxic effect on extraocular muscle fibers in the rat (ref. 2, this study), a recent report by Porter et al concludes that a single retrobulbar dose of bupivacaine or lidocaine causes only relatively minor effects on the fibers of monkey extraocular muscles. The lesions produced in the latter study were most commonly seen in singly innervated muscle fibers with a low mitochondrial content. Affected muscle fibers were concentrated in the global layer of the muscle. These authors postulated that mitochondrial-rich muscle fibers were spared by virtue of their ability to handle the increased concentrations of Ca++ during the early period following exposure to anesthetic. The difference in response to retrobulbar injections of local anesthetics between the rat and the monkey cannot be resolved on the basis of available published data, but recent work has shown a different pattern of damage between injected limb muscles in rats and thumb muscles in monkeys. In the monkey, the distribution of damaged muscle fibers is not so compact or as homogeneous as it is in the rat.

Clinical experience has shown that after standard retrobulbar injections of anesthetics most patients show no or only minor disturbances of extraocular muscle function, but when smaller doses are injected directly into the rectus muscles, the incidence of oculomotor disturbances is sharply increased. It remains to be determined whether extraocular muscle fibers will show more massive patterns of breakdown after a direct injection than after a retrobulbar injection, in which access of the anesthetic to the extraocular muscle fibers would be controlled by diffusion and the epimysial connective tissues of the muscles.

Key words: regeneration, extraocular muscle, mepivacaine, rat, ultrastructure

References

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