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Effect of Copper and Silver Nanoparticles on Trunk Muscles in Rainbow Trout (*Oncorhynchus mykiss*, Walbaum, 1792)

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Abstract

Nanotechnology has been developing rapidly for the last 20 years. Silver and copper nanoparticles are commonly used in various industries. Popularization of nanoparticles (NPs) led to increase of their concentration in the aquatic environment. Therefore, it is necessary to investigate the effects of nanoparticle contamination on fish growth.

In this study, to define nanoparticles impact on juvenile freshwater fish survivalability and growth, rainbow trout (Oncorhynchus mykiss, Walbaum, 1792) were treated with silver (AgNPs) or copper (CuNPs) nanoparticles suspended in water. During the experiment body weight and length were measured. After the treatment trunk muscle tissue was sampled for histological and genetic analysis.

The highest mortality and the lowest body weight was observed in juvenile rainbow trout treated CuNPs. Histological analysis showed disruptions in muscle structure in both experimental groups. Differences between AgNPs and CuNPs were observed in expression of genes associated with the growth of muscle tissue. These results indicate that metallic nanoparticles can affect growth and survivability of juvenile freshwater fish.

Keywords: nanoparticles, muscle, growth, rainbow trout

Introduction

Nanotechnology, the industrial branch that deals with nanoparticles, is still young and promising (Savolainen et al., 2010) and has been developing rapidly for the last 20 years (Farré, Gajda-Schrantz, Kantiani & Barceló, 2009; Sajid et al., 2015). Silver nanoparticles (AgNPs) are used in various industries, eg. as a disinfectant of manufacturing areas, including those in contact with water. Copper nanoparticles (CuNPs) exhibit similar antibacterial properties to AgNPs and are also in broad use, e.g. in industrial filter systems (Griffitt, Hyndman, Denslow & Barber, 2009). Toxicity of the both nanoparticles are referred to as “toxic” or “highly toxic” for fish at various life stages (Johari, Kalbassi, Soltani & Yu, 2013). In recent years NPs were frequently used in aquaculture of fish and seafood production for nanofiltration or food packaging (Can et al., 2011; Rather et al., 2011). It is disturbing that NPs are also used in the production of fish feeds (Handy et al., 2011).

Popularization of NPs leads to an increase of their concentration in the environment, including aquatic environments. Unfortunately, the knowledge about the risks that come with use of NPs is limited. The presence of NPs in the environment may have serious ecological consequences and may affect human and animal health (Handy, Henry, Scown, Johnston & Tyler, 2008; Sajid et al., 2015). Research on aquatic organisms (Shaw & Handy, 2011; Handy et al., 2011) revealed that NPs are toxic in both high and low concentrations. In fish, signs

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of chronic toxicity were observed, along with histopathological changes, similar to those caused by other xenobiotics (e.g., heavy metals and pesticides) (Poleksic et al., 2010; Boran, Capkin, Altinok & Terzi, 2012). The organs most endangered by waterborne NPs are gills, intestine, and liver (Al-Bairuty, Shaw, Randy & Henry, 2013; Handy et al., 2011; Ostaszewska, Chojnacki, Kamaszewski & Sawosz-Chwalibóg, 2016), however the epidermis (Li et al., 2009) or muscles (Zhou, Wang, Gu & Li, 2009; Ashouri, Keyvanshokooh, Salati, Johari & Pasha-Zanoosi, 2015) which have major effect on swim efficiency, may also be affected.

The aim of the present study was to determine the effect of silver and copper nanoparticles on growth and muscle tissue histology in rainbow trout, which are popular fish in aquaculture and food industry. The possible impact of nanoparticles has been increasing for trout farms where groundwater is usually utilized for their needs. Verification of the impact of the NPs on the growth, in the most intensive growth phase, will help to determine the likely consequences for the aquaculture of this species in the nearest future.

Materials and Methods

This protocol has been evaluated and approved by the Third Warsaw Local Ethics Committee for Animal Experimentation at Warsaw University of Life Sciences.

