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Report

An *In Vitro* Model to Evaluate Muscle Damage Following Intramuscular Injections

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An isolated rat muscle preparation was developed to screen for muscle damage (myotoxic potential) following intramuscular injections. Myotoxicity is evaluated by the total cumulative efflux of the enzyme creatine kinase from the extensor digitorum longus muscle into the incubation medium over a 2-hr period or by the slope of the cumulative creatine kinase efflux curve. The system allows for rapid screening of compounds and/or formulations regarding their myotoxic potential and is not sensitive to fluctuations of *in vivo* creatine kinase levels caused by animal handling or patient conditions. A good rank-order correlation was obtained between this *in vitro* technique and the *in vivo* myotoxicity of a number of pharmaceutical formulations, as indicated by circulating creatine kinase levels and histological observations.

KEY WORDS: intramuscular injection; muscle damage; *in vitro;* rat; extensor digitorum longus (EDL); creatine kinase.

INTRODUCTION

The intramuscular route of drug administration is frequently employed in drug therapy, particularly for drugs which require prompt action but which, for a variety of reasons, are not suitable for intravenous or oral administration (1–3). A common sequela with this route of administration is patient discomfort and skeletal muscle damage. Many classes of therapeutic agents, including anesthetics, antibiotics, antiarrhythmics, barbiturates, benzodiazepines, and phenothiazines, have been shown to cause pain and muscle damage (myotoxicity) following intramuscular administration (4–12). This muscle damage can frequently be attributed to the therapeutic agent and/or the solvent system used in the formulation.

Both *in vivo* and *in vitro* methods have been used for the screening of agents and/or formulations for their potential to cause muscle damage, i.e., the myotoxic potential (13–20). As indices of myotoxicity, the *in vivo* methods have utilized largely increases in the circulating levels of creatine kinase (an intracellular sarcoplasmic enzyme) or histological evidence of skeletal muscle damage. These methods are tedious and labor intensive, with each study generally requiring between 1 and 7 days. In addition, the results obtained are highly variable because of the presence of substantial intrinsic fluctuations and differences in the baseline and agent-induced increases in creatine kinase levels. Animal handling and sampling artifacts often increase such variabilities, leading to difficulties in the interpretation of the data obtained (15,21–23). *In vitro* methods have utilized (as indices of myotoxicity), the cytotoxicity of MRC-5 fibroblasts as measured by histological examination (18) or creatine kinase release from a rat skeletal muscle cell line (L6) (19). These methods are deficient in that histological measurements are semiquantitative at best and there may be questions concerning the similarities between isolated cell cultures and whole muscle fibers. Hemolysis of erythrocytes has also been used as an *in vitro* method of screening parenterals for their myotoxicity (18,20). However, data interpretation from these methods might be hampered because the ratio of test solution to erythrocyte is extremely high (e.g., 50 to 1) (18) and the test solution may interfere with the determination of hemoglobin absorbance by shifting the hemoglobin visible spectrum (24). In addition, the degree of tissue damage (i.e., hemolysis) was assessed only by gross observation (20).

We report herein a rapid and simple *in vitro* screening method for the myotoxic potential of pharmaceutical products, utilizing a readily isolatable rat skeletal muscle. The efflux of creatine kinase from the muscle was used as an index of myotoxicity. We showed that this technique gave a good rank-order correlation with published *in vivo* literature data. This *in vitro* technique appeared useful in the rational development of intramuscular formulations for the minimization of skeletal muscle damage following injection.

MATERIALS AND METHODS

Test Solutions

Four commercially available solutions were obtained and tested for their myotoxic potential. These were lidocaine (xylocaine, Astra), Valium (diazepam, Hoffman–La Roche), Lanoxin (digoxin, Burroughs Wellcome), and dilantin (phenytoin, Parke-Davis). Since the latter three formulations

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shared the commonly used organic cosolvent system, *viz.*, 40% propylene glycol-10% ethanol in water, this cosolvent system was also tested for its myotoxic potential. Propylene glycol and ethanol were obtained from Fisher Scientific Company and Aaper Alcohol and Chemical Company, respectively. Dextrose injection (5%), sodium chloride injection (0.9%), and sterile water for irrigation were obtained from Travenol Laboratories. All other chemicals were at least reagent grade and obtained from J. T. Baker Chemical Company.

