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1 Effect of carcinogen 1,2-dimethylhydrazine treatment on fibre types in 2 skeletal muscles of male Wistar rats

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14 Short title: Effect of carcinogen on rat muscle fibres

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1 Summary

2 The cancerogen 1,2-dimethylhydrazine (DMH), widely used in the experimental animal model of
3 carcinogenesis, affects various organs, but its effect on muscle fibres is unknown. To evaluate the effect of
4 15-week DMH treatment on the fibre size and myosin heavy chain (MyHC) isoforms, which substantially
5 determine fibre types and their contractile characteristics, pure and hybrid fibre types were
6 immunohistochemically determined according to the MyHC isoform expression in soleus, extensor
7 digitorum longus, gastrocnemius medialis and lateralis muscles of DMH-treated and control male Wistar
8 rats. Whereas the size of fibres was mostly unaffected, the MyHC isoform expression was partially affected
9 in both gastrocnemius samples, but not in the soleus and extensor digitorum longus of DMH-treated rats. The
10 lower proportions of hybrid fibre types and especially that of type 1/2x in most gastrocnemius samples of
11 DMH-treated rats resulted in a shift towards a single MyHC isoform expression, but the extent and pattern of
12 the MyHC isoform shift varied across the different gastrocnemius samples. Such variable response to DMH
13 treatment across muscles indicates that each muscle possesses its own adaptive range.. These findings are
14 essential for an accurate evaluation of skeletal muscle characteristics in DMH animal model.

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16 **Key words:** Carcinogen, Immunohistochemistry, Myosin heavy chain isoform, Rat, Skeletal muscle

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1 **Introduction**

2 The 1,2 dimethylhydrazine (DMH) or azoxymethane (AOM) model is a well-established and widely used
3 model of experimental colon carcinogenesis (Corpet and Pierre 2005). DMH and its metabolite AOM are
4 carcinogens that induce multistep development of colon carcinogenesis in rodents (Perše and Cerar 2011).
5 The susceptibility for DMH/AOM-induced colorectal carcinogenesis is species-, strain-, sex- and age-
6 dependent (Kobaek-Larsen *et al.* 2000). The stage and the number of the colonic lesions depend on the total
7 amount of the carcinogen administered and the latency period. In long-term studies DMH is administered
8 weekly for 15-20 weeks in a relatively low concentration (20 mg/kg), and 6-10 weeks after the last
9 application the animals are scored for all the steps of colonic lesions (dysplastic crypts, adenomas,
10 adenocarcinomas) (for review see (Perse and Cerar 2005, Perše and Cerar 2011). At that point colonic
11 lesions are small enough that do not affect the animal's health status but nonetheless make it possible to
12 evaluate the effect of the tested factor on the multistep development of colon carcinogenesis (Bruce 2003). In
13 our laboratory the effect of various factors on DMH-induced colon carcinogenesis is usually evaluated 6
14 weeks after the last application of DMH (20 mg/kg per week for 15 weeks) (Perse *et al.* 2009, Perše *et al.*
15 2012).

16 Although DMH and AOM are considered highly specific colon carcinogens, they affect other organ systems
17 as well, like the liver (Netto *et al.* 1992, Perse *et al.* 2009) and may cause tumours of the Zymbal glands (i.e.
18 modified sebaceous glands at the base of external auditory canal) in a small percentage of animals (Shetye *et*
19 *al.* 1994). Further, it was reported that the oxidative status in the circulation (Devasena *et al.* 2006) and heart
20 (Perse *et al.* 2009) were affected. Moreover, in a mouse DMH model it was shown that the catabolism and
21 energy production of skeletal muscle were affected by a high-mobility group box 1 protein, released by
22 tumour cells. This protein not only increased the proliferation of tumour cells, but it also activated muscle to
23 supply glutamine as an energy source to cancer cells (Luo *et al.* 2014).

24 Skeletal muscles are known to be susceptible to various factors, such as a changed pattern of neural
25 discharge, muscle activity, loading and hormonal status. Since the skeletal muscles are not only a locomotor
26 organ, but are the metabolic engine of the body and have immunogenic and endocrine functions as well
27 (Nielsen and Pedersen 2008, Pedersen *et al.* 2007), it would be of a great importance for all the studies that
28 employ this experimental model to evaluate which skeletal muscle components are affected by DMH
29 treatment. As the metabolism, size and contractile characteristic of muscle fibres are interrelated, we

1 postulated that not only metabolism (Luo *et al.* 2014) but muscle fibres could be affected by DMH treatment
2 as well. However, the effect of DMH treatment on the muscle fibre characteristics has not yet been studied.
3 Skeletal muscles can adapt to changed conditions with a shift in the expression of myosin heavy chain
4 (MyHC) isoforms, which are one of the most relevant markers of fibre types and their contractile
5 characteristics (Baldwin and Haddad 2001, Schiaffino *et al.* 2013). There are four major MyHC isoforms
6 that determine slow or type 1 fibres and fast type 2a, 2x (2d) and 2b fibres in rat skeletal muscles (Pette and
7 Staron 1997, DeNardi *et al.* 1993). In addition to the major four pure fibre types, hybrid fibres co-expressing
8 mainly two MyHC isoforms are present as well (Talmadge 2000, Baldwin and Haddad 2001, Caiozzo *et al.*
9 2003). The shift in MyHC isoform expression leads to fibre type transitions (Pette and Staron 1997, Baldwin
10 and Haddad 2001, Schiaffino *et al.* 2013). Beside the MyHC expression even the size of fibres can be
11 modulated by various factors. Namely, resistance training, overload and certain humoral factors (androgens,
12 β-agonists, IGF-1) induce fibre hypertrophy, whereas decreased activity or disuse, denervation, decreased
13 loading and increased level of glucocorticoids result in fibre atrophy (Blaauw *et al.* 2013).