Materials and Particles Characterization

Preparation of silver nanoparticles (576832, Sigma Aldrich, UK) suspension was based on manufacturer's specifications to obtain particles with diameter <100 nm, density of 10.49 g/cm^3 and purity of 99.5%. Copper nanoparticles (684007, Sigma Aldrich, UK) preparation was based on manufacturers specifications preparation of particles with size <50 nm, density of 8.94 g/cm^3 and purity of 99.5%. The distribution and the size of the particles were measured by transmission electron microscopy (TEM; JEOL JEM-1220 TE 80 KeV, JEOL Ltd., Japan) with camera Morada 11 Mpx (Olympus Corporation, Japan).

AgNPs and CuNPs 50 mg/L stock suspensions were diluted in Mili-Q water by sonication (30 minutes; Sonicator, 250W, 40kHz, 25°C, Ultron U-507 Ultron, Poland). The suspended solutions were filtered throught the 200 nm nylon membrane filter (Whatman®, UK). The zeta potential of the suspended particle solutions was then measured using Zetasizer Nano-ZS90 (Malvern, UK) after 2 minutes, 25°C, pH 7.

Experimental Conditions

Rainbow trout fry (total length 23.75 +/- 1.15 mm; body weight 0.012 +/- 0.002 g) were obtained from commercial rainbow trout farm in Darzy Bor (Poland) and transported to the experimental hatchery of Department of Ichthyobiology, Fisheries and Aquaculture Biotechnology, Warsaw University of Life Sciences (Poland). Since the 3rd day post hatching (dph) fish were maintained in the 20 L glass tanks with 5 cm^3 airstones, 50 fish per tank. Fish were fed *Artemia nauplii ad libitum* for the first 4 days. Gradually the diet was switched to commercial Aller Futura (AllerAqua, Denmark). The 28-day experiment was carried out in triplicates. Rainbow trout fingerlings were treated with 1.5 mg/L AgNPs and 0.15 mg/L CuNPs dissolved in water. The concentrations were selected based on preliminary studies conducted in Department of Ichthyology, Fisheries and Aquaculture. Temperature, pH, oxygen saturation, total ammonia and water hardness were measured every day before and after water change. Photoperiod was set: 12 h light, 12 h dark.

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Histological and Morphometrical aAnalysis

After 28 days of experiment, muscle tissue from caudal region was sampled according to Valente et al. (1999), from 12 individuals from each group. Before sampling fish were anaesthetised with buffered MS222 (Sigma Aldrich, UK). Animals were measured (total length with ± 0.1 mm accuracy and weight with ± 0.001 g accuracy). Samples were preserved in Bouin solution and subjected to standard histological procedures. Paraffin embedded samples were cut transversely into 5µm-thick sections using the Leica RM2265 microtome (Leica Microsystems, Germany). Standard H&E staining was used. Proliferating cells were detected by immunohistochemical method according to Ostaszewska, Dabrowski, Hliwa, Gomółka & Kwasek (2008), using anti-PCNA antibody in 1:300 dillution (clone PC10, DAKO, Poland).

The morphometric measurements were performed using Nikon Eclipse 90i microscope equipped with NIS-Elements 10 software (Nikon Corporation, Japan). All measurements were performed on cross sections of each of the individuals from all investigated groups (50 measurements): total cross sectional area, total cross sectional area of white muscle tissue, total cross sectional area of red muscle tissue, number of white muscle fibers in cross sectional area, number of red muscle fibers in cross sectional area. The average area of single white muscle fiber and the average area of single red muscle fiber were calculated based on 500 measurements. The number of proliferating myonuclei was determined by immunohistochemical reaction with PCNA antibodies.

