

The Effect of Low Dose Dexamethasone on Skeletal Muscle Regeneration *in vivo*.

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Abstract

Effects of the synthetic corticosteroid Dexamethasone [9 α -Fluoro-16 α -methyl-prednisolone (Dex)] on skeletal muscle regeneration *in vivo*, were assessed histologically and autoradiographically after crush injury to tibialis anterior muscles of 52 mature male BALB/c mice. Dex was administered intramuscularly, with doses ranging from 1 μ g/kg to 100 μ g/kg of bodyweight, one day before, and for 9 days after injury. To label replicating muscle precursor cells, tritiated thymidine (³H-Tdr) was injected at either 48, 72 or 120 hours after injury: muscle samples were taken 10 days after injury when labelled precursors had fused to form myotubes and labelling of myotube nuclei was assessed. Histological observations revealed no differences in the appearance of regenerated lesions apart from a reduction in the proportion of persistent necrotic material in the 1 and 10 μ g/kg Dex groups. Autoradiographic data revealed a greater proportion of labelled myotube nuclei resulting from ³H-Tdr injection at 72 and 120 hours after injury in the 1 μ g/kg and 10 μ g/kg groups, in comparison with the other test group and controls: however these values were not significantly different ($P > 0.05$). We conclude that low doses of Dex do not markedly improve the regeneration of skeletal muscle *in vivo* nor significantly increase muscle precursor replication. This information is relevant to the current interest in corticosteroid therapy as a means of transiently improving the strength of muscles in patients with Duchenne muscular dystrophy.

Keywords: skeletal muscle, regeneration, injury, dexamethasone, proliferation, autoradiography.

BAM 1 (2): 139-144, 1991

It is well documented that regeneration of skeletal muscle occurs in response to a wide variety of physical and chemical trauma, and that the process of regeneration is similar regardless of the method of injury [1, 6]. The survival, replication and fusion of a population of muscle precursor cells (myoblasts) is essential to ensure successful regeneration. The muscle precursors (widely termed satellite cells in mature skeletal muscle [5]), continue to proliferate until many either fuse with each other to form multinucleated myotubes, or with the ends of damaged myofibres [30]. A variety of hormones and growth factors affect the proliferation and fusion of muscle precursors *in vitro* [9], although little is known about the role of these factors in muscle regeneration *in vivo* [13].

Several *in vitro* studies have shown that corticosteroids can increase the proliferation of myoblasts [10,16] and fibroblasts [11, 10, 18]. This response is dose dependent: at low doses (10^{-7} M) the synthetic corticosteroid Dexamethasone (Dex) increases the number of myoblasts *in vitro* by up to 800% after 7 days, whereas high doses (10^{-4} M) of Dex completely inhibit myoblast proliferation [16]. Under serum-free conditions in tissue culture, addition of a corticosteroid to the media is an absolute requirement for the growth of satellite cells [2]. Dex is widely used *in vitro* [2, 17] as it is more potent than

the naturally occurring corticosteroids in promoting myoblast growth [15, 16]. The effect of corticosteroids on increasing cell proliferation occurs primarily during the phase of growth in which cell numbers are increasing logarithmically, for both myoblasts [16] and fibroblasts [3, 18]. Promotion of cell proliferation by Dex *in vitro* is dependent upon the presence of other growth factors, and may be mediated by modulation of cellular receptors for fibroblast growth factor [10], epidermal growth factor [3, 18], or the insulin-like growth factors [38]. Although muscle precursor cell proliferation *in vitro* is convincingly enhanced by corticosteroids such as Dex, there is a paucity of data on the effects of corticosteroids on myogenesis *in vivo*, either in embryonic or mature muscle. We found only two studies on the effect of Dex on skeletal muscle regeneration *in vivo* [32, 33]. The earlier study showed that there was no effect of pharmacological doses of Dex on muscle precursor proliferation; however the work of Steiss [33] showed that administration of a low dose of Dex, similar to levels used in *in vitro* experiments, resulted in a transient increase in myotube numbers in minced muscle autografts in rats.