14 Since the DMH animal model is frequently used in dietary and exercise studies (Perse *et al.* 2009, Perše *et*
15 *al.* 2012), it is essential to evaluate the potential effect of DMH treatment on skeletal muscle fibres for an
16 accurate and valid interpretation of the results. The aim of this study was thus to evaluate the effect of long-
17 term DMH treatment on skeletal muscles by analysing the expression of MyHC isoforms
18 immunohistochemically and the fibre type size morphometrically. Since each muscle has its own
19 characteristic range of possible extent of fibre type transitions, assumed to be related not only to the muscle
20 intrinsic myogenic or genetic constraints (Schiaffino and Reggiani 2011) but also sex-dependent (Drzymala-
21 Celichowska *et al.* 2012), various hind limb muscles of male rats were analysed.

22

1 Materials and methods

2 Animals and experimental protocol

3 Male Wistar (HsdRccHanTM: WIST) rats were used in the study. They were acclimatized and housed at
4 Medical Experimental Centre (Ljubljana, Slovenia) 4 per cage (1825 cm² floor space) on Lignocel ¾
5 bedding material (Germany) at 22-23°C and 55± 10% humidity, 12 h light/dark cycle (illumination between
6 07:00 p.m. and 07:00 a.m.) and had free access to food (Altromin 1324, Germany) and tap water. Twelve-
7 week-old rats were randomly divided into two groups, namely a DMH-treated (n=6) group and an untreated
8 control group (n=5). The rats were treated either with DMH (20 mg/kg, dissolved in 0.001 M EDTA; pH 6.8)
9 or saline (0.9 % NaCl) instead of DMH, both administered subcutaneously once a week for 15 consecutive
10 weeks (Perse et al., 2009; Perše et al., 2012). The animals of both groups were euthanized 6 weeks after last
11 DMH or saline treatment at age of 33 weeks. The care and use of animals was approved by the National
12 Ethic Committee of the Republic of Slovenia (Licence No. 34401-61/2007/7) and conducted in accordance
13 with the European Convention ETS 123, Directive 86/609/EEC regarding the protection of animals used for
14 experimental and other scientific purposes and Slovenian legislation on the protection of animals used in
15 research.

16 The body weight of the rats was recorded once a week, water and food intake was recorded three times per
17 week during the entire experiment. Before and during the experiment blood was taken for measurement of
18 red and white blood picture and serum lipid profile. Faecal corticosterone levels were measured during the
19 experiment. At autopsy all internal organs, including abdominal fat were removed, weighed and
20 macroscopically examined (Perse et al. 2009; Perše et al. 2012).

21

22 Muscle samples and MyHC isoform immunohistochemistry

23 After euthanasia four skeletal muscles, known to differ in the fibre type proportions, were excised from the
24 right hind limb of both experimental groups: slow soleus (SOL), fast extensor digitorum longus (EDL), and
25 the heterogeneous gastrocnemius lateralis (GL) and medialis (GM) muscles. For the latter two muscles a
26 non-random distribution of fibre types is known, with the prevalence of slower ones in the deep region and a
27 gradual change to the superficial region with the predominance of faster ones, (Matsakas *et al.* 2006). GL
28 and GM samples were therefore further divided into a superficial part (GLs, GMs) and a deep part (GLd,

1 GMd), whereas the intermediate, mixed part of GL (GLi), containing more fast fibre types than GLd and less
2 than GLs, was also separately analysed. Whole muscle samples were frozen in liquid nitrogen and stored at -
3 80° C until being processed for immunohistochemistry.

4 Fibre types were determined according to the expression of MyHC isoforms demonstrated with a set of
5 monoclonal antibodies specific to MyHC isoforms: BA-D5 (MyHC-1) and SC-71 (MyHC-2a), BF-F3
6 (MyHC-2b) (Schiaffino 1986), and 6H1 (MyHC-2x) (Lucas *et al.* 2000). The supernatants of BA-D5, SC-71,
7 and BF-F3 antibodies were produced in the local laboratory from corresponding cell lines provided from
8 Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), whereas
9 that of 6H1 was purchased from the Developmental Studies Hybridoma Bank (DSHB, University of Iowa,
10 USA).

11 Serial muscle cryosections (10 µm) were pre-incubated in phosphate buffered saline, containing 0.5% bovine
12 serum albumin (PBS/BSA) and rabbit serum (1:40), for 30 minutes. The appropriate dilutions of antibodies
13 with PBS/BSA were determined (BA-D5 1:500; SC-71 1:200; 6H1 1:50; BF-F3 1:20). Subsequently, the
14 sections with primary antibody were incubated in a humidified box overnight at 4°C. As a control to each set
15 of the analysed samples, a slide with serial sections was simultaneously incubated in PBS/BSA, but without
16 the primary antibody. The reactivity of the antibodies BA-D5, SC-71 and BF-F3 was revealed with
17 horseradish peroxidase conjugated secondary antibody (P260, Dako), diluted (1:100) in PBS/BSA,
18 containing rabbit serum (1:40). To reveal the secondary antibody binding, the sections were incubated in
19 0.05% diaminobenzidine tetrahydrochloride hydrate (DAB) and 0.01% H₂O₂ in 0.2 M acetate buffer (pH 5.2)
20 for approximately 7 minutes in the dark (Gorza 1990, Smerdu and Soukup 2008). The binding of 6H1 was
21 demonstrated using NovoLink Polymer Detection System and following the instructions of the producer
22 (Leica Biosystems, Newcastle, UK). The control sections were respectively incubated either with the
23 secondary antibody or NovoLink Polymer Detection System.

24

25 *Muscle section analysis*

26 Serial muscle sections were analysed using a computer-assisted system for image analysis, developed in our
27 laboratory in the collaboration with colleagues from Academy of Sciences of the Czech Republic (Karen *et*
28 *al.* 2009). Briefly, in each muscle section stained with different antibody specific to MyHC isoform, the
29 same selected area was registered by a digital camera, connected to a microscope. Thereafter, the registered

1 images were adjusted to each other (*Muscle Reg program*) and then on the average 170 fibre contours were
2 outlined manually in one of the registered images using a program *Ellipse* (ViDiTo, Košice, Slovakia). The
3 set of registered and adjusted images with the superimposed fibre contours was used as an input data to
4 computer program (*FibClasM program*) to determine the labelling pattern of fibres with different antibodies.
5 Finally, using several macros (Microsoft Excel), fibre types were determined according to the labelling
6 pattern of fibres. In addition, the average diameters of fibre types were computed from the fibre contours.

