Acta Scientiarum
http://www.periodicos.uem.br/ojs/
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v43i1.52612

Primary culture macrophages from fish as potential experimental model in toxicity studies with multiwalled carbon nanotubes

Isabella Ferreira Silva[1], Letícia Fagundes Papa[1], Pedro Gontijo Carneiro[2], Mariane Cristina Schnitzler[2], Silmara Nunes Andrade[3], Rosy Iara Maciel Azambuja Ribeiro[4], Fernando Pilla Varotti[3], Ralph Gruppi Thomé and Hélio Batista Santos[1*]

[1]Laboratório de Processamento de Tecidos, Universidade Federal de São João Del Rei, Avenida Sebastião Gonçalves Coelho, 400, 35501-296, Divinópolis, Minas Gerais, Brazil. [2]Laboratório de Química Orgânica e Nanoestruturas, Universidade Federal de São João Del Rei, Divinópolis, Minas Gerais, Brazil. [3]Laboratório de Bioquímica Medicinal, Universidade Federal de São João Del Rei, Divinópolis, Minas Gerais, Brazil. [4]Laboratório de Patologia Experimental, Universidade Federal de São João Del Rei, Divinópolis, Minas Gerais, Brazil. *Author for correspondence. E-mail: hbsantos@ufsj.edu.br

ABSTRACT. Multiwalled carbon nanotube (MWCNT) has been broadly used in several sectors of society. This material when exposed to the environment might reach the aquatic animals and cause toxic effects. Here, it was evaluated the MWCNTs toxicity in melanomacrophages primary culture that was submitted to 1 μ gm L^{-1} MWCNTs for 24 hours. After exposition to MWCNT, 48 and 59% liver and spleen melanomacrophages were healthy, respectively. The control group presented 85% viability. Phagocytosis activity of melanomacrophages was observed by presence of black inclusions in cytoplasm. The findings indicate MWCNT was cytotoxic to melanomacrophages, where its release and effect into aquatic environment must be more studied. Finally, the melanomacrophages present large potential as experimental model for evaluation of carbon-based nanomaterial toxicity.

Keywords: carbon nanotubes; liver; spleen; melanomacrophages; Nile tilapia.

Received on March 13, 2020.
Accepted on November 3, 2020.

Introduction

The contamination of aquatic ecosystems by nanomaterials has caused great concern on the part of global health agencies (Jackson et al., 2013; Zarbin & Oliveira, 2013; Avant et al., 2019). In the coming decades, the release of carbon nanotubes (CNTs) into the environment will increase due to increase production and use of these nanocompounds in various sectors of society (Zarbin & Oliveira, 2013). However, the adverse effects of CNTs on the environment, mainly aquatic organisms, are little known (Jackson et al., 2013; Avant et al., 2019). The CNTs are cylinder with hollow internal cavity and with one end closed. According to the number of graphene layers, two classes of CTNs are identified: single-walled carbon nanotube with a single layer of graphene (SWCNT) and multiple layers of graphene known as multi-walled carbon nanotubes (MWCNTs) (Jackson et al., 2013; Zarbin & Oliveira, 2013).

The absorption of nanocomposites in fish might be through the digestive tract and the gill surface, being carried by the blood up to the target organs as spleen and liver (Marijić & Raspor, 2006; Lee, Choi, Kim, & Lee, 2015). The liver has been used in several toxicological studies because it is a key organ in the detoxification of xenobiotics (Ribeiro et al., 2011; Manrique et al., 2014). On the other hand, the spleen is a central component of the immune system, playing an important role in the response against invasion of pathogens and functioning as a selective filter of the vascular system (Gomes et al., 2015; Oliveira et al., 2018). In some fish species, head kidney, liver, and spleen present accumulation of pigmented macrophages called melanomacrophage centres (MMC) (Sales et al., 2017; Steinel & Bolnick, 2017).

The MMCs have been used in several studies with teleosts submitted to different xenobiotics as biomarkers. Thus, the increase in the number of MMCs, the area and types of pigments are correlated with the action of stressors (Gomes et al., 2015; Oliveira et al., 2018; Rastgar, Ardeshir, Zabihi, Movahedinia, & Salati, 2019). However, studies that evaluate the adverse effect of nanocomposite such as MWCNT in

primary culture from fish melanomacrophages were not developed yet. Macrophage primary culture from cranial kidney and spleen from fish have been developed to characterize this cell type both morphologically and biochemically (Belosevic, Hanington, & Barred, 2006; Hodgkinson, Fibke, & Belosevic, 2017). The main characteristics of the macrophage are adherence, irregular shape and CSF-1 expression (Belosevic et al., 2006). Interestingly, the melanomacrophages show autofluorescence due to present lipofuscin pigment (Diaz-Satizabal & Magor, 2015). Finally, a great repertoire in synthesis and release of pro-inflammatory cytokine by melanomacrophages have been recorded (Belosevic et al., 2006; Hodgkinson et al., 2017).

