In Vitro-In Vivo Myotoxicity of Intramuscular Liposomal Formulations

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Purpose. The first objective was to study the *in vitro* myotoxicity of empty liposomes and to examine whether liposome size, charge and fluidity affect liposome myotoxicity. The second objective was to investigate the effect of liposomal encapsulation on the *in vitro* and *in vivo* myotoxicity of loxapine compared to the loxapine commercial preparation (Loxitane®).

Methods. The *in vitro* myotoxicity of empty liposomes and loxapine liposomes was evaluated by the cumulative efflux of the cytosolic enzyme creatine kinase (CK) from the isolated rat extensor digitorum longus (EDL) muscle over a 2 hour period. In the *in vivo* studies, the area under plasma CK curve over 12 hours was used to evaluate muscle damage.

Results. The *in vitro* myotoxicity for all empty liposomal formulations was not statistically different from negative controls (untreated control muscles and normal saline injected muscles). However, these empty liposomal formulations were significantly less myotoxic than the positive controls (muscles injected with phenytoin and muscle sliced in half). *In vitro-in vivo* studies showed that the liposomal encapsulation of loxapine resulted in significant ($P < 0.05$) reduction in myotoxicity (80% *in vitro* and 60% *in vivo*) compared to the commercially available formulation which contains propylene glycol (70% V/V) and polysorbate 80 (5% W/V) prepared at equal concentration.

Conclusions. Results indicate that empty liposomes do not induce myotoxicity. Furthermore, liposomal size, charge and fluidity do not affect myotoxicity. In addition, *in vitro* and *in vivo* studies have demonstrated that liposomal encapsulation of loxapine can reduce myotoxicity compared to a formulation containing organic cosolvents.

KEY WORDS: myotoxicity; muscle damage; intramuscular; liposomes; loxapine; and creatine kinase.

INTRODUCTION

A common problem associated with IM drug administration is skeletal muscle pain and/or damage at the injection site (1). Liposomes have been reported to reduce drug-induced myotoxicity (2). These lipid drug carriers are composed of naturally occurring substances in the body. They are thought to be compatible with muscle tissues and could reduce the interaction between the drug and the muscle fibers, thereby reducing the severity of muscle damage upon IM injection. In the utilization of liposomes to reduce muscle damage, certain variables may play a role in the residency time of liposomes at the injection site and their fate following IM injection. These factors include: size, charge and fluidity of liposomes. Large multilamellar liposomes (0.3–2 μm) reside at the injection site longer than small liposomes (0.15–0.7 μm) (3). Positively charged stearylamine-liposomes have been reported to exert cytolytic activity following intravenous administration in rabbits (4). This effect was attributed mainly to the interaction between the positively charged liposomes with the negatively charged erythrocyte membrane leading to cell damage. Fluidity of phospholipid membranes can be influenced by the presence of cholesterol. In the liquid state, cholesterol reduces the freedom of the acyl chains which causes phospholipid membrane condensation with a reduction in area, closer packing and decreased fluidity leading to more stable liposomes (5).

The first objective was to study the *in vitro* myotoxicity of empty liposomes with respect to size (large vs. small), charge (positive vs. negative) and fluidity (presence vs. absence of cholesterol). Neutral liposomes were excluded from this study because their size was constantly changing due to aggregation. This work represents in our views the first and only systematic and complete evaluation of liposomal factors and their impact on muscle damage following IM administration. The second objective was to investigate specifically whether liposomal formulations, owing to their ability to enhance solubility without the use of cosolvents, could be used to reduce the *in vitro* myotoxicity of poorly water soluble drugs. In plasma, increased levels of CK activity is considered a marker for skeletal muscle damage (6). Increased plasma CK levels have been reported to be well correlated with other indices used to monitor muscle damage (viz., hemolysis and histological evaluation) (7). Therefore, the third objective of this study was to use the rodent model to investigate the *in vivo* myotoxicity of loxapine liposomes following IM administration. We believe the use of the rodent model to study *in vivo* myotoxicity contributes to the novelty of this work. Traditionally, the use of other larger animal models (viz., rabbit and dog) has been described. However, the rodent model proves to be superior to other animal models due to a variety of different reasons. In rabbits, pronounced fluctuations in CK activity had been reported because of the escape reaction before and during treatment (8). In addition, Olling and co-workers (9) reported that repetitive blood sampling from the ear vein leads to increased levels of CK which might interfere with CK level following IM injection. In dogs, Aktas and co-workers (10) reported numerous false negatives and false positives in plasma CK levels following IM injection. Our *in vitro-in vivo* results provide an insight on how liposomal formulations can be utilized, without the use of cosolvents, to reduce muscle damage upon IM injection of very poorly water soluble drugs (e.g., loxapine) which often incorporate organic cosolvents (e.g., propylene glycol and ethanol).