7

8 *Statistics*

9 The proportions (%) and the average diameters (μm) of fibre types were determined within each muscle or
10 muscle region (SOL, EDL, GMd, GMs, GLd, GLi, GLs) of control (n=5) and DMH-treated rats (n=6).
11 Thereafter the mean proportion and average diameter of each fibre type was determined for each of the
12 analysed muscles of both groups. Descriptive statistics were used to calculate means, standard errors of the
13 means, and the ranges of all variables. To determine whether the differences between the two groups were
14 significant the means of the same fibre type in the homonymous muscles of both groups were compared
15 either by *t*-test or *Mann-Whitney* test, depending on the distribution of the standard deviations of means,
16 which was determined by a *Shapiro-Wilks* test. The data are presented as a mean \pm standard error of the
17 mean (SEM). The differences with $p < 0.05$ were considered as significant, whereas the differences with $p =$
18 0.05 - 0.1 were considered as trends for differences. Data were analysed using SPSS Statistics program
19 Version 20.

20

1 Results

2 *Health status of animals*

3 The general health status, locomotion, behaviour, and body weight of DMH-treated rats did not differ from
4 those of the control rats throughout the experiment. No colonic lesions were found in the control group,
5 while all rats in the DMH-treated group developed small, microscopically visible dysplastic colon lesions
6 (6.75 ± 0.82 foci of dysplastic crypts, 0.5 ± 0.27 adenomas, 0.38 ± 0.26 adenocarcinomas). DMH-treated rats
7 exhibited no signs of cancer-induced cachexia or muscle wasting (Perse *et al.* 2009, Perše *et al.* 2012).

8 The body weights of rats were comparable in both groups during the entire experiment, as well of food,
9 calorie and water intake. Autopsy showed no significant differences in the relative weights of internal organs
10 or abdominal fat between groups. No differences were found in the serum lipid profile (cholesterol, LDL,
11 HDL, trygliceride), blood parameters, serum LDH levels, or corticosterone levels between both groups
12 (Perse *et al.* 2009, Perše *et al.* 2012), demonstrating that the rats in both goups had comparable clinical and
13 physical status..

14 *Fibre types*

15 Four pure fibre types, expressing a single MyHC isoform (1, 2a, 2x, or 2b), and hybrid fibre types, co-
16 expressing two or even more MyHC isoforms were determined for each muscle sample (Fig. 1 and 2). In the
17 analysed muscles 11 hybrid fibre types (1/2a, 1/2ax, 1/2axb, 1/2x, 1/2xb, 1/2ab, 1/2b, 2ax, 2ab, 2axb, 2xb)
18 were determined according to the pattern of MyHC isoforms' co-expression. Some of the hybrid fibre types
19 were very scarce and present only in few muscle samples (Table 1). In fact, all 11 hybrid fibre types were
20 found only in the EDL of the control animals, whereas only hybrid types 2xb, 1/2x, 2ax, and 1/2a were more
21 frequent, present in more than one third of the analysed muscle samples. Therefore, to reveal an eventual
22 effect of DMH treatment on the muscle fibres, the average proportions and diameters of pure fibre types,
23 four of the most numerous hybrid fibre types, 2xb, 1/2x, 2ax, and 1/2a, whose average proportion was above
24 2% and were present in the majority of muscle samples, and of pooled all hybrid fibre types (H) of control
25 and DMH-treated rats were compared (Fig. 3 and 4).

26

27 *Fibre type transitions*

1 DMH treatment induced significant differences in the average proportions of some fibre types in GLd, GLi,
2 GMd and GMs samples only (Fig. 3). The proportion of type 1 and 2a fibres significantly increased in GLd,
3 whereas that of the hybrid 1/2x fibres significantly decreased (Fig. 1). These results and a trend of type 2x
4 proportion decrease imply transitions from faster fibre types, especially from type 2x, towards slower ones (1
5 and 2a) in GLd. Similar but less pronounced trend was evident in GLi as well. The proportions of the three
6 most numerous hybrid fibre types, 1/2x, 2ax, and 2xb, decreased significantly, which had obviously resulted
7 in a significant increase of type 1 and also in a slight increase of type 2a and 2x proportions. Like in GLd and
8 GLi, in GMd of DMH-treated rats the proportion of hybrid type 1/2x significantly decreased and was
9 accompanied with a trend of type 2a and 2x proportions increase implying trends of transitions towards these
10 two fast fibre types. In GMs only the proportion of hybrid type 2xb and pooled hybrid fibres decreased
11 significantly. To summarize, in all gastrocnemius samples DMH treatment induced either a significant
12 decrease or a trend of pooled hybrid fibre types to decrease (Fig. 3, H). On the contrary, in the SOL and EDL
13 muscles of DMH-treated rats there were no significant shifts in the fibre types, while in EDL muscle there
14 was only a trend for the type 1 and 1/2x proportion decrease.

15

16 *Changes in the fibre type size*

17 To evaluate whether DMH treatment affected the size of the various types of fibres, the average diameters of
18 the four pure and four of the most numerous hybrid fibre types within homonymous muscles of both animal
19 groups were compared (Fig. 4). The average diameters of most fibre types did not differ significantly
20 between the two groups, only those of type 2b in GMd and EDL and that of type 2ax in GLs increased
21 significantly in the DMH-treated group. Nevertheless, the average diameters of most fibre types had
22 tendency to increase in DMH-treated rats, especially those of type 2a in GLi, type 2a and 2x in GLs, and type
23 2xb in EDL.