Nile tilapia, *Oreochromis niloticus*, is an important fish species for world aquaculture, since it presents several characteristic as rich protein resource, high growth rate, resistance to several disease types, omnivorous and fast reproduction. Moreover, *O. niloticus* presented as a useful experimental model due to its easy management practices, laboratory maintenance, good adaptation to environmental changes and susceptibility to various stress conditions (Gomes et al., 2015; Oliveira et al., 2018).

This study aimed to evaluate the toxicity of MWCNTs in primary culture macrophages from liver and spleen of Nile tilapia. In addition, we recorded the different responses of the melanomacrophage types after exposed to MWCNTs.

Material and methods

Chemical characterization of MWCNT

In this study we used the multiwalled carbono nanotubes Baytubes®, developed by Bayer Material Science, Germany. Initially, the Multi-Walled Carbon Nanotubes were characterized by X-ray diffraction, Raman spectroscopy, Thermogravimetric Analysis, Scanning electron microscopy and inductively coupled plasma optical emission spectrometry. X-ray diffraction measurements of the MWNTs were obtained on a Shimadzu 6000 XRD diffractometer using Cu-Kα radiation (λ = 1.5418 Å) at 40 kV, 40 mA and 0.02 ° resolution at 2θ.

The Raman spectra were obtained in Renishaw Spectrophotometer, coupled to an optical microscope with 1 micrometer spatial resolution, using 514 nm laser as an excitation source and 20 mW of power. The spectra were obtained with 30 scans in the 3500-250 cm^{-1} region with counting time of 10 seconds.

The thermalgravimetric analyzes were performed using Shimadzu equipment, model DTG-60H, operating at a temperature ranging from 23 to 900°C. The analyzes were obtained in a nitrogen atmosphere, with gas flow rate of 50 milliliters per minute, using about 3.5 g of sample per analysis.

The images were obtained in Scanning Electron Microscopy Model Pro X (Phenom-World) working at 10 kV. The metals quantification was reached employing the ICP-OES (Perkin Elmer Optima 8300) technique. The digestion protocol applied was the 3050B (U.S.EPA), using 0.01 g of multiwalled carbon nanotubes.

Melanomacrophage primary culture

Twenty healthy juvenile males Nile Tilapia (10 cm total length and 15 g body weight) were obtained from a fish farm in Sete Lagoas, Brazil. The fish were maintained temporarily in tanks with dechlorinated tap water at 25ºC under a 14:10h light: dark cycle for two weeks before the experiment. They were fed with commercial food twice a day. The present work was approved by the Committee on Ethics on the Use of Animals, *Universidade Federal de São João Del-Rei* under protocol 013/2016. Animal handling and laboratory procedures were conducted following the ethical principles established by the Brazilian College of Animal Experimentation (COBEA).

After acclimatization, the animals were anaesthetized using eugenol 1 mg mL^{-1}. The sanitation of the body surface was performed with iodine and 70% alcohol. Liver and spleen were removed and maintained in Leibovitz's (L15) incomplete culture medium. The organs were cut into pieces using sterile stainless steel scissors and forceps. After maceration, the samples were transferred to Falcon tube and centrifuged at 250 g for 10 min. at 4ºC. The supernatant was removed and cells were plated in 6-well microplates with Leibovitz's L-15 medium supplemented with fetal bovine serum (FBS) (10%) and 0.1% gentamicin. Thus, cells were maintained in a humidified environment (95% relative humidity) at 25°C with 5% of CO_2.

Characterization of liver and spleen melanomacrophages

The morphological characterization of the liver and spleen melanomacrophages were performed using three methods: I) inverted microscopy observing the melanomacrophage adherence and morphology in plate; II) the melanomacrophage culture was analyzed in plate at fluorescence microscope for observation of the autofluoresce from melanomacrophages, and III) the melanomacrophages were cultured in glass histological coverslips in the bottom of the wells. Thus, the melanomacrophages adhered to coverlips permitted the cellular stained and morphology identification. Thus, the melanomacrophages were stained by Giemsa for morphological characterization and Perls' assay was used to identify melanin, hemossiderin and lipofuscin pigments present in this cellular types.

Evaluation of the MWCNTs toxcity in melanomacrophage primary culture

Initially, a stock solution of 1 μg mL^{-1} of MWCNTs in dimethylsulfoxide (DMSO) was made. This was added to the Unique® Ultrasonic Cell Disrupter for 2 cycles of 30 seconds, with 70% power, to de-agglomerate the MWNTs. Afterwards, they were submitted to the Ultrasound Bath, Unique®, for 30 minutes. After 48 hours incubation, the melanomacrophage from liver and spleen were submitted to MWCNTs to 1 μg mL^{-1} for 24 hours. The control group did not receive MWCNT, only vehicle 0.1% DMSO.

After exposition to MWCNTs, the cellular viability was recorded using Trypan blue exclusion method (Luna et al., 2016). Briefly, the Leibovitz's (L15) medium was changed and added 100 μ L of the Trypan blue dye and the plate was incubated in at CO_2 5% at 25°C for 20 minute. Next, the cells were analyzed and counted in inverted microscopy where they were classified as viable (Trypan blue-negative) or dead (Trypan blue-positive). For this analysis, 100 melanomacrophages were counted by well and percentage was obtained among viable and dead cells. Moreover, morphological effects after toxicity to MWCNTs were recorded using Perls' assay.

