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Review Article

An overview of bupivacaine-induced morphological changes: a novel animal model of skeletal muscle injury

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Abstract: Skeletal muscle (SKM) injury is a common clinical problem that lacks effective treatment methods. Thus, establishment of an appropriate animal model of SKM injury will provide a foundation for the discovery and verification of effective therapies. Several treatment methods have been employed for SKM injury in experimental and clinical studies. However, few studies have reviewed a method of establishing a unified standard for SKM injury in animal models. In the present investigation, we provide an overview of a bupivacaine-induced animal model of SKM injury from a morphological perspective to provide a review of this available and effective approach.

Keywords: Animal model, bupivacaine, morphological changes, skeletal muscle injury

Introduction

Many factors, such as muscle strains, cardio toxins and sports trauma, cause skeletal muscle (SKM) injury, the health cost of which is more than 790 billion USD in the United States per year [1, 2]. Hence, it is necessary to elucidate the mechanisms of SKM that cause permanent disability due to inadequate regeneration of injured SKM [3, 4]. Developing efficient therapies for SKM injury will reduce social and economic burdens, as well as alleviate personal-psychological pressures [5].

There are few animal models of SKM injury that have been fully characterized within the literature [6]. By contrast, several SKM-injury animal models have been used to analyze the therapeutic effects of various treatment methods [7-14]. Therefore, reviewing an accepted and easily implemented animal model of SKM injury is critical for better elucidating mechanisms of SKM injury and for developing and evaluating therapeutic strategies to assist an aging society that has a high incidence of SKM injury [15-17].

Bupivacaine is a local anesthetic that has been used as a myotoxic drug to induce SKM injury in animal models since 1968 [18]. Numerous papers on bupivacaine-induced SKM injury have been reported [19-26]; however, there are no reviews that delineate a standard for bupivacaine-induced SKM injury models.

Therefore, the purpose of this review is to provide an overview of a bupivacaine-induced animal model of SKM injury from a morphological perspective to facilitate the reproducibility and future applicability of the model. This report will serve to support an animal model of SKM injury and facilitate the reproducibility and future applicability of the model.

Morphological changes of SKM after bupivacaine injection

Thirteen studies have addressed acute or chronic morphological changes following intramuscular injection of bupivacaine [18, 27-38]. Detailed information was extracted from these studies and is listed in **Tables 1**-**3**.

Publication year and location of study

Thirteen studies were conducted between 1968 and 2015. Only two [28, 38] studies were performed at medical research centers, while

Table 1. Basic information and interventional methods of the reviewed studies

Study	Place	Special/Weight	Injured SKM	Intervention (type/amount/concentration/syringe)	Control method
Sokoll [18]. 1968	University of Lund	200-220 g M/W	EDL	s.c. 0.5 ml 0.5% 25 G	NS
Benoit [27]. 1970	Tufts University School of Medicine	200 g M albino rats	Gracilis/posticus muscle	s.c. 0.5 ml 0.5% NR	NS
Hall-Craggs [30]. 1974	University College London	175-265 g F/W	TAS	i.m. 0.5 ml 0.5% 23 G	NS
Schultz [37]. 1978	University of Wisconsin, Madison	4/24 month quail	PLM	i.m. 0.5 ml 0.75% Hypodermic needle	NI
Foster [29]. 1980	University of Michigan	55-80 gm F/SD	TAS	s.c. with 0.2 ml 0.75% 27 G	NS
Newman [33]. 1983	University of Oxford	200-250 g M/SD	Calf muscle	i.m. 3 ml 0.5% NR	NS
Komorowski [31]. 1990	University of Michigan	NR monkey	APBM	i.m. 350 μl/kg 0.75% 22 G	Mepivacaine/Lidocaine+Epinephrine/NS/NI
Polit [36]. 2006	University of Athens	180-250 g M/W	Soleus muscle	i.m. 1 ml 0.5% 0.5 mm needle	NS
Plant [35]. 2006	University of Melbourne	30 g M C57BL/10 mice	EDL	i.m. 100 μl 0.5% 30 G	Notexin/NI
Vignaud [38]. 2007	INSERM, U787, Paris, F-75013 France	15-20 g M/mice	TAS	i.m. NR	Venom/cardiotoxin/NI
Cherng [28]. 2010	National Defense Medical Center	320-400 g M/W	TAS	i.m. 0.2 ml 0.25/0.5/1% 20 G	NS
McNeill [32]. 2011	Federal University of São Paulo	300 g M/W	GNM	i.m. 0.5 ml 0.5% 26 G	NI
Oz Gergin [34]. 2015	Erciyes and Cukurova University	180-200 g F/W	GNM	i.m. 100 μl 0.5% 27 G	Levobupivacaine/Ropivacaine/NS