Genetic Analysis

Total RNA was extracted by Chomczynski & Sacchi method (1987) from three individuals from each investigated group. RNA extract was digested with DNase I (A&A Biotechnology, Poland) according to the manufacturer protocol, purified with Clean-Up Concentrator kit (A&A Biotechnology, Poland), then stored in -80°C. The quality of total RNA was evaluated by 1% agarose gel electrophoresis and the concentration was measured by NanoDrop 2000 (Thermo Scientific, USA). 1 µg of purified total RNA was used to synthesise single-strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the protocol for anchored oligo-d(T) primers. The Real Time PCR was performed in the LightCycler 480 II thermocycler (Roche, Germany). The primers to investigated genes (*igfI*, *igfII*, *mstn1a*, *mstn1b*) were designed based on available sequences for rainbow trout (Tab. 1). The reactions were run in triplicates, using SYBR Green I PCR Master Mix (Roche Diagnostics, Germany) in 10µl of total volume. No template control (NTC) and –RT were applied. PCR protocol: initial denaturation at 95ºC for 5 minutes followed by: 40 cycles of denaturation at 95ºC for 10 s, annealing at 60ºC for 15 s, and extension at 72ºC for 1 min. A melting curve analysis was performed to check the homogeneity of PCR products. The Cq values were calculated using the second derivative method. The Real Time PCR efficiency was calculated from serial template dilutions (1 µg – 123 ng) for each primer pairs. The expression level in each sample was normalized using β-actin as a reference gene and compared to the mean gene expression in the control group (calibrator). The relative quantification was determined among samples using Pfaffl method (2001).

Statistical Analysis

All data were expressed as mean values ± standard deviation (SD). Statistical analyses were performed using Statistica 10 (StatSoft) with the significance value set at 0.05. Histological analysis data were calculated using parametric ANOVA tests, with Fisher NIR post hoc test. Significance of the data obtained from

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immunohistochemical and genetic analysis were calculated using non parametric test – Mann Whitney U test.

Results

Characterization of the Nanoparticles

The shape and size of the silver and copper NPs before treatment were the same as previously descibed by Ostaszewska, Chojnacki, Kamaszewski & Sawosz-Chwalibóg (2016). The size of AgNPs was 6 - 16 nm (average 9.56 ± 1.85 nm), CuNPs was 3 - 9 nm (4.78 ± 1.22 nm). Silver and copper nanoparticles in the suspension formed aggregates 89.27 ± 20.03 nm and 98.51 ± 9.80 nm respectively. The zeta potential of the AgNPs stock solution was 53.6 ± 5.0 mV, while the zeta potential of the CuNPs stock solution was 29.5 ± 0.7 mV.

Survivability and Growth Rate of Fish

The highest growth rate was observed in the control group. Both, AgNPs and CuNPs groups showed lower growth rate, comparing to the control group. Body lengths of fish from the control and nanoparticles groups also showed significant differences. The highest body weight was observed in AgNPs, the lowest in CuNPs. The highest survivability was observed in the control group (Table 2).

Histological Analysis

Muscle structure in the control group shows normal anatomy of this tissue (Fig. 1 A). Muscle fibers were separated by connective tissue (endomysium and perimysium) and poorly vascularized. In all analyzed groups mosaic pattern of muscle fibers distribution (Fig. 1 A, B, C) and PCNA-positive myonuclei were observed (Fig. 1 D, E, F). In both CuNPs and AgNPs few histopathological changes, without characteristics of inflammation were observed. In the AgNPs and CuNPs groups white and red muscle fibers had larger extracellular space between muscle fibers, compared to the control group. The most striking morphological alterations were observed in red muscle tissue. In the control group all fibers had circular, sharp shape with a high number of myonuclei (Fig. 2 A). In the AgNPs group red muscle fibers were splitting and had some internal myonuclei (Fig. 2 B). In the CuNPs group a lot of muscle bounds were shrinked (Fig. 2 C, D) and some of them had eosinophilic reaction (Fig. 2 C). In some parts of myotome the absence of single muscle bounds was observed (Fig. 2 D).