Results and discussion

Chemical characterization

The image below shows the X-ray diffraction for the MWCNTs (Figure 1). An intense peak in the region of 26° in 2θ, related to the distance between the concentric sheets of graphene in the multilayer NTs of the graphite at d = 0.34 nm can be verified. It can also be observed the presence of peaks in 44.7°. These can be explained by interference of the XRD analysis itself, in which the sample port used for XRD was composed of aluminum, with a corresponding peak at 44°.

The Raman spectroscopy of the MWCNTs presented 4 main bands: 1344, 1578, 2684 and 2926 cm^{-1}. The 1344 cm^{-1} band also called the D-band originates from the induced modes of graphite disorder, and its second harmonic, the G'-band, appears due to a double-resonance process, appearing on the spectrum at 2684 cm^{-1}. The G-band, (1578 cm^{-1}), is associated with the vibration modes of graphite. By observing only the D and G bands, a high intensity ratio between them can be noted. This indicates that these nanotubes can contain a large amount of defects in their structure. The D-band spectrum, which is associated with the presence of disorders or finite-size effect on sp^2 carbons, does not necessarily imply that MWCNTs have disorder in their structures. The intensities of the D and G bands are dependent on the polarization of the MWCNTs themselves, in which they can vary according to the RAMAN spectrum polarization (Rao et al., 2000).

The thermogravimetric analysis shows a main decomposition stage, which occurs between 500 and 680°C, which is the characteristic of the decomposition of CNTs with multiple walls. Observing the DTG curve, we see the one main stage of loss, beginning at 572°C, which can be attributed to the burning of CNTs with greater amount of structural defects. At higher temperatures, between 601 and 617°C the weight loss was attributed to the burning of CNTs with the least number of defects in structure and higher number of concentric layers. It is important to note that these starting CNTs were acquired and used without any prior purification treatment. No peaks corresponding to the burning of amorphous carbon, fullerenes or metals were significantly detected. According to the manufacturer, we observed this material is 95% pure.

In scanning electron microscopy image, it was noted that there was a cluster of MWCNTs, enveloped and arranged in bundles. It was also observed some 'light spots' that can evidence the presence of the catalysts particles coming from the synthesis of MWCNTs. The metal particles observed in SEM are from the synthesis

of CNTs, but are not observed in the XRD and Thermalgravimetric techniques due to the limit of detection of these analyzes. It is known that the study of the presence of catalysts is extremely important because they can cause cellular damages in consonance with the MWCNTs.

