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MORPHOMETRIC ANALYSIS OF BICEPS MUSCLE TISSUE OBTAINED FROM RATS ACUTELY EXPOSED TO CARBON-TETRACHLORIDE

Milorad Antić[1], Vladimir Antić[2], Ivana Graovac[1], Jovana Čukuranović Kokoris[1], Ivan Ilić[3], Miljana Pavlović[1], Vladimir Živković[1], Dušan Sokolović[4]

Skeletal muscles comprise around 40% of total body weight, and they are essential for locomotion and body posture. Under experimental conditions, mild damage occurring due to excessive reactive oxygen species production could be mimicked with acute exposure of rats to carbon-tetrachloride. The aim of the present study was to evaluate morphometric changes occurring in rat biceps muscle 24 h after the injection of carbon-tetrachloride (CCl4). Biceps muscle tissue samples, obtained from control and CCl4-damaged groups, stained with hematoxylin and eosin were used to measure muscle fiber area (MFA), muscle fiber perimeter (B), muscle fiber circularity (MFC) and muscle fiber roundness (MFR). The obtained data were compared using Students t-test for two independent samples. Morphometric analysis revealed that the parameters such as MFA, B and MFC were statistically significantly altered (increased) in the group exposed to CCl4. At the same time, the MFR remained almost identical to that of the control group. The obtained results are in agreement with gross microscopic analysis and follow the tissue edema pattern. These data could be useful in future studies that are following changes in the skeletal muscles after CCl4 application. Acta Medica Medianae 2023;62(3):5-10.

***Key words**: biceps muscle, carbon-tetrachloride, edema, morphometry*

[1]University of Niš, Faculty of Medicine, Department of Anatomy, Niš, Serbia
[2]University of Niš, Faculty of Sports and Physical Education, Niš, Serbia
[3]University of Niš, Faculty of Medicine, Department of Pathology, Niš, Serbia
[4]University of Niš, Faculty of Medicine, Department of Biochemistry, Niš, Serbia

Contact: Dušan Sokolović
81 Dr Zoran Djindjić Boulevard, 18000 Niš, Serbia
E-mail: dusantsokolovic@gmail.com

Introduction

Skeletal muscles comprise around 40% of total body weight, and they allow body movement (locomotion) and maintain posture through processes of contraction and relaxation. These processes are dependent on morpho-functional organization of the skeletal muscle (1), which when disrupted might lead to poor and hampered locomotion. The process of contraction and relaxation is highly dependent on cell energy sources (ATP) and on calcium ion concentrations (2). Muscle damage could be concomitant with some liver disorders (3), but also with some non-disease states which are associated with an increase in reactive oxygen species (ROS) production (4). The non-disease states also include excessive physical activity. Interesting feature of the skeletal muscles is that they are able to recover, both in structure and function, in a period of couple of weeks after their injury (5).

Carbon tetrachloride (CCl4) is an industrial pollutant that is utilized for the production of paints and extinguishers. Half-life of CCl4 in the working atmosphere is relatively long and can be somewhere between 30 and 100 years (4). It is a lipophilic organic solvent which has been in use for experimental research for decades. In experimental animals, single injection of a relatively high dose of CCl4 is associated with a significant tissue damage arriving from excessive ROS production. These ROS are formed initially from the biotransformation of CCl4 by the tissue cytochrome P450 (predominantly in liver). Molecules that are generated include trichloromethyl free radicals and trichloromethyl peroxide which further interact with various cellular structures. The generation of these highly reactive molecules occurs after body is exposed to

CCl4, which can be either through inhalation or skin absorption (in working environment) or by an injection (in experimental animals). Damage is mainly seen in tissue highly expressing cytochrome P450, which include liver and kidneys (6), however, distant tissue damage is arriving from circulating ROS generated elsewhere.

The aim of the current study was to morphometrically investigate the changes occurring in bicep muscle myofibers in rats acutely exposed to carbon tetrachloride and to compare the obtained results with the ones obtained from the control group. Also, these findings will be brought in connection with the pathological appearance of the muscle tissue, as well as with biochemical findings.