MATERIALS AND METHODS

Materials

Egg-phosphatidylcholine (PC) and egg-phosphatidylglycerol (PG) were purchased from Avanti Polar Lipids (Alabaster, AL.). Stearylamine (SA), cholesterol (CH) and Sephadex G-50 (medium) were purchased from Sigma Chemical Company (St. Louis, MO.). Loxapine base was a gift from American Cyanamid Company. Phospholipids used were of highest purity (>99%) and all other substances used were of analytical grade.

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Preparation of Empty Liposomes

Positively charged liposomes were composed of PC:SA (9:1 M) or PC:SA:CH (7:1:2 M), while negatively charged liposomes were composed of PC:PG (7:3 M) or PC:PG:CH (4:3:3 M). Liposomes were prepared at a lipid concentration of 25 mg/mL by the thin film hydration method. Using a high pressure extrusion device (Lipex Biomembranes, Inc., Vancouver, BC), extrusion through two polycarbonate membranes of 2 μm pore size and two membranes of 0.4 μm with two membranes of 0.2 μm pore size was used to produce four large (L) liposomal preparations (1.5–2.0 μm) (viz., LPCSA, LPCPG, LPCSACH and LPCPGCH) and four small (S) liposomal preparations (0.2–0.5 μm) (viz., SPCSA, SPCPG, SPCSACH, and SPCPGCH), respectively. Liposome particle size was analyzed using laser light scattering (Nicomp Instruments, Inc., Menlo Park, CA.).

Preparation of Loxapine Liposomes

Loxapine was incorporated in PC:PG (7:3 M) at a drug to lipid molar ratio of 1:2 and lipid concentration of 100 mg/mL. Dried lipids and loxapine were dissolved in tertiary butanol. Tertiary butanol was dried using rotary evaporation at 40°C (*in vitro* studies) or freeze drying at −40°C (*in vivo* studies) to form a thin film on the vessel wall. The dry lipids were dispersed in isotonic phosphate buffer (0.1 M, pH 6) and shaken for 2 hours at room temperature under nitrogen. The resulting liposomes were freeze-thawed 5 times to obtain equilibrium transmembrane solute distribution. The freeze thawed liposomes were extruded 5 times to produce a homogeneous population of liposomes with an average diameter of 1.1 μm. To separate free drug from encapsulated drug, liposomes were passed down a Sephadex G-50 minicolumn preequilibrated with isotonic phosphate buffer (0.1 M, pH 7.4) (11).

Measurement of Encapsulation Efficiency

The HPLC assay to determine the entrapped amount of loxapine in liposomes was a modification of a method of Cheung (12). To lyse loxapine liposomes, an aliquot (10 μl) was diluted 100 times with 80% (V/V) methanol solution. After centrifugation, supernatant (50 μl) was injected directly onto the column. The encapsulation efficiency was determined as the ratio of liposomal drug load to the initial drug load and calculated to be 48–57%.

