No changes in muscle fibre type composition in rat multifidus muscle following lesion of the lumbar intervertebral disc

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SUMMARY

The multifidus muscle has been proposed to play an important role in the development and recurrence of low-back pain (LBP). In line with this, fibre type composition has been found to be altered in humans with LBP. This study aims to investigate the changes in muscle fibre type composition of the multifidus muscle after stab disc lesion in the rat.

Data were obtained from 24 male Wistar rats randomly assigned to the intervention group, in which the L4/L5 intervertebral disc was stabbed, or the control group, in which no intervention was applied. At 7, 14 and 28 days post-intervention, two fascicles of the multifidus muscle between L3 and S1 were removed bilaterally for analysis of fibre type composition. The rats' multifidus muscle consisted for the largest part of type IIB fibres in both the intervention (53 ± 10% across all time points) and the control group (53 ± 9% across all time points). We found no effects of disc lesion on the proportion of type I, IIA, IIX and IIB fibres. These results indicate that the fibre type composition of the multifidus muscle is not affected by disc lesion within the time period (28 days) studied. A different functional role of the multifidus muscle in the rat compared to humans, reflected in the high proportion of fast muscle fibres in the rat's multifidus muscle, may explain our findings. Differences between species in fibre type composition should be taken into account when using rats as a model to investigate the mechanisms causing (chronic) LBP in humans.

Key words: Low-back pain – Animal model – Spine – Degeneration – Muscle fibre type

INTRODUCTION

Chronic low-back pain (LBP) is a common disorder in humans (Heidari et al., 2015; Shraim et al., 2015). Despite its high prevalence, the mechanisms causing chronic LBP are not fully understood. Mechanical dysfunction of the spine, resulting in instability, has been suggested to be involved in the development of LBP (Panjabi, 1992). Three systems are involved in stabilizing the spine: the passive system including ligaments and intervertebral discs, the active system comprising the trunk musculature and the neural

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Submitted: July 31, 2020. **Accepted:** February 4, 2021

system (Panjabi, 1992). Dysfunction of one of these systems can lead to spinal instability and potentially contribute to LBP.

The three systems may also affect each other. Cortical and spinal cord excitability have been found to be changed after disc lesion (Hodges, 2009). This likely leads to an altered activation of parts of the active system (D'hooge et al., 2013; Hodges, 2009; Hodges et al., 2013; Hodges et al., 2006; MacDonald et al., 2011; MacDonald et al., 2009, 2010; Stokes and Young, 1984). Possibly mediated by such changes in activation, disc degeneration seems to induce degenerative changes in the multifidus muscle (Maas et al., 2018). The multifidus muscle is thought to play an important role in stabilization of the spine (Ward et al., 2009). Degeneration of the multifidus muscle may in turn cause increased and uncontrolled disc deformation, which eventually results in disc degeneration (Panjabi, 1992). Deformation of the intervertebral disc (IVD) has been related to low back pain, indicated by an odds ratio of 1.12 to 3.32 between degeneration and LBP (van Tulder et al., 1997). The interdependence of the stabilizing systems suggests that the multifidus muscle may have an important role in the development and rehabilitation of LBP.

In addition to microscopic structural alterations (Cagnie et al., 2015) and atrophy (Danneels, 2000; Hides et al., 2008; Hides et al., 1994) in the lumbar muscles, a change in the fibre type composition has been found in patients with LBP. In human skeletal muscle, three different fibre types can be identified based on their structural and functional characteristics: type I, type IIA and type IIX fibres. These fibre types form a continuum of increasing force production, increasing speed and decreasing endurance capacity respectively (Schiaffino and Reggiani, 2011). Specifically, type IIX fibres appear to occupy a larger area in patients with LBP than in control participants (Cagnie et al., 2015; Goubert et al., 2016; Mannion, 1999). The studies with human participants have a cross-sectional design, making identification of causal relationships impossible. Animal models appear warranted to unravel potential mechanisms underlying chronic LBP. Rats are widely used animal models to investigate surgically induced IVD degeneration (Shi et al., 2018). In contrast to humans, rat muscles have an additional fibre type—i.e., type IIB. Muscle fibres of this type are functionally comparable to the type IIX fibres found in humans. The type IIB fibres in rats have a greater cross-sectional area, can generate more force and shorten at higher velocities than the same type of fibres in humans (Schiaffino and Reggiani, 2011).