Table 2. Outcome measures and morphological changes of the injured muscle

Study	Outcome measures	Morphological changes
Sokoll [18].	H&E	Atrophied and normal fibers were seen at 4 d. Marked atrophy of superficially located muscle fibers at 7 d by twice daily injections. CMF was seen during the recovery phase. Deeply located muscle fibers have atrophied, and the diameter was still reduced after the cessation of twice-daily bupivacaine injections for 7 d.
Benoit [27].	H&E	Hyalinized fibers with pyknotic nuclei were scattered among normal fibers at 15 min. Damaged fibers were filled with macrophages at 24 h.
Hall-Craggs [30].	H&E	Fibers were reduced in caliber with leucocytic infiltration at 1 d. Intense infiltration and macrophages were seen at 2 d. Macrophages invaded and degeneration phenomenon appeared at 3 d. Basophilic cytoplasm, striations and myotubes with PPN appeared at 4 d and 5 d. Young muscle fibers with transverse striations were seen at 6 d. Fifteen days after injection, fibers showed ectopic nuclei and reduced caliber.
Schultz [37].	H&E	Injured muscle was stained with methylene blue in sodium borate. Complete breakdown and fragmentation of myofibers with cellular invasion after injection. Numerous basophilic cells appeared beneath the basal lamina of degenerating fibers at 17 h. Regeneration process appeared subsequently.
Foster [29].	H&E	Damaged muscle fiber was broken down by a synchronous phagocytic reaction and the regenerating process was completed at 30 d, but with numerous central nuclei persisting. Vascular supply and nerves were not affected.
Newman [33].	H&E	Small cell infiltrations with numerous polymorphonuclear leukocytes appeared at 1 d. Inflammatory infiltration formed by macrophage and fragmentation of fibers were seen at 2 d with CMF displayed at 3 d. Regeneration was apparent at 5 d. Diameter of the fiber had increased and small cell infiltration almost resolved at 10 d.
Komorowski [31].	TEM	Breakdown phenomenon was seen at 2 h. Phagocyte-mediated fragmentation of the degenerating muscle fibers was intense during 3 d and 4 d. Myotubes appeared at 6 d and matured at the 2nd week.
Polit [36].	H&E TEM	No significant variation was observed by H&E. TEM: Necrotic fibers infiltrated with mononuclear cells on the day 1. Numerous replicating myoblasts began to fuse the injured place at 3 d. Myotubes appeared at 5 d, and the regenerating fibers with PPN appeared at 7 d. The diameter of the regenerating fibers was increased at 14 and 21 d.
Plant [35].	H&E	The proportion of CSA occupied by degenerating fibers was 24%, 51%, and 33% at 1, 2, and 3 d, respectively. The proportion of CMF in the total muscle CSA was ~23% at 7 and 10 d after bupivacaine injection.
Vignaud [38].	H&E	Numerous CMF filled 80-100% of the CSA. Bupivacaine injection resulted in near destruction of the muscles.
Cherng [28].	H&E	Muscle damage was decided by the concentration of bupivacaine. Specifically, 0.25% caused mild focal mononuclear cell infiltration. Next, 0.5% induced severe muscle damage with neutrophil and lymphocyte infiltration. Finally, 1% caused severe muscle damage with marked neutrophil and lymphocyte infiltration.
McNeill [32].	H&E	Intense inflammatory response was seen at 5 d. CMF appeared at 14 d. The higher presence of normal muscle fibers appeared at 21 d and the muscle was almost normal at 28 d.
Oz Gergin [34].	H&E TEM	H&E: Serious inflammation was observed at 2 d. Mononuclear leukocyte infiltration and necrobiotic changes were observed. Inflammatory edema and a wavy appearance were visible. TEM: Mitochondrial swelling and membranous whorl formations were remarked in sub-sarcolemmal areas. Disruption of the myofibrillar organization and intracytoplasmic edema areas in sarcoplasm were seen. Numerous macrophages were observed around the muscle fibers.

Table 3. Comparison between single and sequential injections

Study	Way of injection	Differences compare to a single injection
Benoit [27].	12-h-interval injections with bupivacaine lasting one week	The degree of the sequential injection was not more serious than a single injection, and each injection appeared to initiate a new injury to the unaffected fibres. Regenerating fibres were unaffected by drug exposure.
Sokoll [18].	12-h-intervals injection with bupivacaine lasting 2/4/7 d	The diameter of superficial muscle was reduced more, and muscle atrophy was deeper when comparing sequential with the single injection method.
Hall-Craggs [30].	Daily injection for 3 d	Degeneration and regeneration processes appeared more active and advanced when comparing sequential with the single injection method.

the other studies were completed at universities (**Table 1**). Among these, four [27, 29, 31, 37] studies were conducted in the USA.