24

1 Discussion

2 In the present study we have demonstrated that 15-week DMH treatment had only a mild effect on the fibre
3 type transitions in some rat skeletal muscles, but had almost no significant effect on the fibre type size.
4 Cancerogenesis itself is among the potential factors that affect the skeletal muscle. Namely, in its progressive
5 stage the cancerogenesis is associated with cachexia, muscle wasting, and fibre atrophy, preferentially that of
6 the fastest type 2 fibres in the fast muscles (Acharyya *et al.* 2005, Ciciliot *et al.* 2013) due to increased
7 degradation of proteins (Llovera *et al.* 1994, Temparis *et al.* 1994, Baracos *et al.* 1995, Lecker *et al.* 2004,
8 Acharyya *et al.* 2005). In our study DMH treatment did not induce fibre atrophy; on the contrary, the average
9 diameters of fibre types had a tendency to increase in DMH-treated rats. Such findings are in accordance
10 with the fact that our experiment was concluded before the rats developed any signs of cachexia or muscle
11 wasting (Perse *et al.* 2009, Perše *et al.* 2012).

12 Our result showed that DMH treatment did not severely affect MyHC isoform expression. Namely, only
13 some significant fibre type transitions, mostly from hybrid towards pure fibre types, were evident in the
14 gastrocnemius samples of DMH-treated rats, but were negligible in SOL and EDL. Such differing responses
15 to DMH treatment across the analysed muscles are not surprising, since each muscle possesses its own
16 “adaptive range” for fibre type transitions (Schiaffino and Reggiani 2011). The muscle’s adaptive capacity is
17 assumed to be related to its original and genetically determined fibre type composition, appropriate to the
18 muscle’s functional role and may be also species-specific (Blaauw *et al.* 2013). However, it can be
19 secondarily modulated by various extrinsic factors such as the type of innervation or electrical stimulation,
20 muscle loading or activity level, and hormonal factors, especially thyroid hormones (Pette and Staron 1997,
21 Baldwin and Haddad 2002, Schiaffino and Reggiani 2011, Blaauw *et al.* 2013, Soukup and Smerdu 2015).

22 The most surprising finding of our study was a pronounced transition of hybrid fibre types towards pure ones
23 in gastrocnemius samples of DMH-treated rats. Namely, in the past it was considered that hybrid fibres are
24 the result of pure fibre types undergoing transitions through the shift in MyHC isoform expression and that
25 the co-expression of MyHC isoforms occurs due to relatively slow protein turnover, i.e. about 30 days. Thus,
26 they were assumed to be more numerous in muscles under changed physiological conditions (Pette and
27 Staron 1997, Talmadge 2000, Baldwin and Haddad 2001, Schiaffino and Reggiani 2011), as found for
28 instance after denervation (Patterson *et al.* 2006). Therefore, higher proportions of hybrid fibre types would
29 be expected in DMH-treated rats. Nevertheless, our findings are in agreement with more recent reports on

1 numerous hybrid fibres in non-transforming muscles (DeNardi *et al.* 1993, Staron *et al.* 1999, Caiozzo *et al.*
2 2003, Stephenson 2001, Glaser *et al.* 2010).

3 Given that the proportions of the pooled hybrid fibre types decreased in DMH-treated rats, we assume that
4 such a decrease may be the result of kind of “instability” or “readiness” of the hybrid fibres to more rapidly
5 adapt with a shift towards the expression of only one of the primary co-expressed isoforms. For instance, in
6 GLd the decrease in the proportion of 1/2x fibres was accompanied with a substantial increase of type 1
7 fibres proportion. Similarly, a resistance training in humans resulted in the reduction of hybrid fibre type
8 proportions with concomitant increase of pure fibre type proportions (Williamson *et al.* 2001). A non-
9 uniform, but muscle-specific transition of hybrid fibres towards pure ones was demonstrated in some
10 maturing mouse skeletal muscles as well, but in some muscles (SOL) hybrid fibres persisted (Brummer *et al.*
11 2013). Moreover, it was demonstrated that different patterns of exercise not only differently affected the
12 proportions of human hybrid type 1/2a and 2ax fibres but the proportions of the co-expressed MyHC
13 isoforms within a single hybrid fibre as well (Kohn *et al.* 2007). These results suggest that the co-expressed
14 MyHC isoforms in a hybrid fibre are under independent regulatory control.

15 Another unexpected finding was the second most numerous hybrid fibre type 1/2x, which does not conform
16 to the proposed way for fibre type transitions following the principle of the “next-neighbour” MyHC isoform
17 co-expression ($1 \leftrightarrow 1/2a \leftrightarrow 2a \leftrightarrow 2ax \leftrightarrow 2x \leftrightarrow 2xb \leftrightarrow 2b$) (Pette and Staron 1997). Finding quite a lot of 1/2x fibres
18 in GLd (18.5%), GMd (8.3%) and GLi (5.4%) samples of control rats, we initially assumed that the antibody
19 specific to MyHC-2x cross-reacted with MyHC-1. Since such staining was neither apparent in all fibres
20 expressing MyHC-1, nor in all muscles, and since 1/2x fibres were less numerous in the above-listed muscles
21 of DMH-treated rats, we excluded the cross-reactivity. Quite high proportions of 1/2x fibres, demonstrated in
22 the hind limb muscles and diaphragm of Wistar-Kyoto and Sprague-Dawley rats by single fibre
23 electrophoresis (Bortolotto *et al.* 2000, Caiozzo *et al.* 2003), imply that in the past their appearance was
24 underestimated in immunohistochemical studies due to the lack of an antibody exclusively specific to
25 MyHC-2x. Therefore, we assume that 1/2x fibres are not only rapidly transforming, the so called “jump”
26 fibres, skipping the gradual transition as previously suggested (Andersen *et al.* 1999, Talmadge 2000), but
27 they more likely represent a physiological fibre type, probably possessing specific contractile characteristics.
28 Thus hybrid fibres should be considered not only as transforming fibres but obviously as a normal
29 physiological fibre type as well. The physiological implications of such single fibre polymorphism are not

1 clearly understood. It is assumed that hybrid fibre types represent a physiological continuum of fibres with a
2 continuum of contractile properties, which expand the functional repertoire of a skeletal muscle and also
3 enable smoother fibre type transitions and fine tuning with changed physiological demands.