In contrast, there have been many studies on the catabolic effects of corticosteroids on skeletal muscle *in vivo*: pharma-

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cological doses of corticosteroids are catabolic in muscle of adult rats [19, 20] and neonatal rats [25], as measured by the urinary excretion of ^3H -methylhistidine [35]. Similarly, the myopathic effects of artificially and naturally elevated levels of glucocorticoids are well documented [4, 21, 31].

Despite the myopathic effects of corticosteroids, recent reports indicate that long-term administration of prednisone extends the period of ambulatory activity of patients with Duchenne muscular dystrophy [7, 23]. It is not known whether the beneficial effects of prednisone in dystrophic muscle, which undergoes cycles of necrosis and regeneration, is due to enhancement of muscle precursor replication and regeneration, to increased catabolism of muscle or to other mechanisms. It is therefore of clinical interest to investigate the effects of corticosteroids on regenerating skeletal muscle *in vivo*.

The increased proliferation of myoblasts following the addition of low doses of Dex *in vitro* [16], and increased myotube formation reported *in vivo* [33], suggest that this corticosteroid may enhance the proliferation of muscle precursors in injured muscle, and that fusion of these precursors results in increased numbers of myotubes. It is the purpose of this study to quantify the effect of Dex on muscle precursor proliferation *in vivo* in regenerating skeletal muscle of mice.

Materials and Methods

Animals and Lesions

Eight week old male BALB/c mice (19-24 g body weight) were used in these experiments. The surgical procedure for

muscle crush injury was based on that described by McGeachie and Grounds [22]. Briefly, 52 mice were anaesthetized with Halothane (BP) and an incision made over the TA muscle between the tibial condyle and the ankle, and the muscle dissected free of the tibia. Two, side-by-side transverse crushes were made in a proximo-distal direction, resulting in an injury approximately 3 mm wide and 5 mm long which did not disturb the overall muscle continuity. Skin wounds were sutured with 7.0 braided silk (Ethicon). No antibiotics were administered.

Dexamethasone Injections

Between 10-11 AM each morning, on the day prior to injury, and from day 1 to 9 after injury, each mouse received a single intramuscular dose of a commercial Dex preparation (containing Dexamethasone Sodium Phosphate - 4mg/ml, David Bull Laboratories, Australia), or a vehicle-only control into the contralateral gastrocnemius. The commercial stock was diluted in sterile physiological saline to give doses of 100, 10 and 1 μg Dex/kg of body weight. The dosage throughout the period of injections was adjusted to the daily body weight of each mouse.

It is known that the levels of circulating corticosteroids (Note: mice do not produce cortisol, and the major physiological corticosteroid in rodent plasma is corticosterone [24]) vary according to a number of factors, including diurnal rhythms and hormonal status [28, 29]: an average level has been calculated as 3 μg /100 ml plasma [8] or approximately 0.03 μg /mouse. The doses of Dex used in this study correspond to those values which elicit a marked increase in myoblast proliferation in tissue culture [15, 16] and have been