Experimental animals and injured SKM models

Detailed information of the animals was reported in all studies, except for animal weight which was not reported in one [31] study. The tibialis anterior muscle in four [28-30, 38] studies, gastrocnemius muscle in three [32-34] studies, and the extensor digitorum-longus muscle in two [18, 35] studies were injured via bupivacaine injections. The soleus muscle, abductor pollicis-brevis muscle, peroneus longus muscle, and gracilis-posticus muscle were all damaged in a single study (**Table 1** [27, 31, 36, 37]).

Interventional and control methods

Interventional methods consisted of single/sequential injections of bupivacaine. Eight [18, 27, 28, 30, 31, 33, 34, 36] studies applied nor-

mal saline, while three [31, 32, 37] studies used blanks as controls. Animal venom and blanks were used as controls in two [35, 38] studies, while one study [38] adopted cardiotoxin as a control. Two [31, 34] studies applied different types of anesthetics or anesthetics plus hormones as controls (**Table 1**).

Bupivacaine injection

(i) The concentration and amount of bupivacaine: a concentration of 0.5% bupivacaine was used most often. Four [18, 27, 30, 32] studies injected 0.5 ml, two [34, 35] studies injected 0.1 ml, and 1 ml [36] and 3 ml [33] were each adopted in one study. Additionally, 0.75% bupivacaine was used in three [29, 31, 37] studies, which included injections with 0.2 ml [29], 0.5 ml [37] and 350 ul/kg [31]. One [28] study applied 0.2 ml bupivacaine with three different concentrations (0.25%, 0.5%, 1%), while another [38] study did not report the concentration or amount of bupivacaine (**Table 1**).

(ii) Injection method: direct intramuscular injections were most frequently applied in previous work, and two [35, 36] studies employing this technique surgically exposed the injured muscle. Two [18, 27] studies applied subcutaneous injections, while another study [29] used percutaneous injections (**Table 1**).

(iii) Specification of syringes: the syringes used ranged from 20-gauge [28] to 30-gauge [35]; two [29, 34] studies used 27-gauge syringes. A 0.5-mm hypodermic syringe was used in one study [36], and a hypodermic syringe was used in another study [37]. Two [33, 38] studies did not list details about the syringes used (**Table 1**).

Outcome measures

Hematoxylin-eosin (H&E) staining was most commonly used to analyze the morphology of the injured muscle. Two [34, 36] studies also performed TEM. One [31] study only reported TEM changes (**Table 2**).

Morphological changes of injured muscle

According to the H&E or TEM images at different time points, the injured muscle experienced inflammation and/or regeneration. At the inflammatory stage, severe inflammatory-cell infiltrations included macrophages and leukocytes. Necrotic fibers with mononuclear-cell infiltration, mitochondrial swelling, disordered lines and bands, and disruption of the myofibrillar organization were observed by TEM analysis. During the regeneration period, centronucleated muscle fibers, basophilic cells, and young muscle fibers appeared; also satellite cells and regenerated myoblasts were also observed from the TEM images (**Table 2**).

Comparison between single and sequential injections

Sequential injections of bupivacaine were applied in three [18, 27, 39] studies. It was indicated that the severity of muscle injury induced by sequential injection was greater than for single injections [18, 39]; one study held the opposite opinion [27].

Comparison of TEM analysis

Different ultra-structural changes were seen according to observed time points from 2 h to 21 d (**Table 2**). Inflammatory reactions were observed during the first 2 d after muscle injury in three [31, 34, 36] studies; detailed information was provided in one [34] study, including the changes of mitochondrial, subsarcolemmal and surrounding supportive tissues. Myoblasts were recognized at 3 d, and myotubes appeared at 5 d, according to the description of the regeneration process. The injured muscle was recoverable but without completed repair until 21 d, as shown by the TEM results.

In all 13 studies, two of the studies analyzed the muscle damage severity according to the morphological changes 2 [34] and 3 [28] d after bupivacaine injection. Although muscle histology is a good static indicator of injury [40], the measurement of muscle function by force evaluation is important to assess injury and recovery comprehensively [41].

Functional analysis of SKM injury after bupivacaine injection

Three studies provided functional analysis of the injured muscle after bupivacaine injection [32, 35, 38]. Muscle mass, maximal twitch force, contraction time, half relaxation time and maximal tetanic force were reported in two

studies [35, 38], while muscle mass and muscle force were analyzed in one investigation [32]. Due to the different muscles and examination times, a statistical comparison was not completed.

Regarding the change of muscle mass, conflicting results were presented. One study [38] showed that the injured muscle mass was reduced at 5 d and increased at 56 d as compared to control muscles. In contrast, muscle mass was 17% greater after bupivacaine-injection during the first 3 d but was not statistically different from non-injected muscle later on [35]. The change of twitch and tetanic force were consistent; the data revealed that the force was decreased after muscle injury and nearly recovered within 28 d [32, 35, 38]. With the recovery of the injured muscle, the contraction and half relaxation time were gradually reduced compared with the control muscles [38].