The multifidus muscle shows structural and functional similarities between rats and humans. In both rats and humans, the multifidus lies superficial of the spine and each fascicle spans up to three segments of the spine. Electromyography recordings in adult rats show tonic activity of the multifidus muscle during both rest and motor activity (Geisler et al., 1996). The continuous activity pattern is in line with the assumption that the multifidus muscle acts as a stabilizer as in humans (Ward et al., 2009). To our knowledge, the effects of disc lesion on fibre type composition have not been investigated in rats.

The aim of the present study was to investigate changes in muscle fibre type composition of the multifidus muscle after stab disc lesion in rats. We hypothesized that an IVD lesion would inhibit multifidus muscle activation, resulting in a transition from slow to fast muscle fibres.

MATERIALS AND METHODS

Animals

24 Adult male Wistar rats (Harlan Laboratories) were randomly divided in an intervention (stabbing the L4/5 disc) group (n=12) and a control group (n=12). Surgical and experimental procedures agreed with the guidelines and regulations concerning animal welfare and experimentation set forth by the Dutch law and were approved by the Committee on Ethics of Animal Experimentation at the Vrije Universiteit Amsterdam (FBW 13-03).

Surgical procedures and experimental setup

In the intervention group, buprenorphine (Temgesic®, Schering-Plough) was administered subcutaneously (0.1 ml/100 g body mass) half an

hour prior to the surgery. After being anesthetized, using isoflurane gas (1-3%), a tenotomy knife (blade length 30 mm, thickness 0.4 mm) was used to penetrate the IVD by 2.5 mm (up to nucleus pulposus) between lumbar vertebrae L4 and L5 using a transperitoneal-ventral approach (Rousseau et al., 2004). After surgery, rats were kept in a large cage (0.55×0.33×0.20 m) with access to food and water *ad libitum*. Up to two days after the surgery, carprofen (Rimadyl®, Pfizer) was administered subcutaneously (once a day, 0.1 ml/100 g body mass) to prevent pain. After administration of carprofen, no behavioural changes were observed. For more details see Maas et al. (Maas et al., 2018). The control group did not undergo surgery.

The control and intervention groups were further randomly divided in three subgroups: 7 days (n=4), 14 days (n=4) and 28 days (n=4). Body mass at the day of tissue harvesting, which is related to age (Huijing and Maas, 2016), was similar between time points (Table 1). At the different time points, the rats were deeply anesthetized with intraperitoneally injected urethane (for details see Maas et al., 2018) and the fascicle running from L3 to L6 and the fascicle running from L4 to S1 were removed as one piece at both sides (Figure 1). After surgery, the rats were euthanized by an intracardiac injection with an overdose Euthasol 20% (Euthasol®, AST farma, Oudewater, The Netherlands).

Excised multifidus fascicles were frozen with liquid nitrogen and stored in cryotubes at -80°C. The fascicles of the left side were sliced transversally (10 µm) in a cryostat (MICROM HM 550, Thermo Scientific™, Waltham, USA) at -22°C. First, 1/3 was cut off at both ends, ensuring that the slices contained both fascicles (Figure 2A). Three slices were put on a Superfrost Plus adhesion slide (Thermo Scientific™, Waltham, USA). The multifidus of one rat of the intervention group (14 days) could not be analysed, because the tissue was not of sufficient quality. Fibre-type composition was assessed by immunofluorescence analysis of myosin heavy chain (MHC) expression (Bloemberg and Quadrilatero, 2012), using primary antibodies BA-D5, SC-71, BF-F3, and 6H1, for MHC-I, MHC-IIA, MHC-IIB, and MHC-IIX, respectively and secondary antibodies Alexa Fluor 488 IgG_{2b}, for MHC-1, 647 IgM for MHC-IIB, and MHC-IIX and 448 IgG_1, for MHC-IIA (all from Fisher Scientific, Landsmeer, The Netherlands). To control for possible differences between the left and right sides, the right multifidus muscle of three rats was also investigated. We found no left-right differences in fibre type composition.