Distribution of muscle fibers was more advantageous in the AgNPs group than in CuNPs. The highest number of small white muscle fibers (<300 μm^2) was observed in the CuNPs group, but fiber distribution in AgNPs was more comparable to the control group. The similar tendency was observed in red muscle fibers distribution. The lowest number of PCNA - positive myonuclei of white and red muscles was observed in the CuNPs group, and the highest in the AgNPs group (Table 3).

Genetic Analysis

Igfs and *myostatins* expression was detected in all investigated groups. The expression of *igfI* was significantly lower than *igfII* in all individuals from experimental groups. In muscle tissue, lower expression of investigated genes was observed in the CuNPs group comparing to AgNPs group, excluding *igfII* and *mstn1b*, with no significant differences (Table 4).

Discussion

The effect of silver and copper nanoparticles on trunk muscle tissue has not been described previously in rainbow trout fingerlings. The results of this study suggest that nanoparticles have negative impact on the survivability and growth of rainbow trout fingerlings, which is confirmed by numerous other studies indicating the negative effect of nanoparticles on aquatic organisms.

Up to date, copper has been defined as more toxic than silver (Griffitt, Hyndman, Denslow & Barber, 2009; Bondarenko et al., 2013), but the size of nanoparticles is the main parameter determining their toxicity (Hua, Vijver, Ahmad, Richardson & Peijnenburg, 2014; Hua, Vijver, Chen, Richardson & Peijnenburg, 2016). The diameter of copper nanoparticles used in this experiment was twice lower than the diameter of silver nanoparticles, which may explain their stronger negative effect on juvenile rainbow trout. Also, the copper nanoparticles in the water release copper ions, which are considered more toxic for fish than CuNPs (Kong, Wang, Lu & Cui, 2014; Black, Reichelt-Brushett & Clark, 2015). This may explain higher mortality and significantly lower overall length and weight of rainbow trout fingerlings, observed in the CuNPs group. Fish body weight is closely related with muscle tissue which contribute to 40-60% of fish body mass. In addition, lower body weight is correlated with muscles histological structure.

Muscle fiber distribution differs depending on the fish life stages (Valente et al., 1999; Dal Pai-Silva, Carvalho, Pellizzon & Dal Pai, 2003a; Dal Pai-Silva, Freitas, Dal Pai & Rodrigues 2003b; Ostaszewska, Dabrowski, Hliwa, Gomółka & Kwasek, 2008; Alami Durante, Médale, Cluzeaud & Kaushik, 2010). The distribution of muscle fibers size changes with age of fish due to the increase in the number of large, hypertrophic muscle fibers. In numerous reports hyperplastic fibers of small cross-sectional area (surface area <300 μm^2) were characteristic for larvae and juvenile fish (Momsen, 2001; Ostaszewska, Dabrowski, Hliwa, Gomółka & Kwasek, 2008; Alami Durante, Médale, Cluzeaud & Kaushik, 2010). In rainbow trout exposed to both AgNPs and CuNPs a high number of hyperplastic fibers (with an area of <300 μm^2) was observed, with the highest number in the CuNPs group. Presumably the observed fiber distribution in this group was caused by an intense growth phase inhibition as it was described by Valente et al., (1999), which showed that the growth of muscle tissue in rainbow trout is not continuous but stepwise.

High percentage of hyperplastic fibers, observed in the CuNPs group, demonstrated high growth potential, higher than in the AgNPs and control groups (based only on the fiber composition, excluding body weight) due to the fact that each one of existing fibers may grow hyperthrophicly in the later life stages. In the CuNPs group muscle fibers with an area exceeding 700 μm^2 were not observed, which indicates near absence of hyperthrophic growth (Valente et al., 1999; Ostaszewska, Dabrowski, Hliwa, Gomółka & Kwasek, 2008; Alami Durante, Médale, Cluzeaud & Kaushik, 2010). High number of small muscle fibers observed in the CuNPs along with a very low number of PCNA positive myonuclei, lack of muscle fibers with the area greater than 700 μm^2, and the lowest expression of *igfI* which is one of the most important genetic factors determining fish growth, rather indicates an inhibition of the muscle tissue growth by CuNPs exposure.