4 Analysing just MyHC isoform expression, we can only speculate about the cellular and molecular
5 mechanisms that could underlay the DMH-induced fibre type transitions. As in the intestine DMH treatment
6 affects various signalling pathways and molecules (Roy *et al.* 2001, Kolligs *et al.* 2002, for review see Perše
7 and Cerar 2011), it might affect even those implicated in the regulation of MyHC gene expression. For
8 example, the Wnt/β-catenin pathway, which interacts with the other, so-called non-canonical Wnt/calcium
9 pathway (Sugimura and Li 2010, Thrasivoulou *et al.* 2013), is substantially affected in the colorectal
10 carcinogenesis (Kolligs *et al.* 2002). The latter is known to regulate intracellular calcium levels (Komiya and
11 Habas 2008), which are one of the main activity correlates that activate the signalling pathways regulating
12 the fibre type transitions (Chin *et al.* 1998, Crabtree and Schreiber 2009, Gundersen 2011, Schiaffino and
13 Reggiani 2011, Blaauw *et al.* 2013). DMH treatment also affects peroxisome proliferator-activated receptor δ
14 (PPARδ) (Chung 2000, Takayama *et al.* 2006), a signalling molecule that is a member of nuclear receptors
15 with multiple functions and is importantly implicated in the regulation of fibre type transitions as well
16 (Mahoney *et al.* 2005, de Wilde *et al.* 2008). Another signalling pathway affected in colorectal
17 carcinogenesis is TGFβ (Chung 2000, Markowitz *et al.* 1995). This or more precisely the myostatin-Smad
18 2/3 pathway is assumed to be implicated in the onset of muscle atrophy (Ciciliot *et al.* 2013, Blaauw *et al.*
19 2013, Schiaffino *et al.* 2013).

20 Another potential mechanism that might be implicated in DMH-induced fibre type transitions is the central
21 nervous system, since the pattern of neural impulses is one of the major factors that determine the muscles'
22 fibre type composition (Schiaffino and Reggiani 2011, Blaauw *et al.* 2013). Some studies demonstrated that
23 DMH affected diurnal levels of neurotransmitters (norepinephrine, dopamine, metabolite of serotonin) in
24 hypothalamic nuclei in rats (Arutjunyan *et al.* 2001) and brain neurotransmitter status by increasing brain
25 epinephrine and decreasing brain serotonin and norepinephrine levels (Pandey *et al.* 2015). Since increased
26 levels of brain serotonin are associated with increased fatigue (Bailey *et al.* 1993), increased daily activities
27 of DMH-treated rats with presumably lower level of brain serotonin would be expected. However, long
28 term effects of increased daily activities reflect in various clinical or biochemical characteristics such as
29 caloric intake, body weight, serum lipids profile, etc. Since on one hand Pandey *et al.* (2015) did not analyse

1 any of these parameters and on the other hand we found no significant differences in various clinical and
2 biochemical parameters (i.e. daily food or caloric intake, body weight gain, abdominal fat, the level of LDH
3 in the serum, the level of corticosterone, relative weight of internal organs, or lipids in the blood) between
4 the control and DMH-treated rats (for more detail see Perse *et al.* 2012), further studies are necessary to
5 elucidate whether the changes in the brain neurotransmitters are caused directly by DMH or are the
6 consequence of the toxic effect of DMH on other organs and tissues. In addition it is also possible that
7 DMH-induced fibre type transitions might result from DMH side effects on the motor system by subtly
8 altering the pattern of nerve impulses to motor units. Nevertheless, further studies are necessary to reveal the
9 cellular and molecular mechanisms underlying the DMH-induced fibre type transitions.
10 In conclusion, the present study showed that 15-week DMH treatment may affect skeletal muscle fibres to
11 some extent, but, as demonstrated, the effect is muscle-specific and should not be generalized. This findings
12 must be taken into consideration not only when evaluating various skeletal muscle characteristics or
13 alterations in DMH-treated animal models, but also in generally, when evaluating the capacity of skeletal
14 muscles to adapt to changed physiological and pathological conditions.

15

16 **Conflict of Interest**

17 There is no conflict of interest.

18

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23

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17

1 **Table 1.** Range of the average proportion (%) per muscle sample and frequency (n) of individual hybrid
 2 fibre types in the analysed muscle samples (totally 77 samples) of control and DMH-treated rats.

Range of the average proportion/muscle sample (%)	Hybrid fibre type		
0–0.2	1/2axb (n = 1/77)	1/2ab (n = 4/77)	1/2b (n = 4/77)
0–0.4	1/2ax (n = 10/77)	1/2xb (n = 9/77)	
0–2.0	2ab (n = 14/77)	2AXB (n = 12/77)	
0–8.0	2ax* (n = 53/77)	1/2a* (n = 33/77)	
0–22.0	2xb* (n = 53/77)	1/2x* (n = 33/77)	

3 n: number of samples in which an individual hybrid fibre type was present

4 *Note that type 2xb, 1/2x, 2ax and 1/2a fibres were the most frequent hybrid fibre types