For further analysis, the stained slides were scanned using a fluorescence microscope (Axiovert 200, Zeiss, Jena, Germany) with a CCD camera (PCO AG, SensiCam, Kelheim, Germany) and the program Slidebook (version 5.0, Intelligent Imaging Innovations, inc., Denver, USA).

Measurements

Four squares, spatially distributed within the muscle section, were selected for analysis (Figure 2B). The number of fibres was counted manually, using ImageJ (http://imagej.nih.gov/ij). Each area contained a total of 60 to 70 fibres, the whole cross-section included about 600 fibres. In some muscles, the quality of the staining for type IIX fibres was poor. Therefore, all fibres not identified as type I, IIA or IIB were classified as type IIX fibres.

Statistics

To test for effects of IVD lesion on muscle fibre type proportion, a two-way ANOVA (SPSS version 24, IBM, Armonk, NY, USA) was applied for each of the fibre types, with between-subject factors time (7, 14 or 28 days) and intervention (disc lesion or

Table 1. Body mass (g) of rats at the day tissues were harvested.

	7-days post-op	14-days post-op	28-days post-op
IVD lesion	282 ± 9	300 ± 10	350 ± 11
Controls	254 ± 18	312 ± 11	360 ± 10

N = 4 for each experimental group and time point. *Note*. Adapted from "Effects of intervertebral disc lesion and multifidus muscle resection on the structure of the lumbar intervertebral discs and paraspinal musculature of the rat", by Maas et al. (2018), *Journal of Biomechanics*, 70: 228-234.

control). If a significant interaction was found, the effects of intervention were tested using an independent samples t-test with Bonferroni correction for each time point and the effects of time for each intervention were tested using a one-way ANOVA and Bonferroni post hoc tests.

RESULTS

For the proportion of type I, IIA, IIX and IIB fibres, neither significant effects of time or intervention were observed, nor a significant interaction (Tables 2, 3; Fig. 3). Rat multifidus muscle consisted for the largest part of type IIB

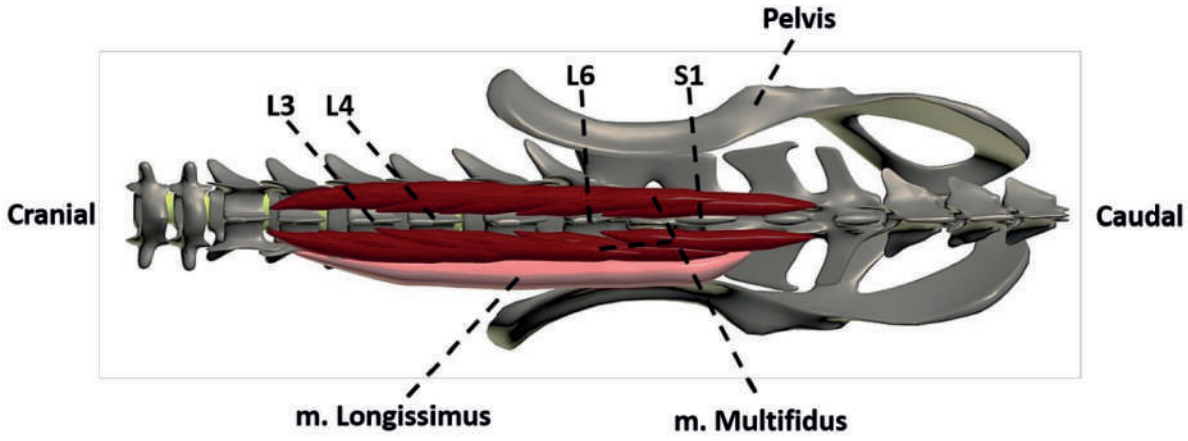

Fig. 1.- Dorsal view of the spine of a rat. The deep red muscle represents the multifidus muscle and the light red muscle represents the longissimus muscle. The fascicles of the multifidus muscle from L3 to L6 and from L4 to S1 were harvested.