It can be concluded that the copper nanoparticles had a negative effect on muscle tissue development, almost completely inhibiting the hypertrophic growth. In contrast to this group, extremely different fiber distribution was observed in the control and AgNPs groups. Both were characterized by a higher number of hypertrophic fibers and PCNA positive myonuclei. Furthermore, in the AgNPs group, the highest number of PCNA positive myonuclei was observed, which may indicate intense synthesis of new muscle fibers and growth of the existing fibers. Interestingly, silver nanoparticles have been described so far as a factor inhibiting cell proliferation (Asharani,

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Hande & Valiyaveettil, 2009; Kruszewski et al., 2011), therefore high number of positive PCNA may respond not only to proliferation but also the ongoing DNA repair process in which the PCNA protein is involved, especially since the highest expression of myostatin, which is responsible for inhibiting muscle tissue growth, was observed in the AgNPs group. Regardless of the hyperplastic muscle fibers presence, along with a very high number of proliferating cells and a higher *igfs* genes expression, compared to CuNPs, indicate that AgNPs does not have such negative impact on muscle tissue as CuNPs.

Based on the results of numerous experiments it may be concluded that fish muscle tissue is more sensitive to heavy metals and their salts, than muscle tissue of other vertebrates (Girial, Krishnakumar, Poornima, Fayaz & Kalaichelvan, 2015). In the case of disruption of muscle tissue growth in fish usually numerous histopathological changes, often correlated with atrophy and cachexia, were observed. Histopathological changes found in the rainbow trout muscle tissue exposed to aqueous AgNPs and CuNPs suspensions were similar to those observed in previous experiments with NPs (Salem, Kenney, Rexroad & Yao, 2010; Al-Bairuty, Shaw, Handy & Henry, 2013; Girial, Krishnakumar, Poornima, Fayaz & Kalaichelvan, 2015). The increase of the intercellular area in muscle tissue, observed in this study was also found in other studies on effects of pesticides and heavy metals on fish (Capkin, Terzi, Boran, Yandi & Altinok, 2010; Patnaik Howrelia, Mathews & Selvanayagam, 2011; Bhuvaneshwari, Padmanaban & Babu Rajendran, 2015; Rajkumar, Kanipandian & Thirumurugan, 2016). In the CuNPs group numerously occurring shrinked acidophilic muscle bounds exhibited unspecific dystrophic changes. Similar results were obtained in experiments on *Mosambiqe* and *Niloticus tilapia* exposed to heavy metals and nanoparticles (Kaoud & El-Dahshan, 2010; Govindasamy & Rahuman, 2012; Suganthi, Murali, He, Basu & Singhal, 2015).

The exposure of juvenile rainbow trout to silver and copper nanoparticles caused not only the histopathological changes in muscle tissue, but additionally changes impairing the proper growth of this tissue. It is necessary to carry out further long-term studies in order to verify the impact of these nanoparticles on fish growth which would allow to determine whether constant exposure of rainbow trout to AgNPs stimulates maintenance of hyperplastic growth potential of muscle tissue.

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Table 1. Real time primer sequences and accession numbers of reference (β actin) and investigated genes.

Gene symbol	Gene Name	qPCR primers	Amplicon	GenBank no.
β-actin	β actin	F: TGACAGGATGCAGAAGGAGA R: ATTTACGGTGGACGATGGAG	191	AJ438158.1
igfI	Insulin-like growth factor I	F: TCAAGAGTGCGATGTGCTGTG R: GGCCATACCCCGTTGGTTTA	186	NM_001124696.1
igfII	Insulin-like growth factor II	F: ATTGCGCTGGCACTTACTCT R: GGAGCGTCTGCTGTTAGACC	150	NM_001124697.1
mstn1a	Myostatin 1a	F: CAGGCACATAGGTATCCGGT R: CGCCAGATCATTTCCCTTCG	163	JN990743.1
mstn1b	Myostatin 1b	F: ATCGACGTGAATGCAGGAGT R: ACGGCCAGATCATTTCCCTT	137	JN990751.1

F – forward; R – reverse

Table 2. Survivability, growth, and specific growth rate factor (SGR) of rainbow trout.