Muscle Extraction and Injection

Male Sprague–Dawley rats (Blue Spruce Farms, Altamont, N.Y.) weighing between 200 and 350 g were sacrificed by cervical dislocation following a stunning blow to the head. The skin and subcutaneous tissue were stripped from the left rear appendage and the leg was severed from the animal above the knee. The underlying extensor digitorum longus muscle was exposed by removal of the tibialis anterior muscle and isolated by cutting the tendons connecting it to the knee and the foot. The same procedure was then applied to the right leg. The total extraction time for the two muscles from each animal was between 10 and 15 min. The muscles were kept moist during the extraction procedure. Prior to injection, the muscles were placed into a weighing vial containing the incubation medium (described below). The wet weight and length of the muscles were recorded. The average wet weight for both the left and the right muscles was 181 ± 32 mg and the length ranged from 4 to 4.8 cm.

To facilitate the injection procedure, the muscles were pinned via the tendons to a dissection board. A specialized brass needle guard was attached onto the needle of a 25-μl Hamilton syringe (No. 702). This device assured a constant injection angle (approximately 30°) and depth (6 mm). Each test solution (15 μl) was injected into the muscle body along its lengthwise axis. Preliminary experiments had shown that this volume was sufficiently large to cause muscle damage without a direct and immediate leakage of the test solution out of the muscle. Following injection, the majority of muscles was observed to have a small welt. The injected muscle was placed into a cylindrical Teflon muscle basket (4.8 cm long and 1 cm in diameter) to maintain complete immersion in the incubation medium. In order to allow adequate oxygenation of the muscle and rapid equilibration of enzyme efflux into the incubation medium, the basket for the isolated muscle was constructed to contained 18 holes (4-mm diameter each) and the bottom was composed of an aluminum wire-mesh screen.

Experimental Procedure

Our method was similar, in part, to that of Dempsey and co-workers (25), who measured the *in vitro* efflux of creatine kinase and lactate dehydrogenase from the isolated mouse gastrocnemius muscle following *in vivo* pretreatment with subcutaneously injected diethylstilbestrol-diphosphate. In contrast, our method examined muscle damage in a completely *in vitro* system. In our study, the muscle basket containing the injected or treated muscle was placed in a 37°C incubation vessel containing 9 ml of a balanced salt solution (BSS; per liter, 6.8 g NaCl, 0.4 g KCl, 1.0 g dextrose, 2.2 g $NaHCO_3$, and 0.005 g sodium phenol red in sterile water for irrigation, with the pH adjusted to 7.4 with 1 *N* HCl). Carbogen (95% O_2–5% CO_2) was bubbled through the incubation medium for the duration of the experiment (4 hr). At 30-min intervals, the BSS was drained from the incubation vessel into a 10-ml vacutainer tube containing 143 U of lithium heparin (Becton Dickinson No. 6484), and 9 ml of fresh prewarmed BSS added to the vessel. The samples were analyzed for creatine kinase activity within 30 min.

Creatine Kinase Analysis

Creatine kinase activity was analyzed using CK reagent No. 47-UV (Sigma Chemical Company) via the reduction of NAD to NADH. All samples and the CK reagent were maintained at 37°C. A 20-μl aliquot of the sample was added to the CK reagent (1 ml) and mixed gently. The reaction mixture was placed into the 37°C bath and incubated for 2.5 min, gently transferred to the cuvette, and placed into the cuvette chamber maintained at 37°C. The difference in absorbance at 340 nm over a 2-min period was determined. Creatine kinase activity was multiplied by a factor of 0.58 to enable comparison with literature creatine kinase, which is frequently measured at 30°C. The assay was validated with standards available from the Sigma Chemical Company. Possible spectrophotometric and kinetic interferences by the test agents were tested in preliminary experiments. None of the solutions tested was found to interfere with the assay.

Data Analysis

The myotoxic potential was assessed either by the total cumulative efflux of creatine kinase into the incubation medium over 2 or 4 hr or by the rate of cumulative creatine kinase released, as determined by linear regression. Statistical analysis was performed using one-way analysis of variance followed by a Tukey's test for differences (if applicable). The results are expressed as the mean and SD for six muscles.