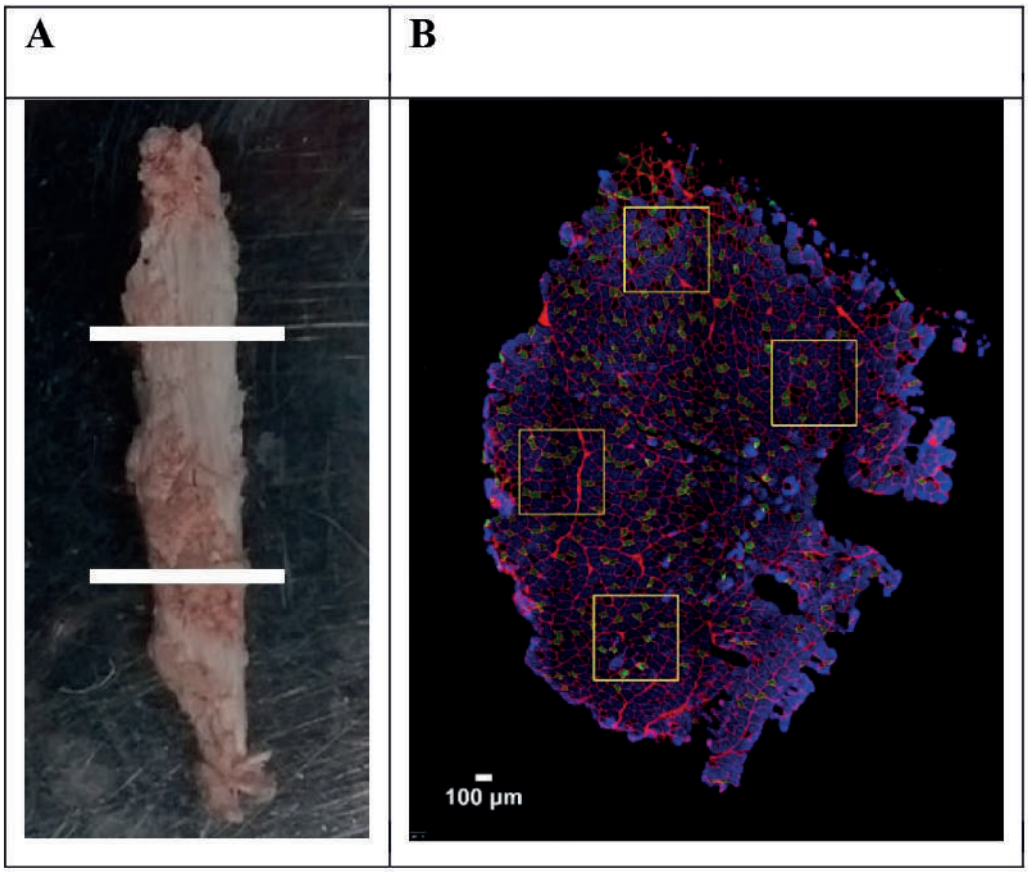

Fig. 2.- A: A typical example of a fascicle of which 1/3 of both sides was removed before the muscle was sliced; **B:** Typical sample of the chosen areas for the determination of the fibre type proportions.

fibres in both the intervention (53 ± 10% across all time points) and the control group (53 ± 9% across all time points).

DISCUSSION

The aim of this study was to investigate the effects of a lumbar disc lesion on fibre type composition of rat multifidus muscle. In contrast

Table 2. Results of the two-way ANOVA for the proportion of the different fibre types, including the degrees of freedom (df), F-value, p-value and power.

Fibre type		df	F-value	p-value	Power
Type I	Time	2,17	0.301	0.744	0.090
	Intervention	1,17	0.986	0.335	0.155
	Interaction	2,17	3.394	0.058	0.559
Type IIA	Time	2,17	0.154	0.859	0.070
	Intervention	1,17	0.037	0.849	0.054
	Interaction	2,17	1.215	0.321	0.229
Type IIX	Time	2,17	1.368	0.281	0.254
	Intervention	1,17	0.660	0.428	0.120
	Interaction	2,17	0.034	0.966	0.054
Type IIB	Time	2,17	0.998	0.389	0.195
	Intervention	1,17	2.147	0.162	0.281
	Interaction	2,17	3.438	0.056	0.565