	C	AgNPs	CuNPs
Survival (%)	99.26±2.02[b]	97.56±0.33[b]	85.50±1.33[a]
Body weight (g)	0.84±0.32[b]	0,86±0.14[b]	0.64±0.05[a]
Body length (mm)	39.87±1.12[b]	37.73±1.47[b]	34.18±0.42[a]
SGR (%)	3.01	2.7	2.44

Different letters indicate statistical differences between groups ($p \leq 0.05$).

Table 3. Total cross sectional area of trunk muscle sections: TCA – total cross section area; TCAW – total cross section area of white muscle tissue; TCAR – total cross sectional area of red muscle tissue.

	C	AgNPs	CuNPs
TCA (μm^2)	1179911.56±296809.83[b]	1173470.90±27149.40[b]	904616.92±15446.41[a]
TCAW (μm^2)	1073887.36±396009.77[b]	720986.07±37074.75[a]	743956.29±12164.66[a]
TCAR (μm^2)	78322.08±1012.96[b]	49637.01±40.29[a]	42901.71±23356.6[a]
PCNA myonuclei in white muscle tissue/mm^2	30.43±1.18[b]	70.24±1.38[c]	2.17±0.22[a]
PCNA myonuclei in red muscle tissue/mm^2	100.00±1.27[a]	217.50±1.19[c]	2.27±0.33[b]
Average area of a white muscle fiber	345.76±262.37[b]	359.14±239.4[b]	254.5±160.7[a]
Average area of a red muscle fiber	302.08±136.21[b]	315.2±130.43[b]	241.26±104.02[a]

Different letters indicate statistical differences between groups ($p \leq 0.05$).

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Table 4. Relative gene expression of *igfI*, *igfII*, *mstn1a*, *mstn1b* (fold change relative to control group) in AgNPs and CuNPs groups.

Gene	AgNPs	CuNPs	p
igfI	0.21±0.12	0.07±0.06	<0.001
igfII	0.64±0.11	0.75±0.51	ns
mstn1a	0.64±0.20	0.32±0.25	<0.05
mstn1b	1.45±0.54	1.00±0.53	ns