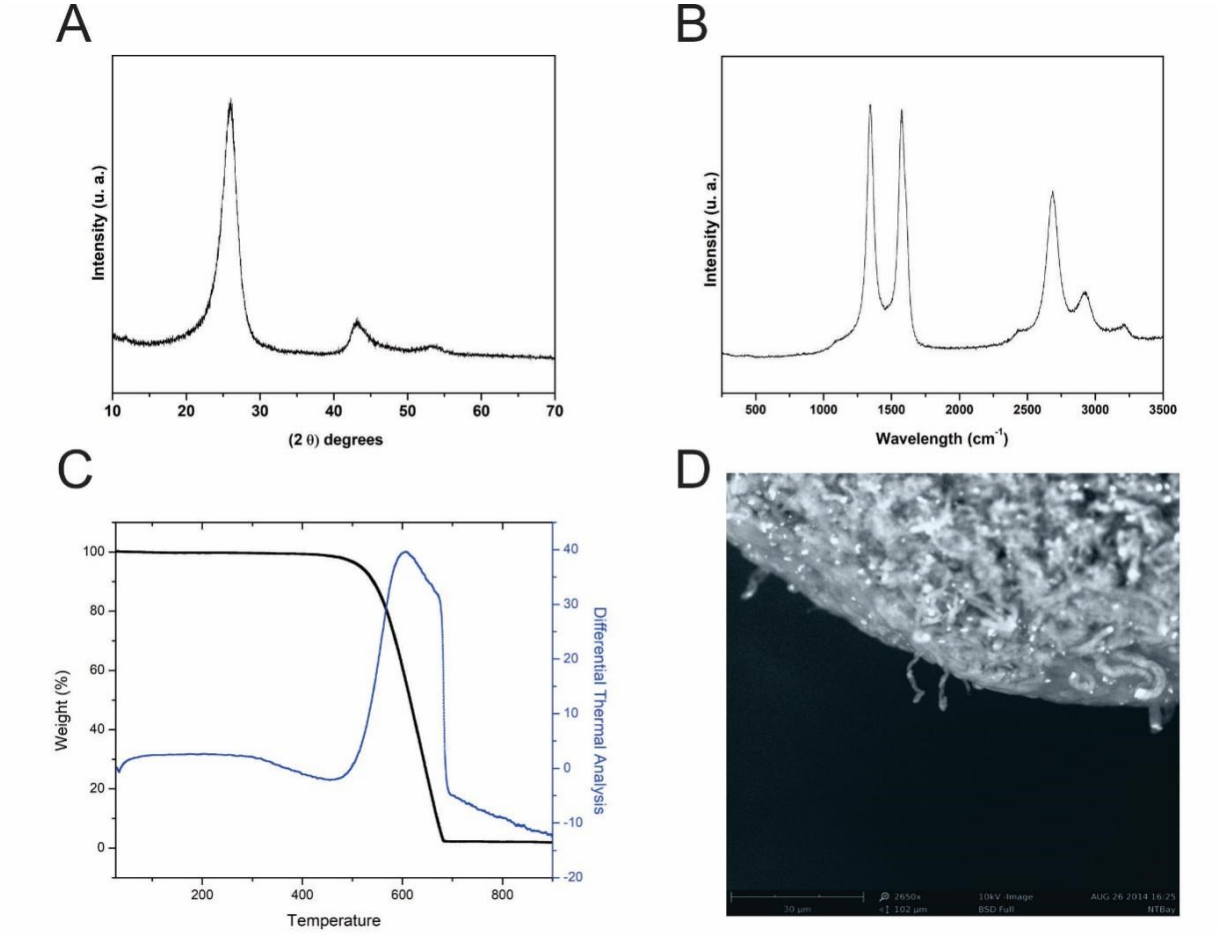

Figure 1. Chemical characterization of multiwalled carbon nanotubes. A) X-ray diffraction pattern of MWCNTs in 2θ. (B) Raman scattering spectra for MWCNTs bundles. C) Thermogravimetric analysis and differential thermal analysis monitored between 30 and 900°C. D) SEM image of MWCNT bundles.

The average concentrations of the determined metal species in ug per gram of MWCNT were determined by ICP-OES analysis. The data obtained in the equipment were treated, being converted to μg g^{-1}, considering the analyzes were carried out in triplicates. The results of quantification of catalytic residual metals obtained the average concentration of 4089.5 μg g^{-1} of aluminum, 1509.4 μg g^{-1} of cobalt, 184.2 μg g^{-1} of copper, 1873.6 μg g^{-1} of manganese, 7932.4 μg g^{-1} of titanium and 149.5 μg g^{-1} of zinc. This result indicates a considerable presence of metals present in the carbon skeleton of the nanotubes.

This type of characterization may be seem unnecessary for a material composed of carbon atoms only. However, it is necessary to remember that metallic catalysts, which justifies the use of this characterization technique, since it is not possible to detect these metals in any of the aforementioned techniques.

Although pure carbon nanotubes have a π-conjugate structure with a highly hydrophobic surface, they are poorly soluble in most organic solvents because of the high molecular weight and their tendency to entangle and form networks through persistent van der Waals interactions. An alternative to overcome this problem is to promote the ds intertwining MWCNTs by energy agitation. Generally, agitation is provided by magnetic stirring, reflux, shear mixing or, more commonly, sonication, both light sonication in bath and sonication of high power using a tip (Di Crescenzo, Ettorre, & Fontana 2014). Also, dimethylsulfoxide, a polar aprotic solvent, is recognized as a solvent with the potential to satisfactorily solvate the MWCNTs and good and relatively stable dispersions by sonicating (Marcus, 1991). For the present study, it was decided not to use surfactants, such as Triton, or dispersants, such as polyethylene glycol or gum arabic. In addition to the fact that they cause interferences in the biological tests, and as false positive, by oxidation of the MTT salt itself

(Belyanskaya, Manser, Spohn, Bruinink, & Wick 2007). Besides, it has been reported that MWCNTs may have direct cytotoxic effects, depending on the level of agglomeration and dispersion of these. Correlating the lack of control in the dispersion, this fact can be determinant for toxicity, since one can lose the control of the concentration to be submitted, being able to be bigger or smaller than the expected one. In addition, Wick et al., 2007 have demonstrated that agglomerates of MWCNTs are less toxic than dispersed, since the presence of larger agglomerates has the obvious consequence of substantially decreasing the surface available for interaction between cells and MWCNT. Therefore, the objective of the dispersion methodology is to obtain the maximum homogeneity of MWCNTs, both to avoid interfering cytotoxic effects from agglomerated MWCNTs and to control the stoichiometry of the concentrations to be submitted to biological tests.

Although it is difficult to create comparison between nanostructures, specially among MWCNTs, which have their own distinct characteristics, making the data not standardized for studies, some conditions have been highlighted as determinants of the toxicity of MWCNTs. One of them is the variation of impurities of the different preparations, being able to cite the presence of metals, like iron and nickel. The toxicity of high concentrations of these metals is well known (Wang, Qu, Huang, Wei, & Wang 2015). Another very important contamination is amorphous carbon, which exhibits comparable biological effects such as graphite or as relevant particles of pollution in ambient air. It has been shown that carbon nanotubes are generally very pure, lose the ability to induce acute toxicity or oxidative stress. Comparing these data with the performed chemical characterization results, the MWCNTs present poorly formed tube and disorder characteristics in the carbon structure, evidenced by thermogravimetric analyzes and Raman spectroscopy. However, as observed in the ICP-OES data, the MWCNTs have purity compromised by the presence of the catalytic residual metals. In this way, it can be inferred that possible cytotoxic effect is due to the presence of these metals adhered to the structure of the nanotubes.