5

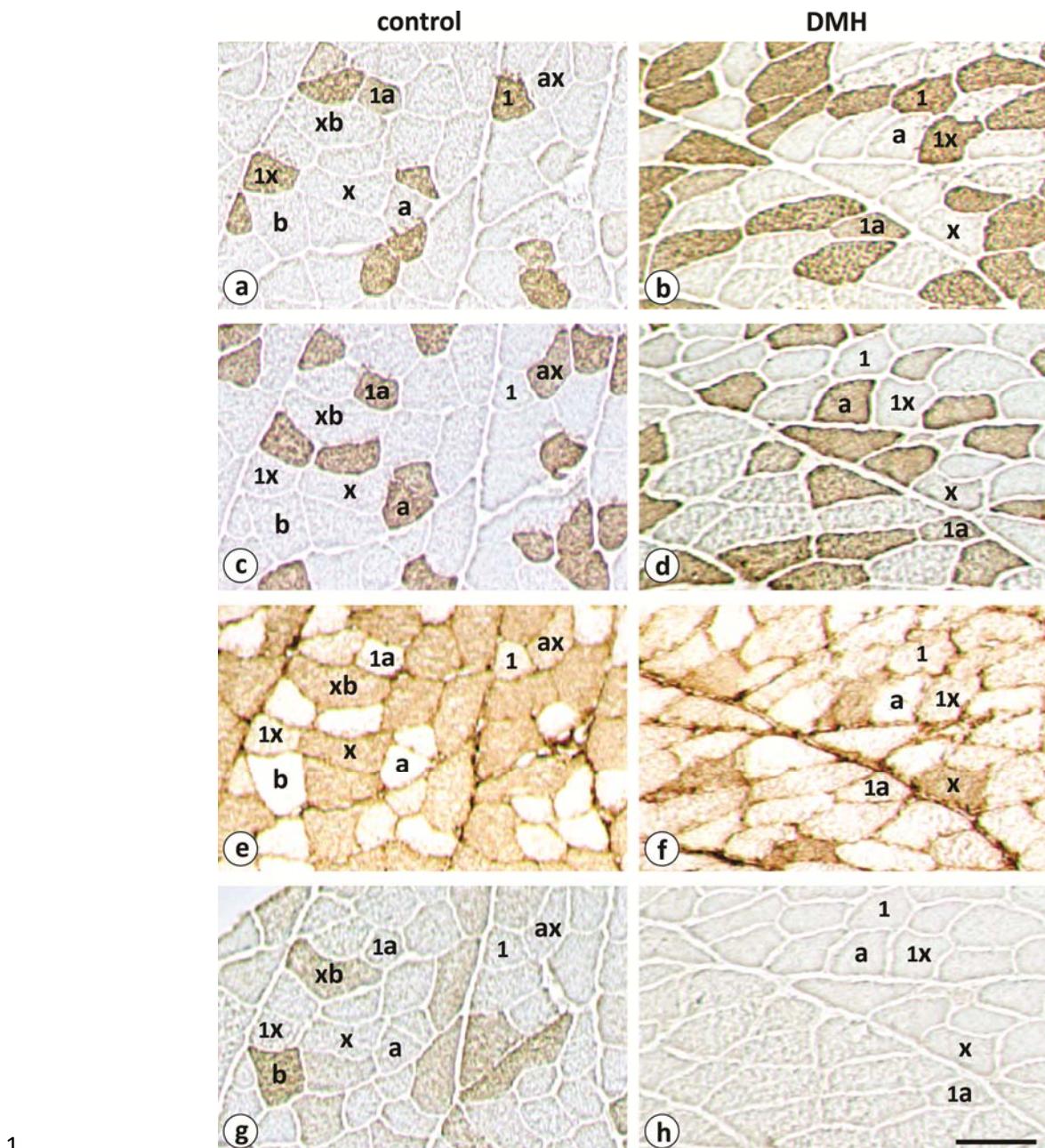
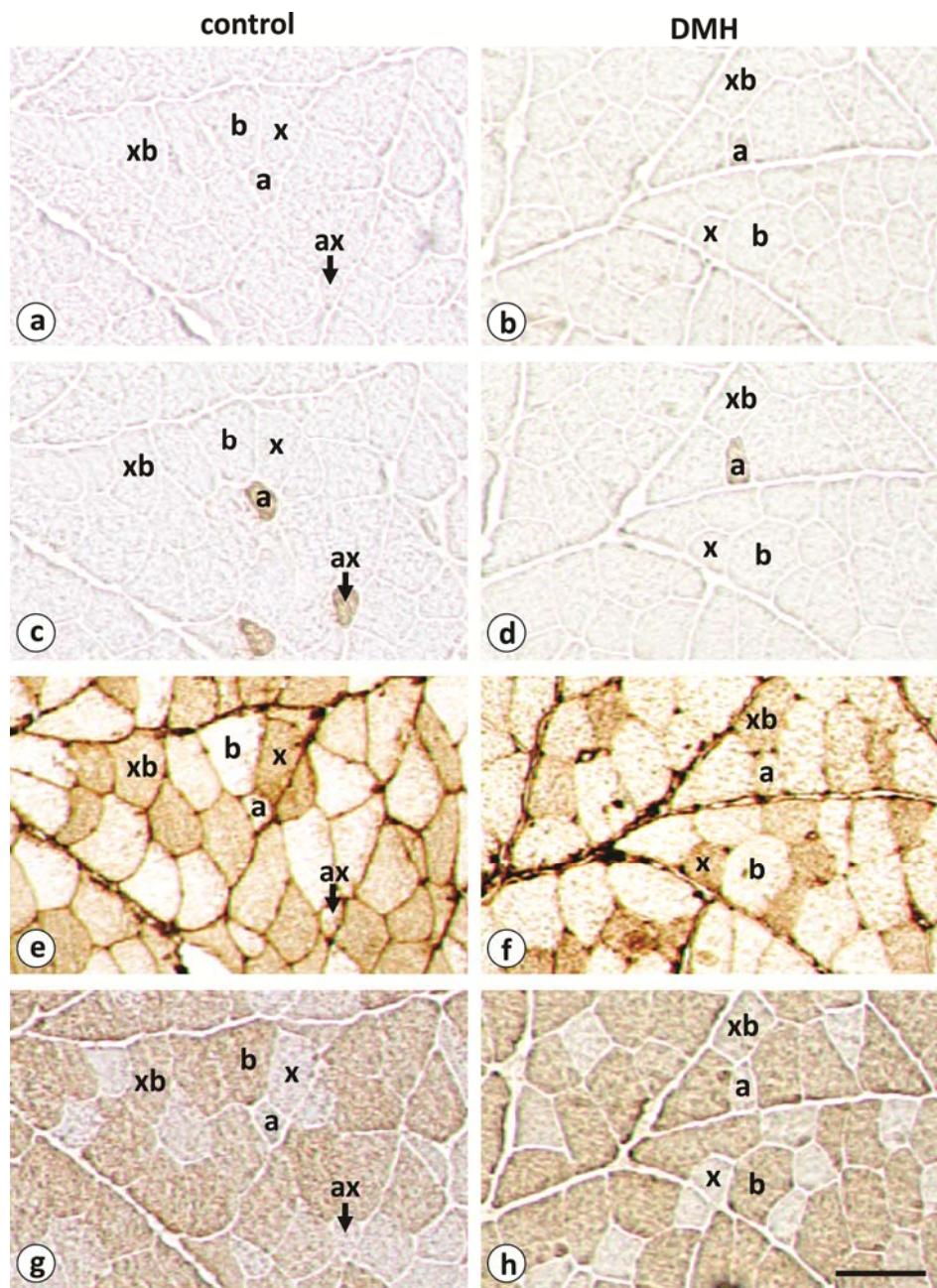