p- significance of differences between groups; ns – no significant differences

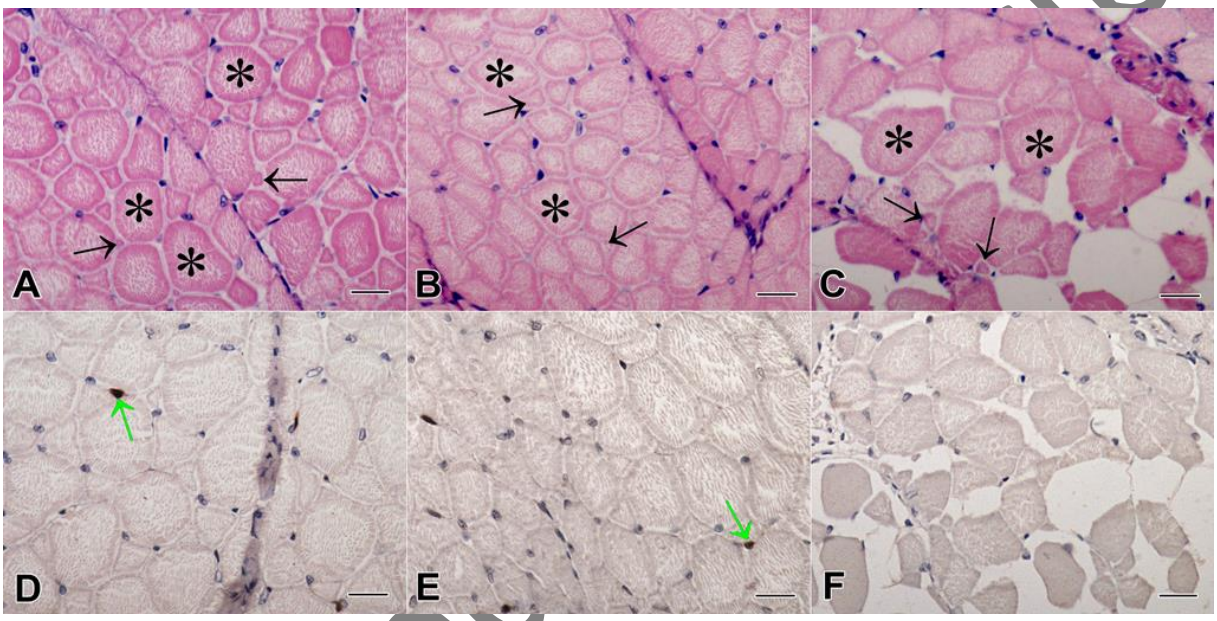

Figure 1. Histological sections of white muscle tissue in control (A and D), AgNPs (B and E) and CuNPs (C and F) group with hypertrophic (*) and hyperplastic fibers (arrow). In figure D and E by green arrow were marked anti-PCNA positive myonuclei, 10μm scale.

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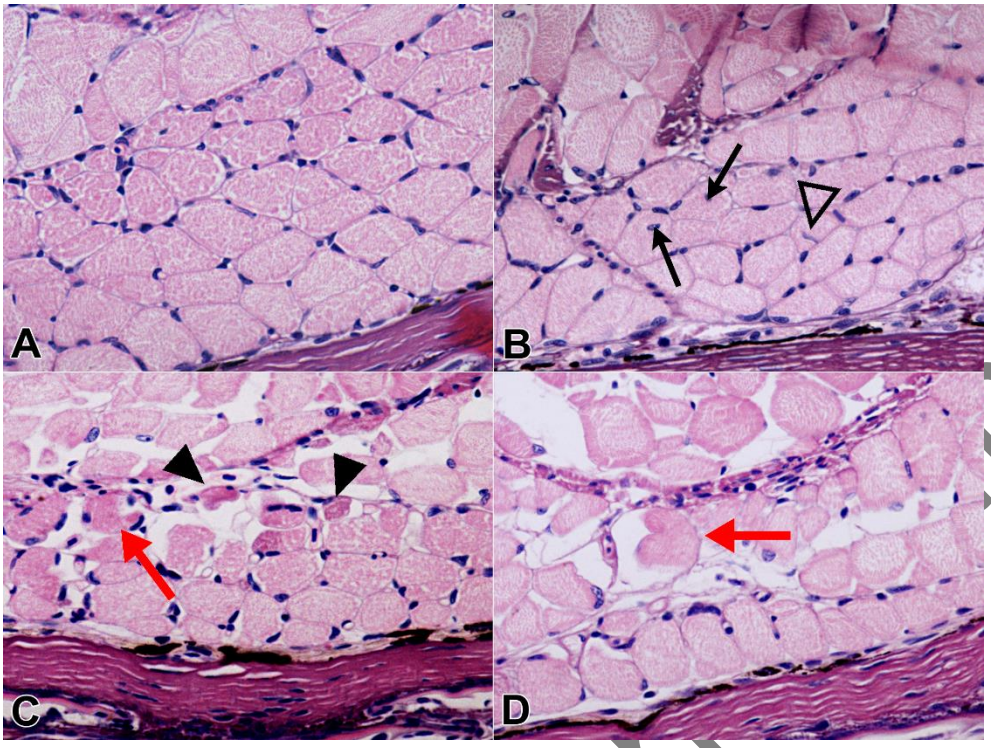

Figure 2. Histological sections of red muscle tissue in control (A), AgNPs (B) and CuNPs (C and D); HE, 10µm scale. Regenerating muscle fiber – black arrow; stripped fibers – black triangle; necrotic fibers – red arrow; acidophilic fibers black arrowhead.