Here, we described the MWCNTs toxicity in melanomacrophges primary culture from liver and spleen in *O. niloticus*. Phagocytosis activity of liver and spleen melanomacrophages was observed by presence of black inclusions in cytoplasm. In mammals, two routes have been described for CNTs after internalization: the first, CNTs would be endocyted by pulmonary macrophages when exposure is by inhalation; the second could be the macrophages resident in the liver and spleen after intravenous injection (Elgrabli et al., 2015). Several experiments verified the toxicity of CNTs in different cell lines aiming to determine nanotoxicity of various nanomaterials (Wang, Sun, Bao, Liu, & An, 2011; Luna et al., 2016). This experiments were performed in cellular culture from mammals and the findings showed that nanoparticules had toxic effects. Macrophages submitted to graphene oxide-silver nanocomposite showed remarkable oxidative stress in this cellular type when compared to control group (Luna et al., 2016). However, studies that verify the toxicity of nanomaterials in defense cells such as fish macrophages have not yet been developed and need to be elucidated.

Characterization of melanomacrophages from liver and spleen in primary culture

In order to verify the toxic effects of MWCNT, it was necessary to know the morphology of the melanomacrophages in the liver and spleen from *O. niloticus*. This characterization was performed in primary culture from melanomacrophage from both organs at 48 hours incubation. Macrophage is an important inflammatory cell acting in different biological processes in the body as phagocytosis, hematopoiesis, inflammatory process and cooperation with the lymphocyte developing adaptive response (Wolke, 1992). Liver and spleen melanomacrohages stained by Giemsa were large in relation to other cellular types. They had irregular shape with cytoplasm filled with large granules, central and evident nucleus (Figure 2). These characteristics were also described in primary culture of macrophage from head kidney of goldfish (*Carassius auratus*), and in rainbow trout (*Oncorhynchus mykiss*) (Belosevic et al., 2006). To confirm the presence of liver and spleen melanomacrophages in culture, the Perls' methods were developed. Melanomacrophages in culture had mainly hemosiderin (blue) pigment in the cytoplasm (Figure 2). The melanomacrophages are clusters of macrophages containing melanin, hemosiderin and lipofuscin pigments (Sales et al., 2017; Oliveira et al., 2018; Rastgar et al., 2019). In goldfish, 10% melanomacrophages from spleen presented hemosiderin using Perls' assay (Diaz-Satizabal & Magor, 2015). Nile tilapia submitted to atrazine showed a quantitative change in the type of pigments from melanomacrophages present in the spleen. In this work, the chronic exposition to atrazine increased lipofuscin in relation to hemosiderin and melanin pigments in melanamacrophage from spleen MMC (Oliveira et al., 2018). In this study, we did not observe difference in pigmentation in relation to melanomacrophage from liver and spleen. Cell culture analysis with 48 hours

incubation under fluorescence microscopy (wavelength 500 to 580 nm) showed that the melanomacrophages of both organs showed green fluorescence (Figure 2). The autofluorescence observed in melanomacrophages was due the presence of lipofuscin, the main pigment in these cells (Agius & Roberts, 2003; Diaz-Satizabal & Magor, 2015).