Fig. 1. Fibre types in the deep portion of gastrocnemius lateralis (GLd) muscle of control (a, c, e, g) and 1,2-dimethylhydrazine (DMH) treated (DMH-treated) rat (b, d, f, h). The fibre types were immunohistochemically determined according to the MyHC expression with monoclonal antibodies: BA-D5 specific to MyHC-1 (A, B), SC-71 specific to MyHC-2a (C, D), 6H1 specific to MyHC-2x (E, F) and BF-F3 specific to MyHC-2b (G, H). Note that individual fibre types are respectively labelled as follows: 1=1, 1/2a=1a, 1/2x=1x, 2a=a, 2ax=ax, 2x=x, 2xb=xb, 2b=b. Scale bar = 100 μm .



1 **Fig. 2.** Fibre types in the superficial portion of gastrocnemius lateralis (GLs) muscle of control (a, c, e, g)
 2 and 1,2-dimethylhydrazine (DMH) treated (DMH-treated) rat (b, d, f, h). The fibre types were
 3 immunohistochemically determined according to MyHC expression with monoclonal antibody BA-D5
 4 specific to MyHC-1 (A, B), SC-71 specific to MyHC-2a (C, D), 6H1 specific to MyHC-2x (E, F) and BF-F3
 5 specific to MyHC-2b (G, H). Note that type 1 fibres were absent and that individual fibre types are
 6 respectively labelled as follows: 2a=a, 2ax=ax, 2x=x, 2xb=xb, 2b=b. Scale bar = 100 μm .
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8