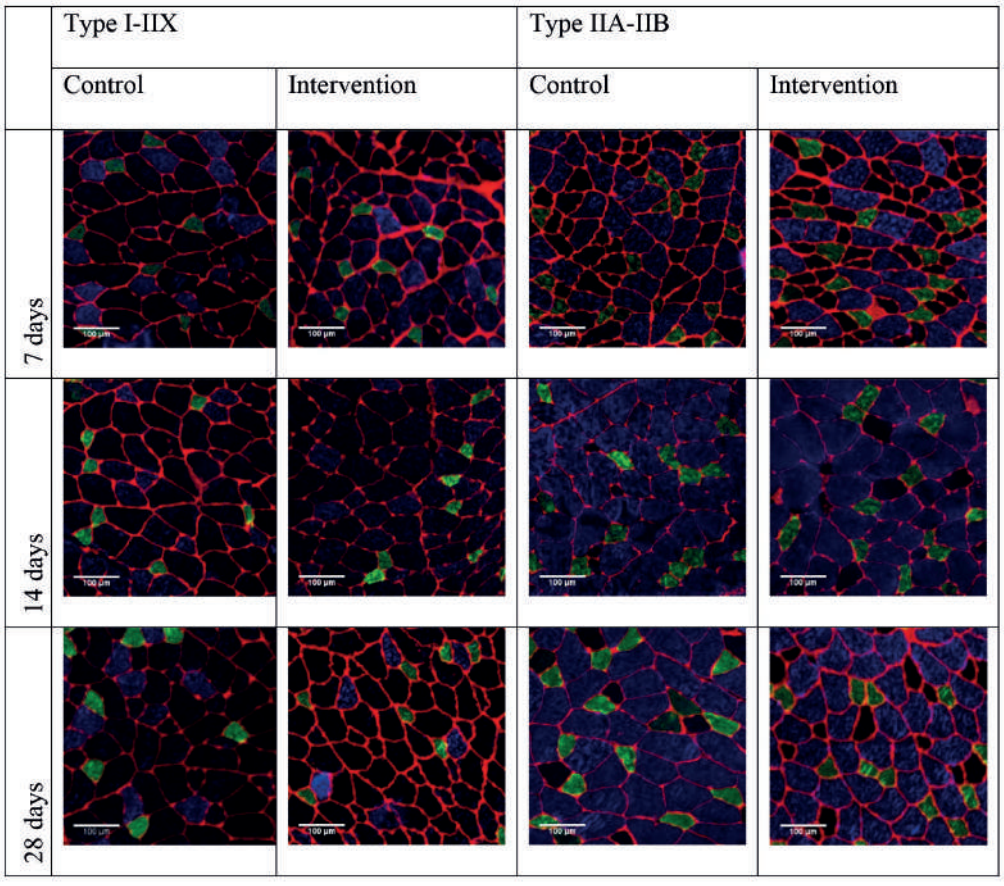

Fig. 3.- Fibre type staining for type I-IIX and type IIA-IIB in the intervention and control group. In the two left panels, green represents type I (antibodies BA-D5 and Alexa Fluor 488 IgG2b) and blue represents type IIX fibres (antibodies 6H1 and 647 IgM). In the two right panels, green represents type IIA (antibodies SC-71 and IgG1) and blue represents type IIB (antibodies BF-F3 and 647 IgM). The red lines represent the connective tissue, stained with wheat germ agglutinin.

with our hypothesis, the disc lesion did not affect the fibre type proportions within the first 28 days after the intervention.

The rats in our study received an anterior stab incision in the IVD to mimic spinal dysfunction, a potential cause of LBP in humans. The area occupied by type IIX fibres appears increased in people with LBP, compared to abled-bodied people (Cagnie et al., 2015; MacDonald et al., 2006; Mannion, 1999). In sheep, a transition from slow to fast muscle fibres at 24 weeks and 6 months after a lateral stab incision has been reported (Hodges, 2014, 2015). In contrast to the abovementioned studies, we did not find a similar transition in the rat model.

There are several possible explanations for our negative preliminary findings. The follow-up time (28 days) might have been too short to detect changes in fibre type composition. However, both overloading and unloading in the rat have resulted in significant fast-to-slow transition even within 14 days (Baldwin et al., 1990; Cornachione et al., 2008; De Souza et al., 2011; Degens et al., 2008; Feng et al., 2016; Fisher and Brown, 1998; Gardiner et al., 1991; Herbison et al., 1984; McCall et al., 2009; Oakley and Gollnick, 1985; Roy et al., 2005; Van der Meer et al., 2011; Watt et al., 1984), demonstrating the plasticity of the muscle tissue of rats. We cannot exclude that time has been a factor, but the plasticity of rat muscles makes it unlikely that the time frame is an explanation for our negative findings.