In Vitro Myotoxicity Studies

Male Sprague-Dawley rats (350–500 g, Charles River, Wilmington, MA.) were sacrificed via cervical dislocation following the administration of an anesthetic cocktail. All protocols were approved by the Animal Care and Use Committee at the University of Florida in accordance with NIH guidelines. The EDL muscles were isolated, removed, injected with test formulation (15 μl) and placed into 9 mL of a balanced salt solution as previously described (13). After injection, the balanced salt solution was drained from the incubation vessel at 30-minute intervals followed by the addition of fresh media. CK activity in the drained solutions was measured spectrophotometrically (λmax 340 nm) using a commercially available kit (Sigma Chemical Company, St. Louis, Mo.). The assay was run at 30°C and validated using a standard (Accutrol Normal). Myotoxicity was assessed by the cumulative release of CK into the incubation medium over a 2 hour period.

In Vivo Myotoxicity Studies

Male Sprague-Dawley rats (250–450 g, Charles River, Wilmington, MA.) were employed for this study. The carotid artery was catheterized with propylene tubing (PE50, Clay Adams, Parsippany, NJ.) under Metofane® anesthesia. The catheter was exteriorized between the scapulae and exposed areas closed using surgical suture (Ethicon, Inc. Somerville, NJ). The rats were allowed to recover for 24 hours before the study to make sure the CK levels reached pre-catheterization baseline levels as originally described by Brazeau (14). Following IM injection (0.3 mL) in the right thigh muscle (musculus rectus), blood samples (0.5 mL) were collected via the carotid artery catheter at 0, 0.5, 1, 2, 4, 8 and 12 hours. Heparinized (100 U/mL) normal saline solution (0.05 mL) was used to fill the catheter just after each blood sample to prevent blood clotting. The blood samples were centrifuged immediately and plasma was stored in the freezer (−20°C) for analysis of CK, while blood cells were reconstituted in heparinized (40 U/mL) normal saline solution (0.25 mL) and vortex mixed to be reinjected into the rat following the next sample to maintain blood volume. Myotoxicity was assessed by the area under the plasma CK curve over a 12 hour period calculated by the linear trapezoidal rule.

Data Analysis

Data are presented as the mean and standard error of the mean with n = 5–8 muscles per treatment for *in vitro* studies or n = 4–5 for *in vivo* studies. Statistical analysis of cumulative CK activity or area under plasma CK curve among the different treatments was performed using one-way ANOVA followed by Tukey's test ($p < 0.05$).

RESULTS AND DISCUSSION

In Vitro Myotoxicity of Empty Liposomes

The cumulative CK release for four large empty liposomal preparations along with two positive controls (muscles injected with phenytoin and muscles sliced in half) and two negative controls (untreated control muscles and muscles injected with normal saline) is shown in Figure 1, while the cumulative CK release for four small empty liposomal preparations along with two positive controls and two negative controls is shown in Figure 2. The injection of phenytoin (Dilantin®) was approximately 45-times more myotoxic than untreated control muscles. This difference in myotoxicity between untreated control muscles versus phenytoin was similar in magnitude to that reported in a previous study (13). Muscles injected with normal saline were used as negative controls to rule out muscle damage caused by needle puncture. The eight empty liposomal formulations prepared at different size, charge and fluidity (viz., LPCSA, LPCPG, LPCSACH, LPCPGCH, SPCSA, SPCPG, SPCSACH, and SPCPGCH) were found to be slightly more myotoxic than untreated control muscles (Figure 1 and Figure 2). Furthermore, the myotoxicity of these empty liposomes were approximately equal to muscles injected with normal saline, which is known