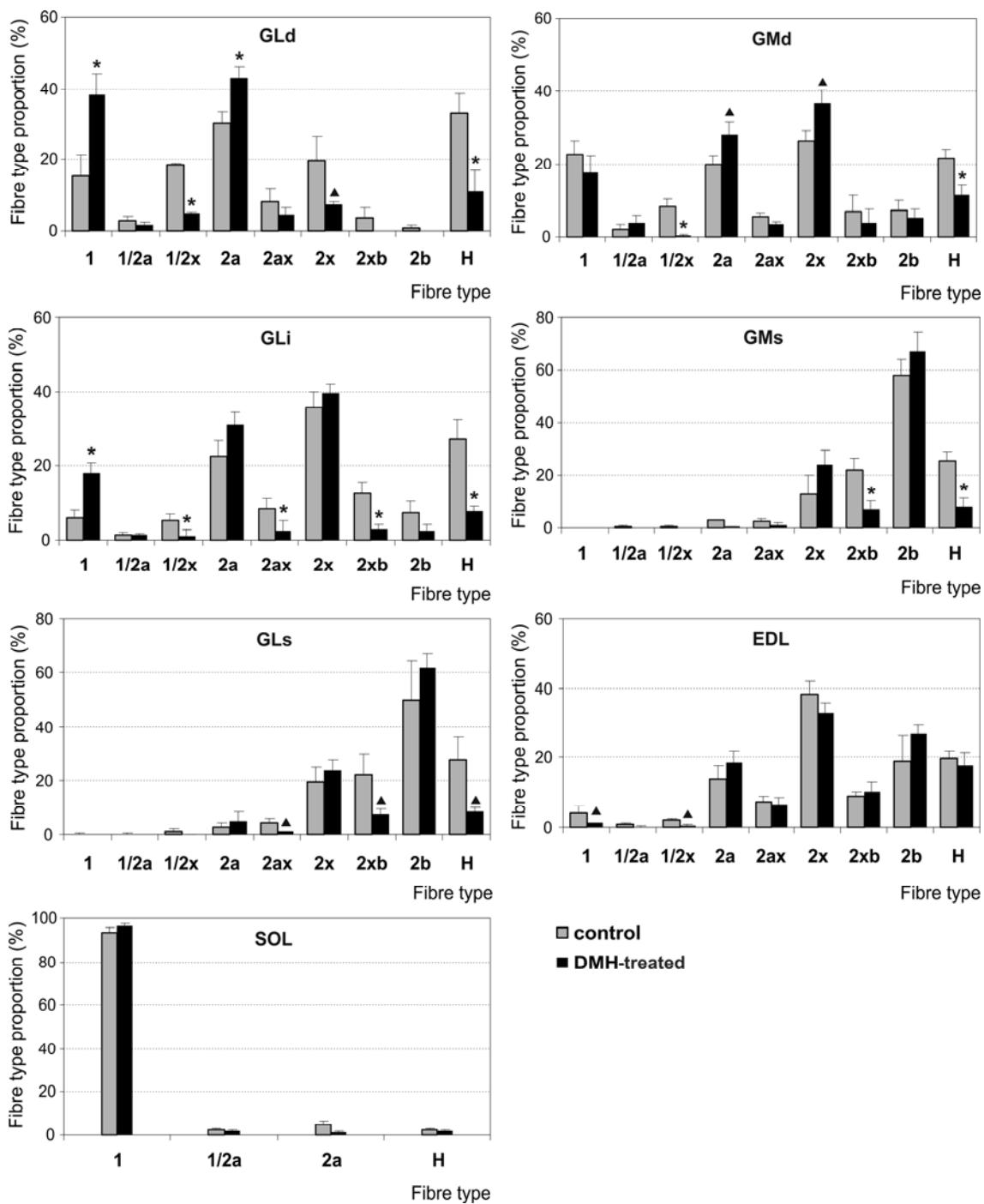
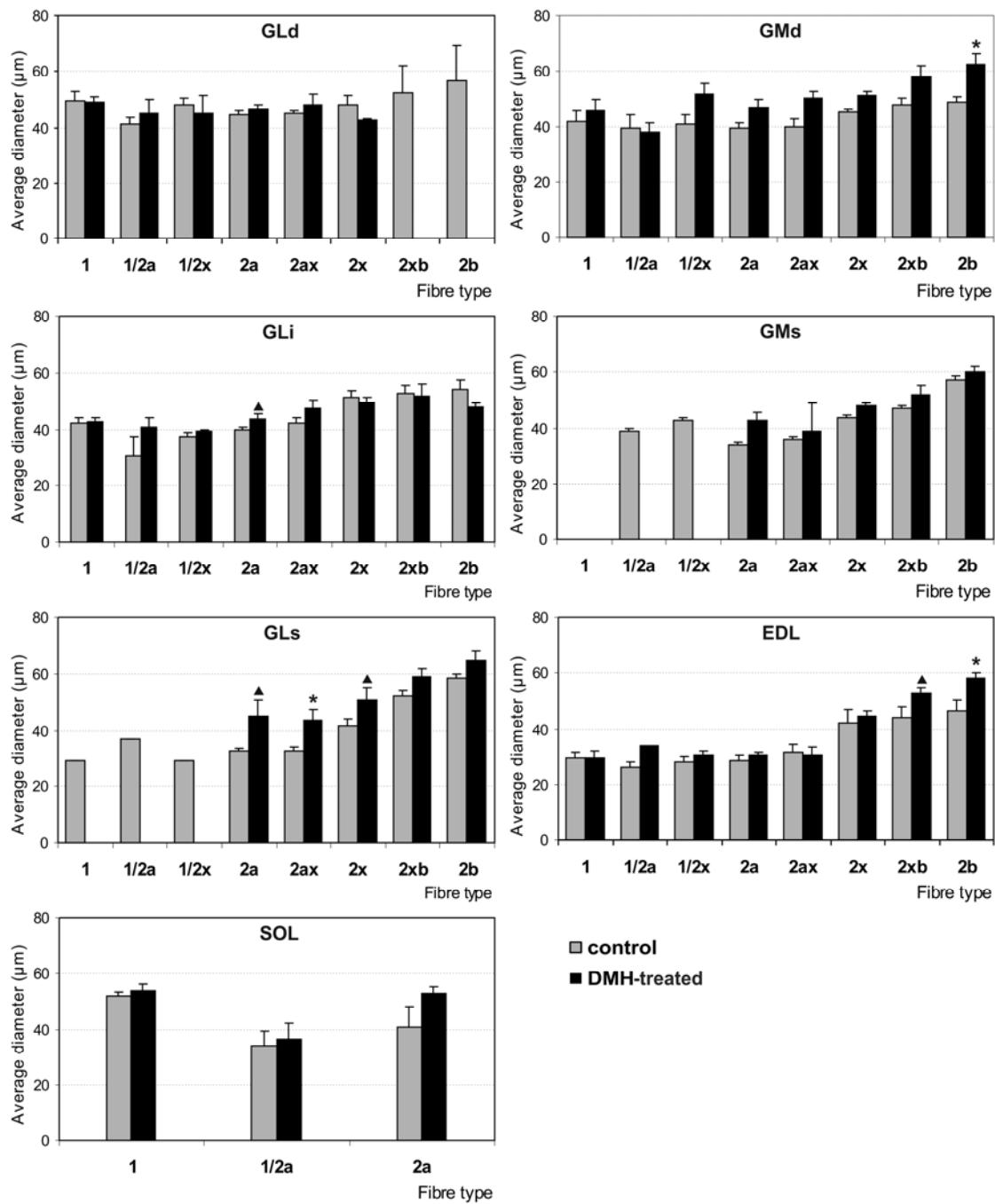


Fig. 3. Average proportions (%) of fibre types in the muscles of the control (n=5) and 1,2-dimethylhydrazine (DMH) treated rats (DMH-treated, n=6). The average proportions of four pure fibre types (1, 2a, 2x and 2b), four of the most numerous hybrid fibre types (1/2a, 1/2x, 2ax, and 2xb), and pooled all hybrid fibre types (H) are presented in the deep, intermediate and superficial portion of gastrocnemius lateralis (GLd, GLi, GLs), the deep and superficial portion of gastrocnemius medialis (GMd, GMs), extensor digitorum longus (EDL) and soleus (SOL) muscles. The values are means \pm SEM, significant differences ($p < 0.05$) between the control and DMH-treated rats are labelled by an asterisk (*) and the trends for differences ($p = 0.05 – 0.1$) are labelled by a triangle (▲).

1



2

3 **Fig. 4.** Average diameters (μm) of fibre types (1, 1/2a, 1/2x, 2a, 2ax, 2x, 2xb and 2b) in the extensor
 4 digitorum longus (EDL), soleus (SOL), superficial, intermediate, and deep portion of the gastrocnemius
 5 lateralalis (GLs, GLi, GLd) and the superficial and deep portion of the gastrocnemius medialis (GMs, GMd)
 6 muscles of the control and 1,2-dimethylhydrazine (DMH) treated rats (DMH-treated). The values are means
 7 ±SEM, significant differences ($p < 0.05$) between the control and DMH-treated rats are labelled by an
 8 asterisk (*) and trends for differences ($p = 0.05 – 0.1$) are labelled by a triangle (▲).