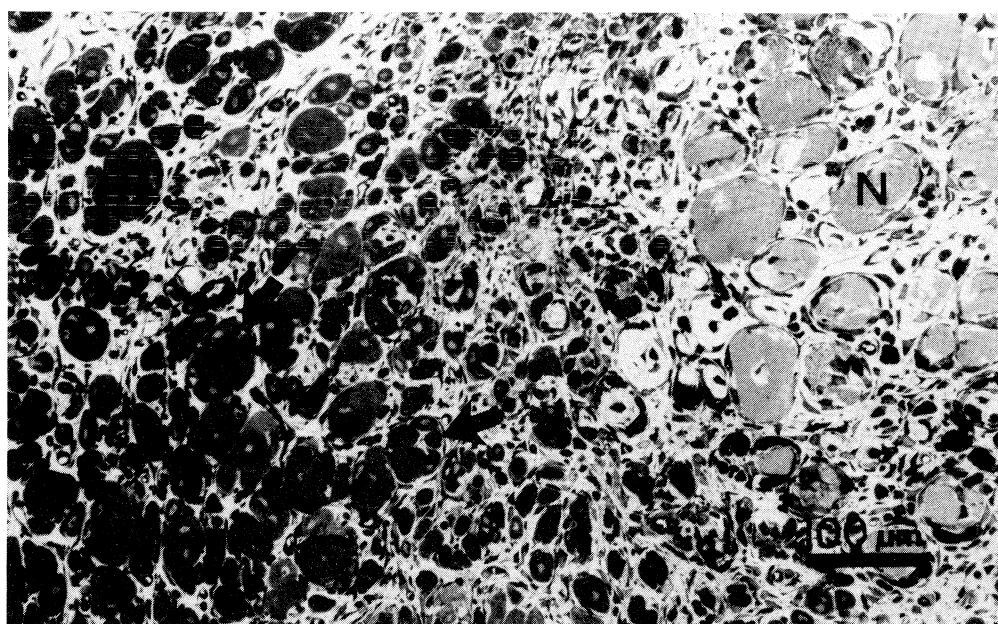


Figure 1. A transverse section from a typical muscle crush lesion examined 10 days after injury. Note the many young myotubes (arrows) with some intervening connective tissue, and a central area containing persistent necrotic myofibres which is devoid of myotubes. This mouse received 1 μg /kg Dex and a single dose of ^3H -Tdr at 72 h after injury.

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Table 1. The proportions of persistent necrotic muscle tissue at 10 days after injury in transverse sections of TA, in response to a range of doses of Dex.

Percentage of persistent necrotic muscle tissue	Dose of Dex ($\mu\text{g/kg}$ bodyweight)			
	100	10	1	0
<10%				2
10-30%				5
>30%				6
Total				13

shown to cause an increase in myotube numbers after long-term administration *in vivo* [33].

Autoradiography and Analysis

Tritiated thymidine (^3H -Tdr; specific activity 5 Ci/mmol, Amersham International) was injected intraperitoneally at a dose of 1 $\mu\text{Ci/g}$ body weight, in order to label muscle precursors synthesizing DNA. Each mouse received a single dose of ^3H -Tdr at either 48, 72 or 120 hours after injury. At least 4 mice from each of the experimental and control groups were injected with ^3H -Tdr at these times. Ten days after injury (when labelled muscle precursors had time to fuse into myotubes), all mice were anaesthetized with Halothane and killed by cervical dislocation. Samples of crush injured mus-

cles were removed, fixed and prepared for resin section light microscopic autoradiography and histology, as previously described [22].

Transverse sections 2 μm thick were placed on glass slides and coated with Kodak NTB2 autoradiographic dipping emulsion (diluted 1:1 in double distilled water). Sections were exposed in light tight boxes at -20°C for 4 weeks, developed in D19 (Kodak), fixed in acid hardener fixer, washed and dried.

The histological appearance of all sections was examined in detail and a quantitative analysis of lesion size, proportion of persistent necrotic muscle tissue and myotube formation was performed by light microscopy. The distribution of autoradiographic grains was analysed with an oil immersion lens ($\times 100$) on a Leitz microscope. The analysis involved counting a minimum of 200 myotube nuclei within each lesion and calculating the percentage which were labelled. Only myotube nuclei with 3 or more grains directly overlying the nuclear area were considered to be labelled [22].

Results

Macroscopically, all injured muscles (10 days after injury) had a pale central area with peripheral whitish patches making the lesion easy to locate. Light microscopy of transverse sections revealed a large central area consisting of cellular and fibrous connective tissue, persistent necrotic myofibres (some of which were calcified), scattered myotubes and numerous mononuclear osmiophilic cells (Fig. 1). Between the area of scar tissue and the surviving myofibres was a 1 to 12 cell thick layer of myotubes. Surviving myofibres accounted for up to 30% of the cross sectional area of muscle sections, and many