Material and methods

Animals and housing

Male Wistar rats (250−300 g) were divided into groups of 6 animals (Institute of Biomedical Sciences, Faculty of Medicine, University of Niš, Serbia). Animals were housed under standard laboratory conditions at room temperature of 22 ± 2 °C. The humidity was 60%, and food and water were free. All experimental procedures, carried according to the Declaration of Helsinki and Europe Community Guidelines for the Ethical Use of Laboratory Animals (2010 EU Directive; 2010/63/EU), were approved by the local Ethics committee.

Experimental procedure

Seven days prior to the experiment, rats were randomly divided into two equal groups, each consisting of seven animals (total n = 14). Rats were treated with CCl4 to induce acute tissue damage according to previously established protocols (4, 6, 7). Control animals, group I (vehicle control), received olive oil as a single dose 24 h prior to sacrifice in a volume of 10 ml/kg. Experimental group of animals, group II (CCl4 control), were administered with CCl4 dissolved in olive oil (1:1) in a dose of 1 ml/kg. One day after the injection, all animals were sacrificed by an overdose of ketamine (Ketamidor, 10%). After that, bicep muscle tissue was dissected, cleaned from surrounding tissue, and separated for histopathological analysis.

Tissue processing and staining

Muscle tissue specimens separated for histopathological study were immersed in

formaldehyde solution (10%, w/v) for fixation. After this process, the tissue was dehydrated with increasing concentration solutions of ethanol (50-100%, v/v), and small tissue segments were cut and embedded in paraffin. Tissue sections, 4−5 μm thick, were obtained from the paraffin molds and routinely stained with hematoxylin and eosin (HE). Tissue damage was scored following previously given scoring system scales (8), where the grade of damage were marked from 0 (absent) to 3 (severely present). Main parameters that were traced included muscle fiber and interstitial tissue edema.

Morphometric analysis

Morphometric analysis of HE stained tissue specimens was performed using ImageJ software (imagej.nih.gov/ij/). At least 10 images were captured of each tissue specimen using digital camera E-450 mounted on BH-2 microscope (Olympus). The magnification used for the tissue analysis and image capturing was 200x. All visible muscle fibers in each image were included in the analysis. The following parameters were measured: muscle fiber area (MFA), muscle fiber perimeter (B), muscle fiber circularity (MFC) and roundness (MFR).

Statistical analysis

The obtained data are presented as mean ± SD. Comparison of the data was performed using Student's t test for two small independent samples (GraphPad Prism, version 7.0; USA). Probability values (p) less than 0.05 were considered to be statistically significant.

Results

Histopathological analysis

Bicep muscle tissue section obtained from a control group consisted of tightly packed homogeneous polygonal muscle fibers, with peripherally located nuclei and normal appearing cell membrane and cytoplasm (Figure 1 and Table 1).

Animals treated with CCl4 displayed abnormal fiber morphology, including occasionally occurring cytokinesis and edematous (swollen) rounded muscle fibers (Figure 2 and Table 1). The stromal compartment was moderately large, with an infiltration of inflammatory cells, primarily neutrophils, lymphocytes, and macrophages, both around blood vessels and between muscle fibers.

Morphometric analysis

Morphometric analysis of the biceps muscle tissue pointed to a significant increase, compared to the control group, in MFA, B and MFC in rats exposed acutely to CCl4 (Table 2). On the other hand, roundness of the muscle fibers (MFR) remained unaltered (Table 2).

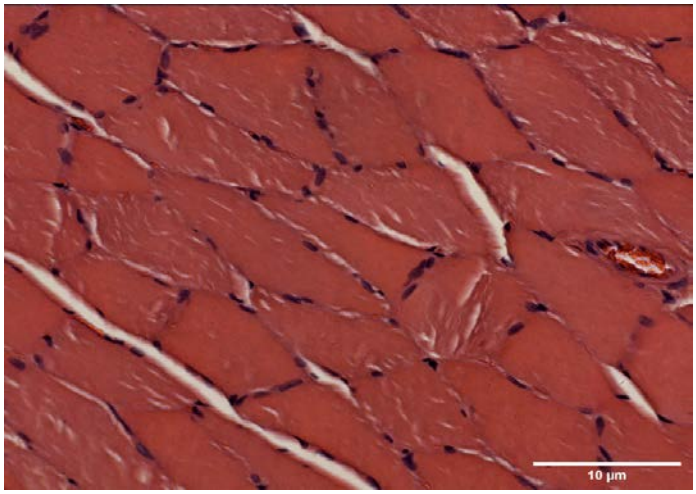

Figure 1. Histomorphological appearance of biceps muscle tissue obtained from rats of the control group