Another reason for our negative findings might be the ability of rats to recover from a disc lesion in a relatively short time. In two out of four rats analysed 28 days after stab incision, the lumbar IVD appeared to be recovered (Maas et al., 2018). Such resilience of the intervertebral discs was not found in sheep (Osti et al., 1990).

Finally, our negative findings may be explained by the functional role of the multifidus muscle of the rat, as reflected in its phenotype. Our findings indicate that the multifidus muscle in control rats contains predominantly (>60%) fast glycolytic (type IIX and IIB) fibres (Table 3), suggesting that this muscle is active predominantly during rapid movements. This differs substantially from the fibre type distribution and muscle function of the multifidus in humans. Human muscle does not express the type IIB myosin heavy chain isoform. In healthy humans, percentages of 54%-74.3% are reported for type I fibres, 16.4%-30.2% for type IIA fibres and 4.6%-22.34% for type IIX fibres (Cagnie et al., 2015; Goubert et al., 2016; Mannion, 1999). The high prevalence of fast glycolytic fibres has also been reported for rodents other than Wistar rats (Schilling, 2009), and follows the ancestral pattern for primates (Neufuss et al., 2014). The orthograde posture and locomotor behaviour of hominoid primates might be related to the slow phenotype of their back muscles (Neufuss et al., 2014). It should be noted that the fibre type composition of the multifidus in sheep, for which a slow-to-fast muscle fibre type transition was found (see above), is similar to rats, i.e., ±73% fast muscle fibres, ±23% slow muscle fibres and ±4% intermediate muscle fibres (Hodges, 2014). This suggests that not only the fibre type composition, but other anatomical or physiological differences (e.g., tissue resilience) are responsible for our negative results.

There are some limitations that should be considered. First, a substantial inter-individual variation was found in muscle fibre type

Table 3. General overview of the percentages of muscle fibre type proportions (mean ± SD) for the intervention and control group at 7, 14 and 28 days after the stab disc lesion.

	7 days		**14 days**		**28 days**	
	Control (n=4)	Intervention (n=4)	Control (n=4)	Intervention (n=3)	Control (n=4)	Intervention (n=4)
Type I	9±7	20±4	19±9	11±5	9±9	16±9
Type IIA	12±5	17±4	14±5	13±10	17±5	13±4
Type IIX	18±8	21±9	13±3	14±2	16±5	18±6
Type IIB	62±13	43±9	56±12	63±7	60±6	54±9

proportions. This will reduce the power to find small differences. A high variability in percentage type I fibres has also been reported for other muscles of the rat (Eng, 2008). The high variation could be a result of an inequal distribution of the muscle fibre types within the cross-section of the muscle of one sample. In this study, the four analysed areas were systematically chosen following predetermined rules (see Methods, Fig. 2), such that the areas were representative for the whole muscle.

Another limitation of this study is the absence of a sham lesion in the control group. It is possible that the surgery damaged the abdominal wall, which may have resulted in reduced physical activity of the rats. Considering that we observed no effects of multifidus resection on the IVD in our previous study (Maas et al., 2018), and no differences with the control group in the present study, we deem confounding by this limitation unlikely.

Variances found within the investigated groups are especially important in case of small sample sizes. The groups studied in this research included 4 (and in one group 3) animals. Despite the limited sample numbers, the relative area of the nucleus pulposus was significantly smaller after the stab incision in the same group of rats 14 days after intervention (Maas et al., 2018). Moreover, there was no main effect of intervention at all time points. Thus, our conclusion that fibre type composition was not affected by a disc lesion was actually based on twelve rats, not four.

We conclude that muscle fibre type composition of the multifidus muscle was not affected by disc lesion in rats within a four-week time period. Differences between quadrupedal mammals and humans in fibre type composition and tissue resilience should be considered when using rats as a model to investigate the mechanisms causing (chronic) LBP in humans.

ACKNOWLEDGEMENTS

We would like to thank Guus Baan for help with the surgical procedures and for the drawing shown in Figure 1. Paul Hodges is acknowledged for the discussions about this work.

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