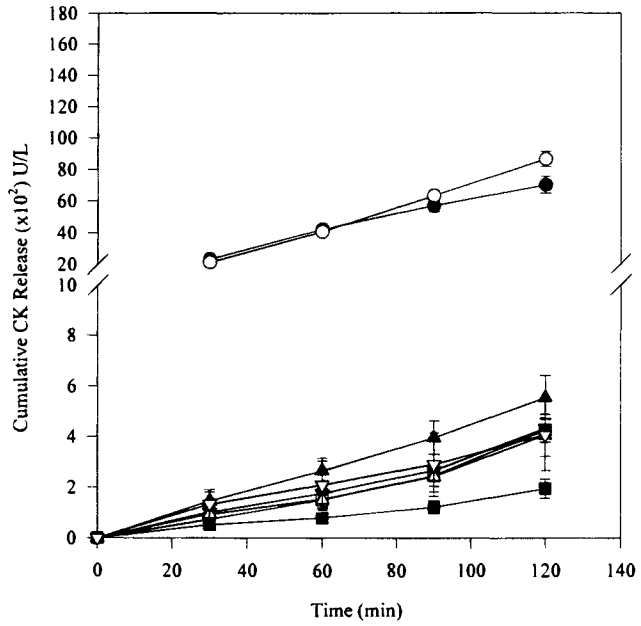

Fig. 1. Cumulative creatine kinase release versus time for muscles injected with phenytoin, 50mg/mL (○), sliced muscles (●), muscles injected with normal saline (□), untreated controls (■), LPCSA (△), LPCPG (▲), LPCSACH (▽), and LPCPGCH (▼). Each point represents the mean and standard error of the mean (n = 5–8).

to be non-myotoxic as measured by enzyme release and histological examination (13). However, these liposomal formulations were significantly less myotoxic than muscles injected with phenytoin or muscles sliced in half. Furthermore, there was no significant difference in myotoxicity between the liposomal preparations which indicates that liposomal size, charge and fluidity do not impact on the extent of muscle damage i.e., all these empty liposomal formulations are tissue compatible.

In Vitro Myotoxicity of Loxapine Liposomes

The ability of liposomes to protect against loxapine-induced muscle damage was investigated using loxapine incorporated into negatively charged liposomes PC:PG (7:3 M). Loxapine was selected because of its limited water solubility. The commercial parenteral formulation of loxapine (Loxitane®) contains loxapine hydrochloride (equivalent to 50 mg/mL loxapine base) dissolved in 70% V/V propylene glycol and 5% W/V polysorbate 80. In previous studies, propylene glycol was found to be extremely myotoxic at this concentration (13).

To determine whether the commercial preparation of loxapine is myotoxic and whether this myotoxicity can be attributed to the solvent system and/or loxapine, the myotoxic potential of the following formulations were investigated: 1) loxapine commercial formulation (Loxitane®-50 mg/mL) and 2) loxapine solvent system (70% V/V propylene glycol and 5% W/V polysorbate 80) (Figure 3). The commercial formulation of loxapine was shown to be 1.7-times more myotoxic than phenytoin and 35-times more myotoxic than muscles injected with normal saline. Furthermore, the loxapine solvent system was found to be as myotoxic as phenytoin and 20-times more myotoxic than muscles injected with normal saline (Figure 3). These results indicate that the commercial formulation of loxapine is indeed myotoxic, with this myotoxicity attributed to both the solvent system and the drug.

To study the effect of liposomes on loxapine myotoxicity, two formulations were studied: 1) loxapine liposomes (12.2 mg/mL) and 2) loxapine formulation at an equal concentration

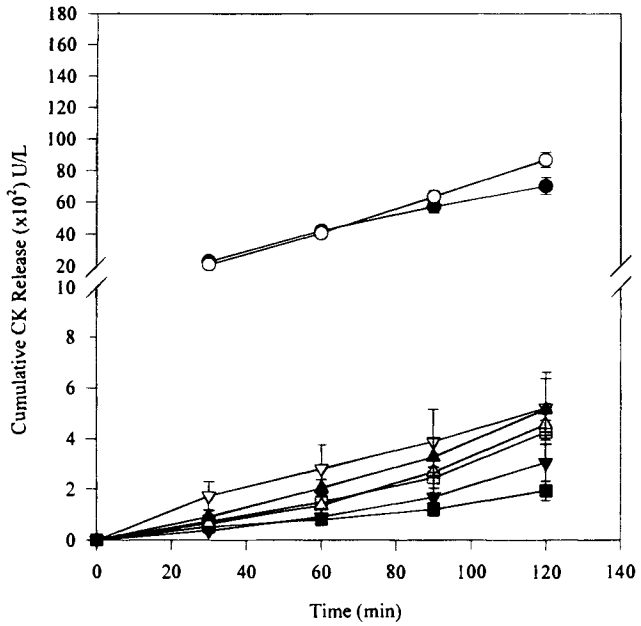

Fig. 2. Cumulative creatine kinase release versus time for muscles injected with phenytoin, 50mg/mL (○), sliced muscles (●), muscles injected with normal saline (□), untreated controls (■), SPCSA (△), SPCPG (▲), SPCSACH (▽), and SPCPGCH (▼). Each point represents the mean and standard error of the mean (n = 5–8).