RESULTS AND DISCUSSION

The experimental system was first tested using untreated control muscles, muscles that were subjected to the injection procedure alone (no test solution), or muscles sliced in half perpendicular to the lengthwise axis (Fig. 1). The sliced muscle exhibited a linear release of creatine kinase into the incubation medium over the 4-hr study period. In both the control and the needle-puncture muscles, however, there was little cumulative creatine kinase efflux into the incubation medium up to 2 hr. From 2 to 4 hr, however, there was a marked increase in the cumulative creatine kinase efflux. This time-dependent change in the slope of the cumulative creatine kinase efflux curve may be due to a loss in viability (probably by unspecific degradative processes) of the isolated skeletal muscle preparation after the first 2 hr, resulting in the efflux of creatine kinase out of the muscle fiber sarcoplasma. From these results, it was decided to examine the cumulative creatine kinase release from 0 to 2 hr only.

Results obtained for several injectable solutions are shown in Figs. 2 and 3. For each injected solution, the mean

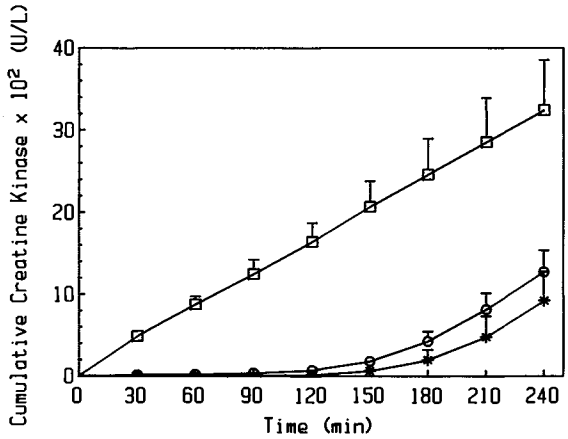

Fig. 1. Cumulative creatine kinase efflux from untreated control muscles (*), muscles punctured by the injection needle only (○), and muscles sliced in half (□).

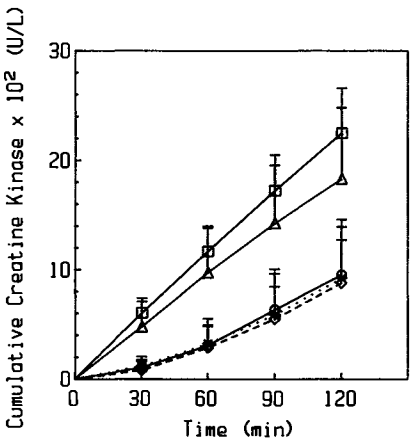

Fig. 3. Cumulative creatine kinase efflux following intramuscular injection with 40% (v/v) propylene glycol–10% (v/v) ethanol in water (◇), lidocaine (*), digoxin (○), phenytoin (△), and diazepam (□).

cumulative release of creatine kinase was linear with respect to time over the 2-hr period. Figure 2 shows the results from those treatments that caused minor creatine kinase efflux, *viz.*, untreated control, needle puncture (no test solution), normal saline, and 5% dextrose. The intramuscular injection of normal saline into animals has been demonstrated to cause no histological evidence of damage to skeletal muscle fibers (15). Furthermore, isotonic solutions such as normal saline and 5% dextrose are commonly considered to cause minimal tissue damage (*viz.*, hemolysis of erythrocytes) when injected parenterally (18,26). Creatine kinase efflux caused by the trauma of the injection procedure alone was less than the efflux following the injection of the two isotonic solutions. These four treatments were not statistically different from each other in total creatine kinase efflux over 2 hr ($P > 0.05$).

Figure 3 shows the mean creatine kinase efflux from those treatments that caused moderate to severe intracellular release. The organic cosolvent system, 40% (v/v) propylene glycol–10% (v/v) ethanol in water, caused markedly less enzyme efflux than two commercial formulations which employed this system, diazepam and phenytoin ($P < 0.05$). The third commercial formulation utilizing this solvent system, digoxin, showed no statistical difference in creatine kinase efflux from the solvent system itself ($P > 0.05$).