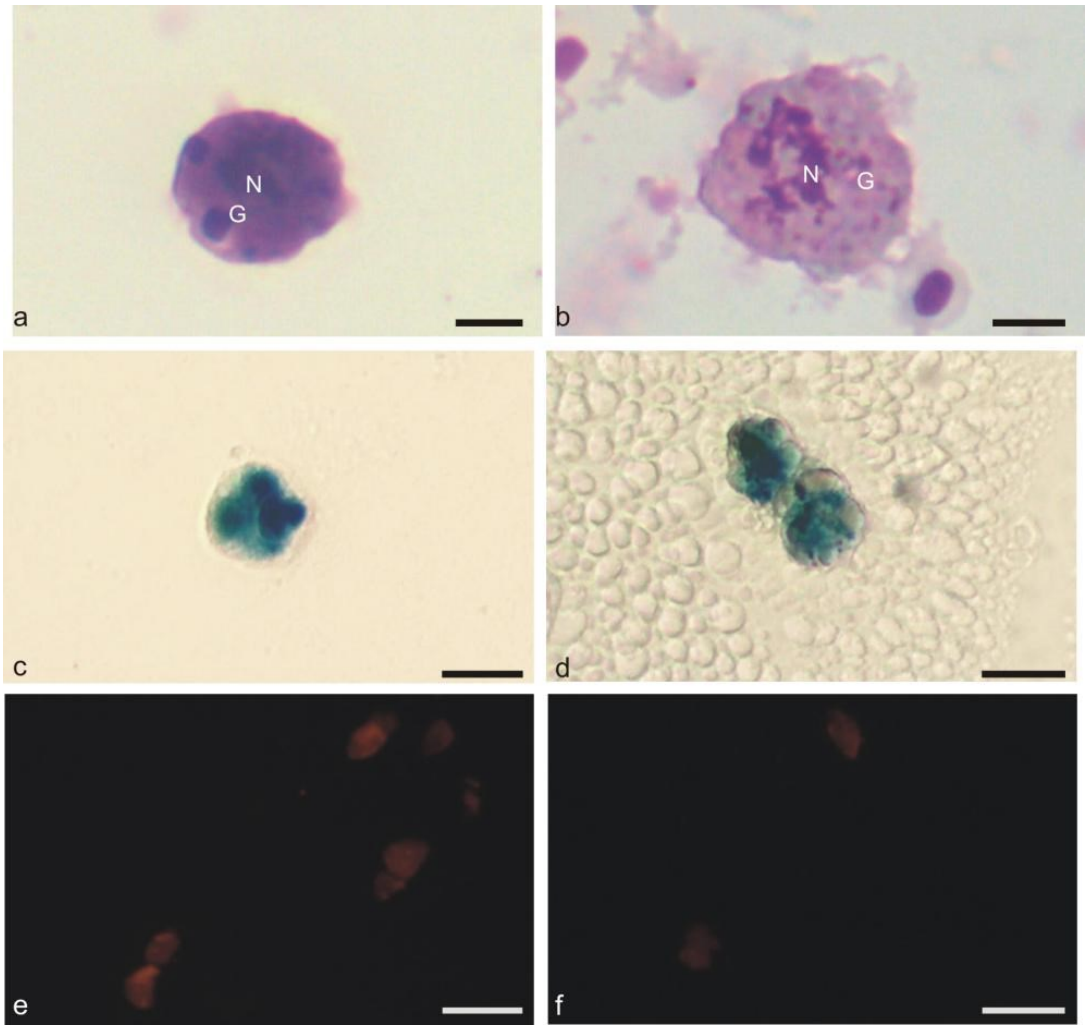

Figure 2. Melanomacrophages from liver (A, C and E) and spleen (B, D and F) stained with Giemsa (A-B), Perls' assay (C-D) and autofluorescence when excited with wavelengths of light at 500 - 580 nm (E-F). Nucleus (N) and granules (G) in the cytoplasm. Bars = 50 µm

MWCNT promoted toxicity in melanomacrophages primary culture

The Trypan blue exclusion test proved to be efficient to verify cellular viability of melanomacrophages after exposition to MWCNTs (Figure 3). This assay permitted to evaluate cellular integrity, while dead cells are more permeable to stain in relation to viable cells (Luna et al., 2016). Previous studies indicate that CNTs released to the environment can reach aquatic animals, thus raising a great concern about the environmental toxicity of these nanocomposites (Lee et al., 2015). In this sense, it is necessary to develop experimental studies with CNTs to know the toxic effects in experimental conditions that can be extrapolated to the natural environment in future. Herein, we chose the macrophages because they are important cells to the immune system being present in important organs of the metabolism and defense of fish by phagocytosis (Steinel & Bolnick, 2017; Oliveira et al., 2018). After exposition to 1 µg mL^{-1} MWCNTs, spleen and liver melanomacrophages presented viability of 59 and 48%, respectively. In the control group, the melanomacrophages presented 85% cellular viability. Few studies have verified the effects of nanocomposites on cultured macrophages. Rat peritoneal macrophages and J774 tumor macrophages submitted to 1.25 µg mL^{-1} of graphene oxide-silver nanocomposite showed a decrease in cellular viability, between 15 and 25% (Luna et al., 2016). Pleural macrophages from rats that were submitted to concentrations of 5 to 50 µg cm^2 of carbon nanotubes showed a decrease in cell viability proportional to the increase in concentrations (Murphy,

Schinwald, Poland, & Donaldson, 2012). From a qualitative analysis of melanomacrophage culture, it was possible to observe that spleen and liver macrophages responded differently to MWCNTs, where the liver melanomacrophages were mostly lysed when compared to spleen melanomacrophages. The relationship between MWCNTs and macrophages damage is known, which previous studies suggest that MWCNTs may damage or rupture the plasma membrane of macrophages, which explain the data obtained (Hirano, Kanno, & Furuyama, 2008; Palomäki et al., 2011).