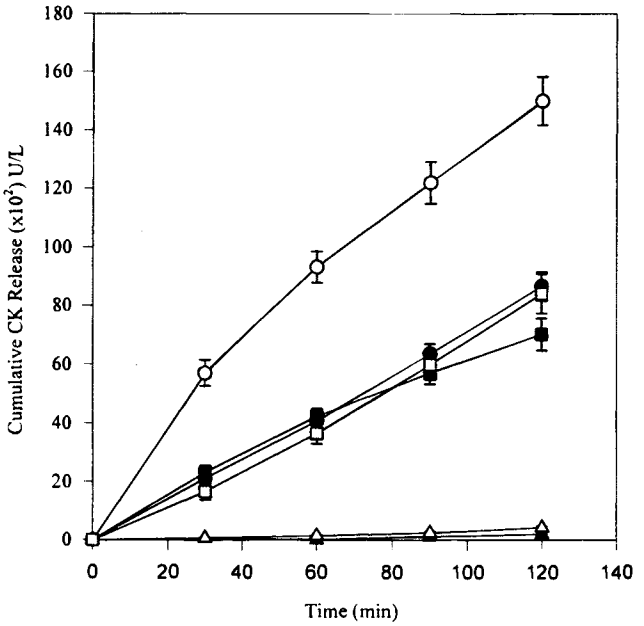

Fig. 3. Cumulative creatine kinase release versus time for muscles injected with loxapine, 50mg/mL (○), phenytoin, 50mg/mL (●), loxapine solvent system (□), sliced muscles (■), muscles injected with normal saline (△), and untreated controls (▲), Each point represents the mean and standard error of the mean (n = 5–8).

as the loxapine liposomes (12.2 mg/mL) and containing the organic cosolvents. Loxapine liposomes were found to significantly reduce muscle damage (80%) when compared to loxapine formulation prepared at equal concentration (12.2 mg/mL) (Figure 4). In addition, loxapine liposomes were shown to be 3-times less myotoxic than muscles injected with phenytoin and 6.5-times more myotoxic than muscles injected with normal saline. These results indicate that a liposomal formulation of loxapine can indeed reduce the severity of muscle damage following IM injection. This can be explained by the fact that the solvent system, which is thought to be primarily responsible for muscle damage, was excluded from the liposomal formulation. In addition, liposomes have been reported to deliver drugs in a sustained release manner (15). Loxapine will, therefore, be released from the liposomes over a period of time which is expected to decrease initial drug-muscle tissue interaction and consequently reduce muscle damage. On the other hand, the fact that these loxapine liposomes were found to be more myotoxic than normal saline controls can be explained by the fact that loxapine itself is myotoxic and should be expected to cause muscle damage to some extent. This effect was seen to be concentration dependent. i.e., a loxapine preparation (50 mg/mL) was found to be more myotoxic than a loxapine preparation at a concentration of 12.2 mg/mL (Figure 4). Results of the *in vitro* myotoxicity of loxapine liposomes is in close agreement with a previous work published by Kadir and coworkers (2) who reported that histological examinations had shown that the IM injection of a negatively charged "gel state" liposomal formulation did not cause any signs of an inflammatory reaction at the injection site.