The myotoxic potential of the different treatments, evaluated by the total cumulative creatine kinase released into the incubation medium, is summarized in Table I. One-way analysis of variance revealed a statistical significance among the different treatments ($F_{9,50} = 27.9$, $P \leq 0.005$). A subsequent Tukey's test suggested that the degree of intracellular enzyme release can be divided into three groups: (i) those treatments causing minor creatine kinase efflux (control, needle puncture, normal saline, and 5% dextrose), (ii) those treatments causing moderate enzyme efflux (40%, v/v, propylene glycol–10%, v/v, ethanol in water, lidocaine, and digoxin), and (iii) those treatments causing extensive enzyme efflux (sliced muscle, phenytoin, and diazepam). Based upon histologic evaluation, Paget and Scott have also

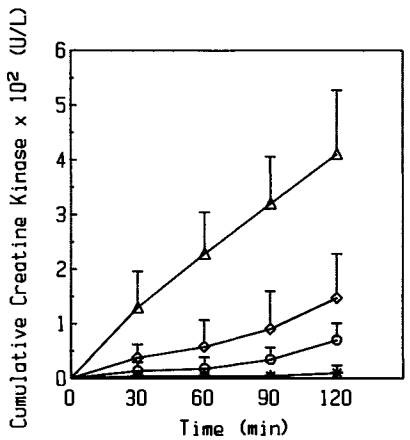

Fig. 2. Cumulative creatine kinase efflux from untreated control muscles (*), muscles punctured by injection needle only (○), muscles injected with normal saline (◇), and muscles injected with 5% dextrose (△).

Table I. *In Vitro* Enzyme Release of Various Pharmaceutical Systems Tested by the Current Technique[a]

Treatment	Cumulative CK released over 2 hr, mean ± SD (U/liter) × 10^2	Rate of cumulative CK release, mean ± SD (U/liter × min)
Control	0.10 ± 0.14	0.06 ± 0.10
Needle puncture	0.70 ± 0.31	0.61 ± 0.21
Normal saline	1.47 ± 0.81	1.20 ± 0.68
5% dextrose	4.10 ± 1.17	3.12 ± 0.75
40% (v/v) PG–10% (v/v) EtOH in water	8.77 ± 5.14	8.86 ± 4.72
Lidocaine	9.19 ± 3.52	8.93 ± 3.19
Digoxin	9.51 ± 5.09	9.66 ± 5.57
Sliced muscle	16.4 ± 2.29	12.8 ± 2.75
Phenytoin	18.3 ± 6.51	15.0 ± 5.01
Diazepam	22.5 ± 4.12	18.3 ± 3.36

[a] CK, creatine kinase; PG, propylene glycol; EtOH, ethanol.

divided the effects of various intramuscular injections into the rat into three categories (27).

The myotoxic potential can also be quantified by using the slope of the cumulative creatine kinase efflux curve as the rate of enzyme release into the incubation medium (Table I). The cumulative creatine kinase curves for the individual muscles were linear over the 2-hr period, with the coefficient of determination, r^2, ranging from 0.6 to 1.0 (data not shown). Similar significant results among the various treatments ($F_{9,50} = 22.7$, $P \leq 0.005$) also occurred if the rate of creatine kinase release was used as the index of myotoxicity. Although our results exhibited relatively large standard deviations, they are no larger than that reported in the literature for other studies employing creatine kinase release as an index of muscle damage (8,9,15,19).

It is difficult to compare the entire set of the present data with literature *in vivo* results, since in vivo studies seldom carried out cross-comparisons of more than three intramuscular formulations within a given experiment. However, our *in vitro* results appear to be in good agreement with the available *in vivo* data from several animal species. For example, treatments that caused minor increases in serum creatine kinase activity in rabbits, *viz.*, control and normal saline, also caused minor total cumulative creatine kinase efflux in the *in vitro* system (16). Likewise, these parallel *in vitro* vs *in vivo* observations were obtained for those treatments that caused significant myotoxicity, *viz.*, lidocaine, digoxin, and diazepam (15). Similar rank-order correlations between *in vitro* observations and *in vivo* results in rats (9,10,17) swine (14,15), dogs (28), and man (6,8,15,29,30) were also obtained. Wilensky and Lowden have shown that intramuscular injection of the commercial phenytoin formulation, its vehicle, or components of its vehicle into the rabbit gastrocnemius muscle caused histological evidence of hemorrhage. In addition, those muscles injected with the commercial formulation showed the presence of phenytoin crystals at the injection site (31). In the present *in vitro* system, some of the muscles injected with the commercial phenytoin formulation displayed a small white area within the muscle body upon removal from the incubation medium. This observation was not seen with the other treatments.

The present *in vitro* system, therefore, could permit rapid quantitative assessment of the myotoxic potential of prospective intramuscular injection solutions. This technique allows the formulator to incorporate potential skeletal muscle damage as a measurable parameter in the formulation of the parenteral injection system. This information together with that on solubility, stability, and injectability will form the basis upon which the final formulation should be chosen.

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