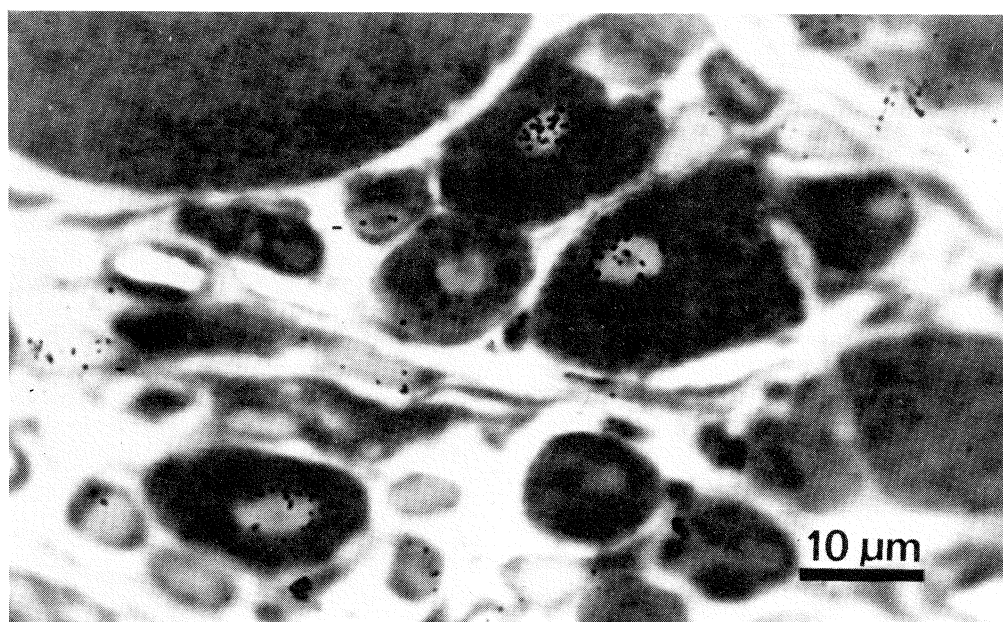


Figure 2. An autoradiograph of myotubes in cross section from muscle removed 10 days after injury. The silver grains overlying the myotube nuclei (arrows) indicate that muscle precursors which fused to form these myotubes were synthesizing DNA at the time of ^3H -Tdr injection (72 hours after injury). This mouse received 100 $\mu\text{g/kg}$ Dex.

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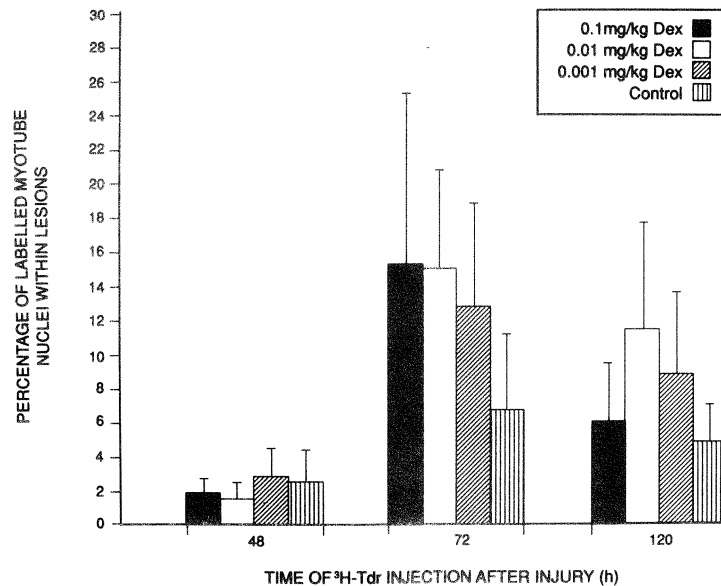


Figure 3. The percentages of autoradiographically labelled myotube nuclei in transverse sections of crush injured TA of mice treated with different doses of Dex. Vertical bars represent the mean plus standard error.

myofibres close to the lesion had central nuclei (Fig. 2). This pattern was seen in all groups, with no marked differences between the histological appearance of lesions, other than the proportions of necrotic muscle tissue.

A quantitative analysis of lesions (Table 1) showed that less necrotic muscle persisted in the 1 µg/kg and 10 µg/kg Dex groups compared with the control group. Furthermore much more necrotic muscle remained in the 100 µg/kg group at 10 days after injury in comparison with all other Dex treated groups and controls.