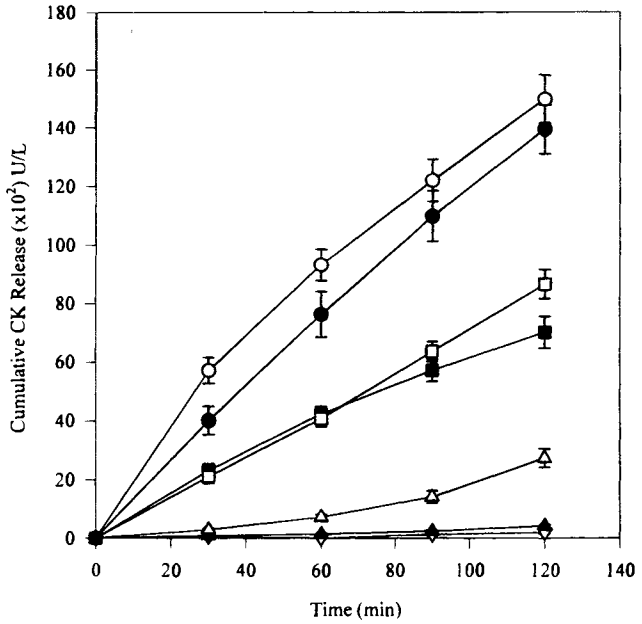

Fig. 4. Cumulative creatine kinase release versus time for muscles injected with loxapine, 50mg/mL (○), loxapine, 12.2mg/mL (●), phenytoin, 50mg/mL (□), sliced muscles (■), loxapine liposomes, 12.2mg/mL (△), muscles injected with normal saline (▲), and untreated controls (▽), Each point represents the mean and standard error of the mean (n = 5–8).

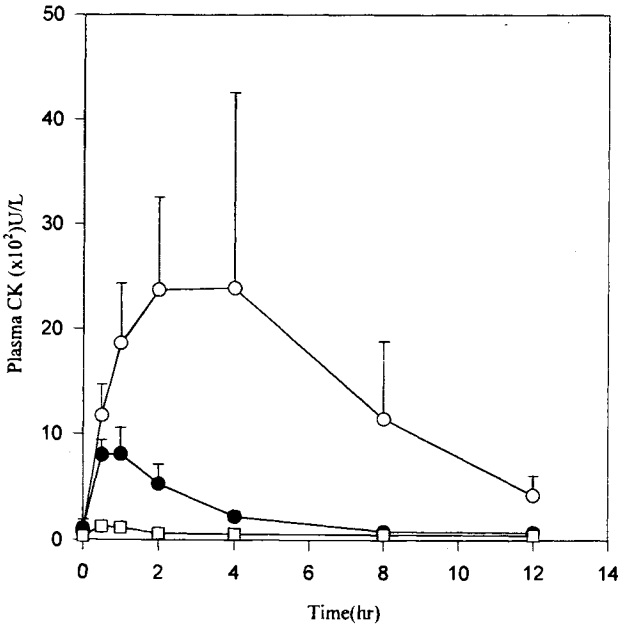

Fig. 5. Plasma creatine kinase versus time for muscles injected with loxapine, 50 mg/mL (○), phenytoin, 50mg/mL (●), muscles injected with normal saline (□), Each point represents the mean and standard error of the mean (n = 4–5).

In Vivo Myotoxicity of Loxapine Liposomes

Similar to the *in vitro* studies, a positive control (animals injected with phenytoin) and a negative control (animals injected with normal saline) were included in the study (Figure 5 and Figure 6). The area under plasma CK for 12 hours (U-