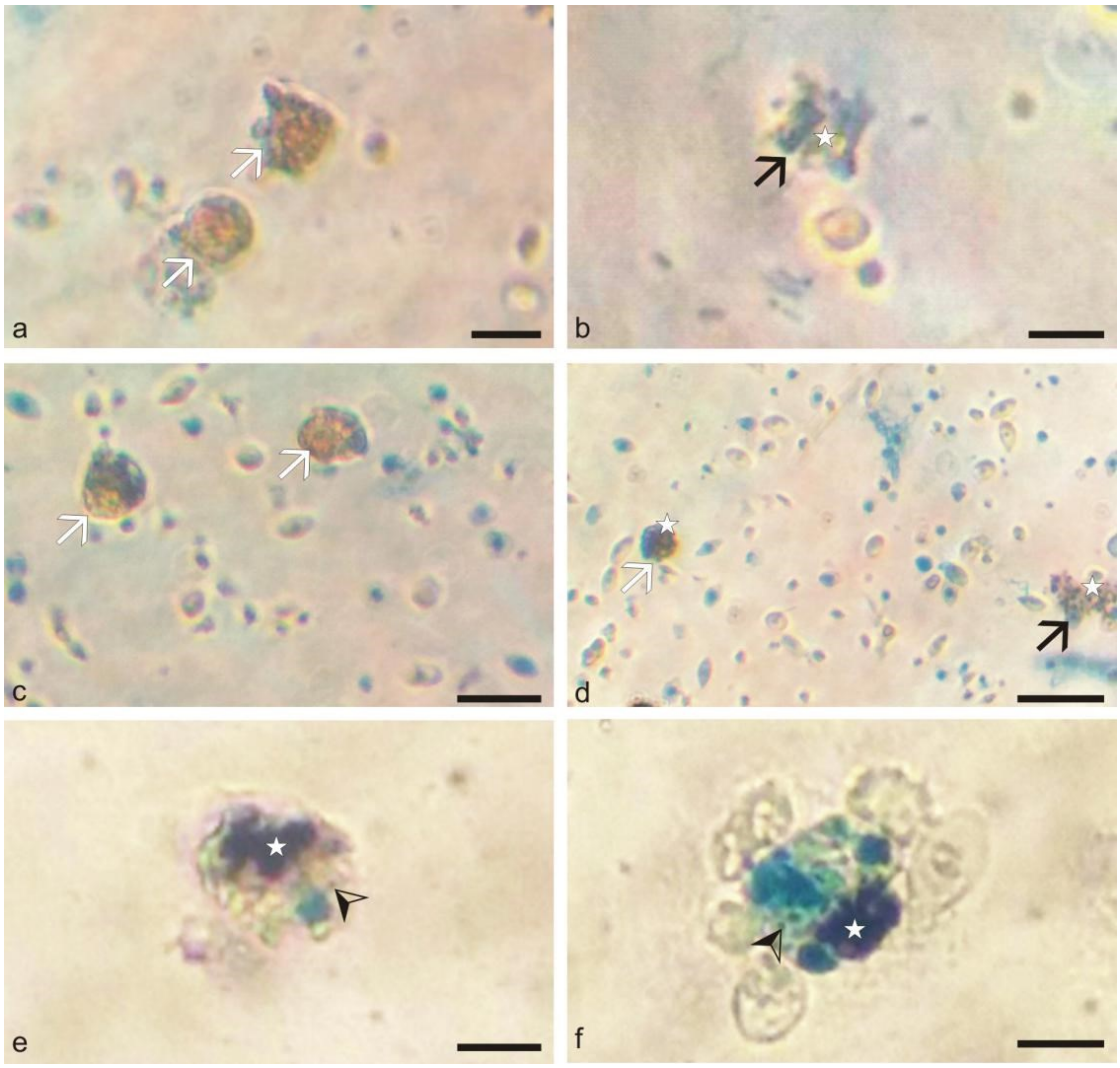

Figure 3. Celullar viability observed using Trypan blue (A-D) and MWCNT internalized by melanomacrophages using Perls method (E-F). Melanomacrophages from liver (A-B and E) and spleen (C-D and F). Control groups (A and C) with viable cells (white arrows) and treatment groups (B, D, E-F) showing cellular death (black arrows) of melanomacrophages. MWCNT endocytosed by melanomacrophages (stars and arrowheads). Bars = 50 µm.

A hallmark of macrophages is the high phagocytic activity of substances foreign to the organism (Steinel & Bolnick, 2017). Phagocytic activity can be observed in light and electron microscopy in the macrophages (Luna et al., 2016; Qiu et al., 2016) Thus, from the coverslips stained with the Perl's assay it was possible to observe that the cytoplasm of the liver and spleen melanomacrophages had black inclusions which might be MWCNTs endocytosed by these cells (Figure 3). Phagocytic activity after exposition to nanocomposites were recorded in rat macrophages and murine macrophages (tumoral lineage J774) and black inclusions were also observed in the cytoplasm of the these cells (Murphy et al., 2012; Luna et al., 2016). Probably MMCs from distinct organ can present different functions, where MMCs from liver could be associated to general metabolism, while MMCs from spleen could be related with inflammatory process (Vigliano, Bermúdez, Quiroga, & Nieto, 2006; Ribeiro et al., 2011). However, the real function of different types of macrophages from MMC still promotes great discussion and remains controversial.

Conclusion

In summary, this study demonstrated the success in the establishment of primary culture of liver and spleen macrophages from *O. niloticus*. Then, in the future we will be able to perform cytotoxic assays with varied xenobiotics using defense cells in fish. In addition, we observed that the cytoplasm of macrophages showed MWCNTs inclusions demonstrating phagocytic activity and that the concentration of 1 μg mL^{-1} of the nanocomposite used was cytotoxic for both macrophages. At last, it was observed that liver and spleen melanomacrophages responded to MWCNTs after 24 hours exposition promoting phagocytic activity.

Acknowledgements

We would like to thank the *Fundação de Amparo à Pesquisa de Minas Gerais* (FAPEMIG CVZ_APQ-03548-16) and the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq process 405822 / 2016-2) for financial support. The authors also thank the *Universidade Federal de São João Del Rei* (UFSJ) for equipment and undergraduate student scholarship.