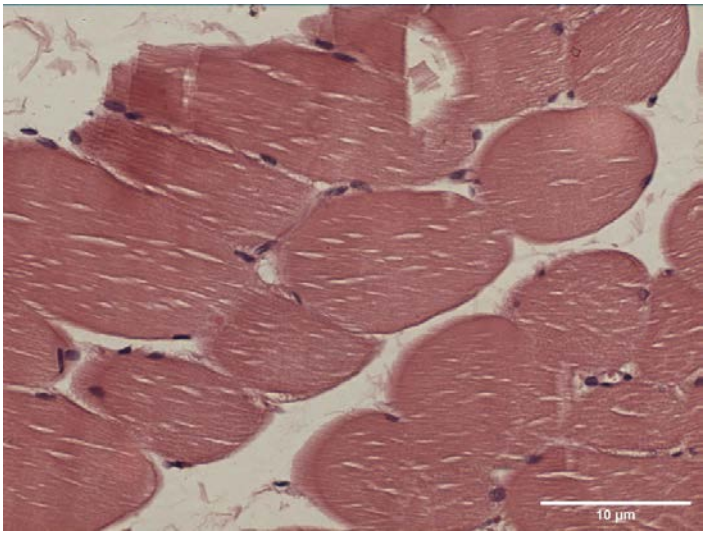

Figure 2. Histomorphological appearance of biceps muscle tissue obtained from rats treated with CCl_4

Table 1. Semiquantitative score obtained from the control and CCl_4-treated animals

Morphometric parameter	Control group	CCl_4 group
Muscle fiber edema	0	2
Interstitial tissue edema	0	2.2
Cytoplasm degeneration	0	1.7

Table 2. Biceps muscle morphometric parameters measured in control and CCl_4-treated animals

Morphometric parameter	Control group	CCl_4 group	*p*-value
Muscle fiber area (MFA; mm^2)	41.5 ± 6.3	68.9 ± 7.5	<0.001
Muscle fiber perimeter (B; µm)	26.3 ± 3.8	32.6 ± 2.4	<0.001
Muscle fiber circularity (MFC; mm)	0.74 ± 0.07	0.87 ± 0.06	<0.01
Roundness (MFR; mm)	0.7 ± 0.01	0.75 ± 0.09	>0.05

Discussion

The biceps femoris is one of the largest muscle in the hind-extremity of an animal and serves multiple functions including thigh abduction, hip extension, and knee flexion (9). Its damage following CCl4 application has been previously proven using a panel of serum biochemical parameters such as lactate dehydrogenase and creatine kinase activity, as well as potassium ion levels (4). Cell and organelle membrane damage occurs after CCl4 exposure due to a chain reaction which is initiated by the removal of a hydrogen atom from an unsaturated fatty acid by ROS (produced by xanthine oxidase) and free radicals formed after CCl4 metabolism (7). These excessive ROS production leads to muscle antioxidant capacities depletion, which results in a more or less pronounced microscopic changes (4).

In animals exposed to CCl4 there was a significant alteration in cytoplasm appearance (score 1.7; Table 1), which was not visible in the control group animals (score 0; Table 1). These microscopic changes might correspond to the damaged cytoplasm structures (proteins and lipids), as well as to the damaged organelles. These changes on the organelles are potentially irreversible, and could lead to cell death, especially if the organelles in question are mitochondria. On a previous occasion, an increase in MDA, a marker of lipid peroxidation induced by ROS, was noted in the muscle tissue of animals acutely exposed to CCl4 (4). The ROS could arrive from different cells, and in the present situation these cells are mainly inflammatory ones such as neutrophils and macrophages, as well as mastocytes (4, 10). These cells are packed with enzymes capable of creating numerous ROS as a response to tissue injury and inflammation.

Using gross microscopic analysis the examination helped us reveal an expanded and rounded muscle fiber profile with eosinophilic cytoplasm (Figure 2), which markedly deviated from the findings in the control group (Figure 1).