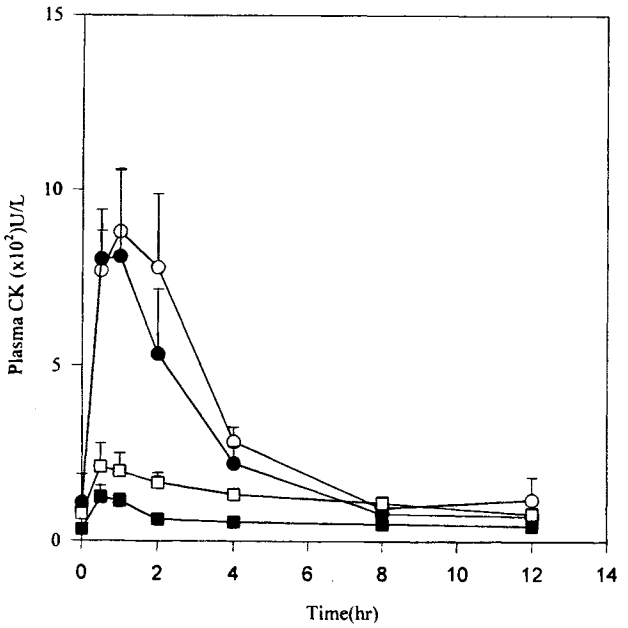

Fig. 6. Plasma creatine kinase versus time for muscles injected with loxapine, 10.2mg/mL (○), phenytoin, 50mg/mL (●), loxapine liposomes, 10.2mg/ml (□), and muscles injected with normal saline (■), Each point represents the mean and standard error of the mean (n = 4–5).

h/L, Mean ± SEM) was used to assess muscle damage. The injection of phenytoin (Dilantin®) was approximately 4-times more myotoxic than an injection of normal saline ($29.7 \times 10^2 \pm 7.6 \times 10^2$ vs. $7.12 \times 10^2 \pm 1.21 \times 10^2$). Peak plasma CK were found to occur at 0.5–2 hours following injection which is in close agreement with a previously published report (14). The myotoxicity of the commercial formulation of loxapine (Loxitane®-50 mg/mL) was studied by comparing plasma CK to the negative control or the positive control (Figure 5). Results indicate that the commercial formulation of loxapine is 6-times more myotoxic than phenytoin ($182 \times 10^2 \pm 104 \times 10^2$ vs. $29.7 \times 10^2 \pm 7.6 \times 10^2$) and 26-times more myotoxic than normal saline injected animals ($182 \times 10^2 \pm 104 \times 10^2$ vs. $7.12 \times 10^2 \pm 1.21 \times 10^2$).

To study the effect of liposomes on the *in vivo* myotoxicity of loxapine, two formulations were studied: 1) loxapine liposomes (10.2 mg/mL) and 2) loxapine formulation prepared at an equal concentration as the loxapine liposomes (10.2 mg/mL) and containing the organic cosolvents. Loxapine liposomes were found to significantly reduce muscle damage (60%) when compared to a loxapine formulation ($15.3 \times 10^2 \pm 1.01 \times 10^2$ vs. $37.2 \times 10^2 \pm 5.38 \times 10^2$) (Figure 6). In addition, loxapine liposomes were shown to be 1.9-times less myotoxic than muscles injected with phenytoin ($15.3 \times 10^2 \pm 1.01 \times 10^2$ vs. $29.7 \times 10^2 \pm 7.6 \times 10^2$) and 2-times more myotoxic than muscles injected with normal saline ($15.3 \times 10^2 \pm 1.01 \times 10^2$ vs. $7.12 \times 10^2 \times 1.21 \times 10^2$) which can be explained by the fact that loxapine itself induces myotoxicity.

CONCLUSIONS

The *in vitro* myotoxicity studies of the empty liposomes with different size, charge and fluidity indicate that liposomes do not cause muscle damage and are safe to inject intramuscularly. In addition, liposomal size, charge and fluidity do not impact on the extent of muscle damage. The *in vitro* myotoxicity studies of loxapine indicate that the commercial formulation of loxapine (Loxitane®-50 mg/mL) is indeed myotoxic and this myotoxicity can be attributed to both the solvent system and loxapine. An important finding in the *in vitro* studies is that loxapine liposomes were found to significantly reduce muscle damage (80%) when compared to a loxapine formulation containing an organic cosolvent system. Results of the *in vivo* myotoxicity studies were in close agreement with the *in vitro* studies. The *in vivo* studies in rats indicate that the commercial formulation of loxapine is myotoxic. In addition, loxapine liposomes were found to significantly reduce muscle damage (60%) when compared to loxapine formulation containing organic cosolvents. Two mechanisms were proposed to explain the ability of loxapine liposomes to reduce muscle damage and these are: 1) the solvent system (70% V/V propylene glycol and 5% W/V polysorbate 80) which is thought to be primarily responsible for muscle damage was excluded from the liposomal formulation, 2) liposomes have been reported to deliver drugs in a sustained release manner, therefore following IM injection of loxapine liposomes, loxapine will be released from the liposomes over a period of time which is expected to cause a gradual exposure of the muscle tissue to the drug and consequently reduce muscle damage.