Autoradiography

The number of autoradiographic grains directly overlying myotube nuclei in muscle 10 days after injury (Fig. 2), varied between 3 and 25, and the percentage of labelled myotube nuclei within lesions ranged from 0.5 to 30.5% (Fig. 3).

No difference in levels of labelling was found between the Dex treated or control groups in mice injected with ³H-Tdr at 48 hours after injury. However, at 72 and 120 hours the mean proportion of labelled myotubes in the 1 µg/kg and the 10 µg/kg groups was substantially greater than in control mice (Fig. 3). Using a Student t-test these values were not significantly different ($p > 0.05$).

Discussion

The histological appearance of regenerated crush injured murine skeletal muscle is similar to that reported in previous studies [22, 14]. The only notable difference is a reduction in the proportion of necrotic muscle tissue seen in the 10 µg and 1 µg/kg Dex treated groups compared with controls.

Whilst almost complete removal of necrotic muscle tissue was seen in 18 out of 26 Dex treated mice (receiving either 1 or 10 µg/kg Dex) and 5 out of 13 controls, new muscle formation was not noticeably improved. These findings concur with those of Grounds (1987) who showed in minced muscle isografts that phagocytosis of necrotic muscle tissue is essential for successful regeneration, but it does not guarantee it. In contrast, mice treated with 100 µg/kg Dex had far more persistent necrotic muscle tissue after 10 days than controls. This is possibly due to suppression of phagocytic activities of macrophages by corticosteroids [36, 37]. Phagocytosis is principally carried out by macrophages which migrate from the vascular bed possibly in response to chemical mediators released from damaged tissue [26]. The access of macrophages to these areas of injury and subsequent phagocytosis of necrotic debris is thus dependent on the viability of the vasculature or revascularization. Where lesions contained areas of incompletely phagocytised muscle debris (Table 1) there were few associated blood vessels. A likely reason for the excessive scar tissue formation, characteristic of all crush lesions in BALB/c mice is the ischaemic conditions found in the centre of muscle lesions [27] which promote the proliferation of fibroblasts over other cell types [34].

The autoradiographic results (Fig. 3) suggest that daily dose of 1 and 10 µg of Dex treatment moderately increase the proportion of muscle precursors replicating at 72 and 120 hours after injury. This increase in numbers of labelled myotube nuclei in crush injured muscles of mice at 10 days corresponds with the data of Steiss (1986), who found an increase in the number of myotubes in minced muscle isografts in rats after 21 days using similar doses of Dex, although

myotube numbers returned to control levels by 60 days. A possible explanation for the transient increase in myotube numbers reported by Steiss (1986), is that muscle precursors close to patent vasculature proliferate in response to the Dex and later fuse, resulting in from 1 to 7 small myotubes forming within the persistent basal lamina of damaged myofibres. However, at later times lateral fusion between the plasmalemmata of these adjacent myotubes [30] would form a single large myotube, and therefore a concomitant reduction of myotube numbers in cross sections of regenerated muscles when compared with earlier times.

These autoradiographic data correspond with the pattern of muscle precursor replication found in previous studies of 44 crush injured BALB/c mice, where the onset of muscle precursor replication was at 30 hours and the peak was between 60 and 84 hours post-injury [22].

Although corticosteroids may be important in foetal or neonatal muscle *in vivo* to enhance the ongoing proliferation of precursors in developing muscle [16], our results indicate that corticosteroids do not significantly increase the proliferation of muscle precursors in mature muscle and do not enhance the regeneration of skeletal muscle after injury. Thus it seems unlikely that the improved function of dystrophic muscle seen with prednisone treatment is due to improved regeneration or enhanced proliferation of muscle precursor cells.

Acknowledgements

This work was supported by the Neuromuscular Foundation (CAM), The National Health and Medical Research Council of Australia (MDG) and the Nicholas and Eliza McCluskey Memorial Bequest (JKM). We wish to thank Mr. A. Minten and Mr. L. Bubrick for technical assistance, and Mrs M. Seats for typing the manuscript.

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