The mentioned changes are the result of water influx into the cell and consequential cytosol dilution i.e. edema development (Miller MA, Zachary, 2017). If the process is not reversed this could lead to a decrease in ATP content and relatively fast switch to the anaerobic processes (11), which muscles could endure for some time due to their specific structure/function (2). After certain time point, when the intracellular lactate levels increase and pH and ATP decrease, cells suffer and more pronounced/irreversible changes occur (11). The described changes causing such massive disturbances would lead cell to oncotic necrosis (11).

Measured morphometric parameters could be perfectly used to estimate cell edema, since they describe cell features which are altered during cell volume expansion following water influx. These include MFA, which represents total skeletal muscle cell size (area), B, a total length of a cell membrane, and MFC, which describes a cell shape compared to a full circle. All three mentioned parameters were found to be statistically significantly increased in the group of animals treated with CCl4. Also, the measured parameters (Table 2) correlate with the microscopic changes and score values for the corresponding groups (Table 1).

Conclusion

The present study revealed that acute application of CCl4 provokes significant, potentially reversible, changes in the skeletal muscle appearance. This was determined based on the light microscopy analysis, and further corroborated using morphometric analysis. Detailed morphometric analysis confirmed the presence of significant muscle fiber edema with increased muscle fiber area, perimeter and circularity. Furthermore, the present analysis showed that the changes are only temporary, and could be potentially reversed since no muscle fiber necrosis or cell apoptosis were seen.

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MORFOMETRIJSKA ANALIZA TKIVA DVOGLAVOG MIŠIĆA (*MUSCULUS BICEPS*) PACOVA AKUTNO IZLOŽENIH UGLJEN-TETRAHLORIDU

Milorad Antić[1], Vladimir Antić[2], Ivana Graovac[1], Jovana Čukuranović Kokoris[1], Ivan Ilić[3], Miljana Pavlović[1], Vladimir Živković[1], Dušan Sokolović[4]

[1]Univerzitet u Nišu, Medicinski fakultet, Katedra za anatomiju, Niš, Srbija
[2]Univerzitet u Nišu, Fakultet za sport i fizičko obrazovanje, Niš, Srbija
[3]Univerzitet u Nišu, Medicinski fakultet, Katedra za patologiju, Niš, Srbija
[4]Univerzitet u Nišu, Medicinski fakultet, Katedra za biohemiju, Niš, Srbija

Kontakt: Dušan Sokolović
Bulevar dr Zorana Đinđića 81, 18000 Niš, Srbija
E-mail: dusantsokolovic@gmail.com

Skeletni mišići čine oko 40% ukupne telesne težine i neophodni su za kretanje i držanje tela. U eksperimentalnim uslovima, blago oštećenje koje nastaje usled prekomerne proizvodnje reaktivnih vrsta kiseonika može se oponašati akutnim izlaganjem pacova ugljen-tetrahloridu. Cilj ove studije bio je da se morfometrijski procene promene koje se javljaju u dvoglavom mišiću pacova 24 sata nakon injekcije ugljen-tetrahlorida (CCl4). Uzorci mišićnog tkiva bicepsa, dobijeni od pacova iz kontrolne grupe i grupe oštećene CCl4, obojeni hematoksilinom i eozinom, korišćeni su za merenje površine mišićnih vlakana (MFA), perimetra mišićnih vlakana (B), kružnosti mišićnih vlakana (MFC) i zaobljenosti mišićnih vlakana (MFR). Dobijeni podaci upoređeni su korišćenjem Studentovog t-testa za dva nezavisna uzorka. Morfometrijskom analizom otkriveno je to da su parametri kao što su MFA, B i MFC statistički značajno izmenjeni (povećani) u grupi pacova izloženih CCl4. U isto vreme, MFR je ostao skoro identičan onom u kontrolnoj grupi. Dobijeni rezultati u saglasnosti su sa mikroskopskom analizom i prate obrazac edema tkiva. Ovi podaci mogli bi biti korisni u budućim studijama koje prate promene u skeletnim mišićima nakon primene CCl4. Acta Medica Medianae 2023;62(3): 5-10.

***Ključne reči**: dvoglavi mišić, ugljen-tetrahlorid, edem, morfometrija*