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REFERENCES

1. O. Svendson. Local muscle damage and oily vehicles: A study on local reaction in rabbits after intramuscular injection of neuroleptic drugs in aqueous or oily vehicles. *Acta. Pharm. et. Toxicol.* **52**:298–304 (1983).
2. F. Kadir, W. M. C. Eling, D. Abrahams, J. Zuidema, and J. A. Crommelin. Tissue reaction after intramuscular injection of liposomes in mice. *Int. J. Clin. Pharmacol. Ther. Toxic.* **30**:374–382 (1992).
3. J. A. Jackson. Intramuscular absorption and regional lymph uptake of liposome-entrapped inulin. *Drug Metab. Dispos.* **9**:535–540 (1981).
4. E. Yoshida, and T. Nakae, Cytolytic activity of liposomes containing stearylamine. *Biochem. Biophys. Acta.* **854**:93–101 (1986).
5. E. Arakawa, Y. Imai, H. Kobayashi, K. Okumura, and H. Sezaki. Application of drug-containing liposomes to the duration of the intramuscular absorption of water-soluble drugs in rats. *Chem. Pharm. Bull.* **23**:2218–2222 (1975).
6. G. Chellman, L. Lollini, A. Dorr, and L. DePass. Comparison of ketorolac tromethamine with other injectable nonsteroidal anti-inflammatory drugs for pain-on-injection and muscle damage in the rat. *Hum. Exp. Toxicol.* **13**:111–117 (1994).
7. C. Surber, and H. Sucker. Tissue Tolerance of intramuscular injectables and plasma enzyme activities in rats. *Pharm. Res.* **4**:490–494 (1987).
8. C. Surber and U. Dubach, Tests for local toxicity of intramuscular drug preparation. Comparison of *in vivo* and *in vitro* findings. *Arzneim.-Forsch./Drug Res.* **39**:1586–1589 (1989).
9. M. Olling, K. VanTwillert, P. Wester, A. B. T. Boink, and A. G. Rauws, Rabbit model for estimating relative bioavailability, residues and tissue tolerance of intramuscular products: comparison of two ampicillin products. *J. Vet. Pharmacol. Ther.* **18**:34–37 (1995).
10. M. Aktas, D. Augusta, D. Concordet, P. Vinclair, H. Letebvre, P. L. Toutain, J. B. Braun. Creatine kinase in dog plasma: preanalytical factors of variation, reference values and diagnostic significance. *Res. Vet. Sci.* **56**:30–36 (1994).
11. D. W. Fry, J. C. White, and I. D. Goldman. Rapid seperation of low molecular solutes from liposomes without dilution. *Anal. Biochem.* **90**:809–815 (1978).
12. S. Cheung, S. Tang, and G. Remington. Simultaneous quantitation of loxapine, amoxapine and their 7- and 8-hydroxy metabolites in plasma by high-performance liquid chromatography. *J. Chromatogr.* **564**:213–221 (1991).
13. G. A. Brazeau, and H-L. Fung. An *in vitro* model to evaluate muscle damage following intramuscular injection. *Pharm. Res.* **6**:167–179 (1989).
14. G. A. Brazeau and C. A. White. Use of an *in vivo* model to evaluate muscle damage from intramuscular (IM) injections. *Pharm. Res.* **8**:S168 (1992).
15. H. A. C. Titulaer, W. M. C. Eling, D. J. A Crommelin., P. A. M. Peeterst., and J. Zuidema. The parenteral controlled release of chloroquine in mice. *J. Pharm. Pharmacol.* **42**:529–532 (1990).