Discussion

Bupivacaine was recommended by the United States Food and Drug Administration (FDA) for use as a local anesthetic, although the side effects have not been fully recognized [42]. The experimental myotoxic effects are robust and reproducible, although only a few case reports of myotoxic complications in patients have been published [43]. Therefore, we reviewed the utility of the bupivacaine-induced SKM-injury rat model herein as well as provided a novel utilization of bupivacaine.

Use of SKM injury induced by bupivacaine-injection

Animal models of bupivacaine-induced SKM injury have been widely used for *in vivo* [20, 44] and *in vitro* experiments [45, 46] since the model was first successfully introduced in 1968 [18]. Subsequently, more and more studies of bupivacaine-induced SKM have been used in the biomedical field [47-49]. A focus of many of these studies was to test the model's success rate by biomedical indices or functional properties of injured muscle [24, 50, 51], but no report has reviewed and summarized the variety of ways for establishing the bupivacaine-induced SKM injury model.

Bupivacaine has been widely used to investigate muscle injury through *in vitro* experiments [25, 39, 45, 46, 52-57]. Different muscular cells have been cultured with bupivacaine at varying concentrations to explain its myotoxicity [46, 53] or effectiveness in treatment [55-57]. While a variety of experiments have focused on human muscle injury produced by bupivacaine injections, these studies contained no morphological analysis [58-60]. Animal models of bupivacaine-induced SKM injury can be used as SKM pain models, although diagnosis and treatment principles differ between muscle injury and pain [61]. These discrepancies are because: (i) the pathogenesis of bupivacaine-induced SKM injury, such as acute muscle inflammation, is the pathological manifestation of muscle pain [62]; and (ii) bupivacaine injection can enhance sarcoplasmic reticular Ca^{2+} release, which plays an essential role in the development of muscle hyperalgesia [63, 64]. Bupivacaine-induced sarcoplasmic/endoplasmic reticulum stress and apoptosis may be the reason for muscle pain [45]. Hence, this type of animal model is vital for related research about pain management.

SKM injury induced by bupivacaine-injection

Animal models of bupivacaine-induced SKM injury using an intramuscular administration route conform to the pathological process of muscle injury and are convenient for research on muscle injury. Bupivacaine can be used as a myotoxic agent to induce SKM injury due to its neuromyotoxic properties [38, 65], ability to reduce muscle energy metabolism [66], ATP activity [33], mitochondrial function [64] and contractile properties [32, 35, 38], all of which are consistent with the morphological and functional changes of injured muscle. Reviewing the H&E/TEM results indicates that a single intramuscular injection with 0.1-0.5 ml 0.5% bupivacaine can establish an animal model of SKM injury.

There are other important considerations when establishing bupivacaine-induced SKM injury models. One crucial aspect to consider is that recent studies evaluated morphological changes, while only three [32, 35, 38] studies analyzed the function of the injured muscle, which is important for assessing the degeneration and regeneration degree of muscle [67]. There is a close relationship between morphological changes and muscular functional properties

[68-72]. Hence, it is reasonable to suggest that analysis of the injured muscle, both from morphological and functional perspectives, should be included in further studies.

H&E and TEM are widely used methods for assessing changes in muscle structure [73, 74]. Other imaging technologies are also recommended such as CT or MRI scanning [75, 76]. Another important factor is that the specification of the syringe should be taken into consideration. Different syringe specifications were used in the reviewed studies, and previous reports suggest that the syringe (size, length, and angle inserted into the muscle) may cause mechanical muscle damage or confound any therapeutic effects [77, 78]. One study, using saline as a control, found that mechanical damage occurred near the injection site [79]. Thus, using the same specification syringe with a small diameter is recommended to avoid mechanical damage when establishing SKM models.

Conclusion

Intramuscular injection of 0.1-0.5 ml 0.5% bupivacaine can establish an animal model of SKM injury. Bupivacaine injection is an appropriate animal model of SKM injury and has wide applicabiltiy in research. However, it is necessary to provide additional functional analysis of this kind of animal model in future investigations.

Disclosure of conflict of interest

None.

Abbreviations

APBM, abductor pollicis brevis muscle; CMF, centro-nucleated muscle fibers; CSA, cross-section area of the muscle; EDL, extensor digitorum longus muscle; F, female; G, the size of the syringe; GNM, gastrocnemius muscle; i.m., intramuscular injection; M, male; NS, normal saline; NI, no injury; PPN, peripherally placed nuclei; PLM, peroneus longus muscle; SD, Sprague-Dawley rat; s.c., subcutaneous injection; TAS, tibialis anterior muscle; W, Wistar rat; NR, not reported.

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