References

Agius, C., & Roberts, R. J. (2003). Melano-macrophage centres and their role in fish pathology. *Journal of Fish Diseases*, *26*(9), 499-509. doi: 10.1046/j.1365-2761.2003.00485.x

Avant, B., Bouchard, D., Chang, X., Hsieh, H. S., Acrey, B., Han, Y., ... Knightes, C. D. (2019). Environmental fate of multiwalled carbon nanotubes and graphene oxide across different aquatic ecosystems. *NanoImpact*, *13*(1), 1-12. doi: 10.1016/j.impact.2018.11.001

Belosevic, M., Hanington, P. C., & Barreda, D. R. (2006). Development of goldfish macrophages in vitro. *Fish and Shellfish Immunology*, *20*(2), 152-171. doi: 10.1016/j.fsi.2004.10.010

Belyanskaya, L., Manser, P., Spohn, P., Bruinink, A., & Wick, P. (2007). The reliability and limits of the MTT reduction assay for carbon nanotubes-cell interaction. *Carbon*, *45*(13), 2643-2648. doi: 10.1016/j.carbon.2007.08.010

Di Crescenzo, A., Ettorre, V., & Fontana, A. (2014). Non-covalent and reversible functionalization of carbon nanotubes. *Beilstein Journal of Nanotechnology*, *5*(1), 1675-1690. doi: 10.3762/bjnano.5.178

Diaz-Satizabal, L., & Magor, B. G. (2015). Isolation and cytochemical characterization of melanomacrophages and melanomacrophage clusters from goldfish (*Carassius auratus*, L.). *Developmental and Comparative Immunology*, *48*(1), 221-228. doi: 10.1016/j.dci.2014.10.003

Elgrabli, D., Dachraoui, W., Ménard-Moyon, C., Liu, X. J., Bégin, D., Bégin-Colin, S., ... Alloyeau, D. (2015). Carbon nanotube degradation in macrophages: live nanoscale monitoring and understanding of biological pathway. *ACS Nano*, *9*(10), 10113-10124. doi: 10.1021/acsnano.5b03708

Gomes, J. M. M., Ribeiro, H. J., Procópio, M. S., Alvarenga, B. M., Castro, A. C. S., Dutra, W. O., ... Corrêa, J. D. (2015). What the erythrocytic nuclear alteration frequencies could tell us about genotoxicity and macrophage iron storage? *PLoS One*, *10*(11), e0143029. doi: 10.1371/journal.pone.0143029

Hirano, S., Kanno, S., & Furuyama, A. (2008). Multi-walled carbon nanotubes injure the plasma membrane of macrophages. *Toxicology and Applied Pharmacology*, *232*(2), 244-251. doi: 10.1016/j.taap.2008.06.016

Hodgkinson, J. W., Fibke, C., & Belosevic, M. (2017). Recombinant IL-4/13A and IL-4/13B induce arginase activity and down-regulate nitric oxide response of primary goldfish (*Carassius auratus* L.) macrophages. *Developmental and Comparative Immunology*, *67*(1), 377-384. doi: 10.1016/j.dci.2016.08.014

Jackson, P., Jacobsen, N. R., Baun, A., Birkedal, R., Kühnel, D., Jensen, K. A., ... Wallin, H. (2013). Bioaccumulation and ecotoxicity of carbon nanotubes. *Chemistry Central Journal*, *7*(1), 154-175. doi: 10.1186/1752-153X-7-154

Lee, J. W., Choi, Y. C., Kim, R., & Lee, S. K. (2015). Multiwall carbon nanotube-induced apoptosis and antioxidant gene expression in the gills, liver, and intestine of *Oryzias latipes*. *BioMed Research International, 2015*(Spec.), 1-10. doi: 10.1155/2015/485343

Luna, L. A. V., Moraes, A. C. M., Consonni, S. R., Pereira, C. D., Cadore, S., Giorgio, S., & Alves, O. L. (2016). Comparative in vitro toxicity of a graphene oxide-silver nanocomposite and the pristine counterparts toward macrophages. *Journal of Nanobiotechnology*, *14*(12), 12-29. doi: 10.1186/s12951-016-0165-1

Manrique, W. G., Claudiano, G. S., Petrillo, T. R., Castro, M. P., Figueiredo, M. A. P., Belo, M. A. A., ... Moraes, F. R. (2014). Response of splenic melanomacrophage centers of *Oreochromis niloticus* (Linnaeus, 1758) to inflammatory stimuli by BCG and foreign bodies. *Journal of Applied Ichthyology*, *30*(5), 1001-1006. doi: 10.1111/jai.12445

Marcus, Y. (1991). The effectiveness of solvents as hydrogen bond donors. *Journal of Solution Chemistry*, *20*(9), 929-944. doi: 10.1007/BF01074953

Marijić, V. F., & Raspor, B. (2006). Age- and tissue-dependent metallothionein and cytosolic metal distribution in a native Mediterranean fish, *Mullus barbatus*, from the Eastern Adriatic Sea. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *143*(4), 382-387. doi: 10.1016/j.cbpc.2005. 05.019

Murphy, F. A., Schinwald, A., Poland, C. A., & Donaldson, K. (2012). The mechanism of pleural inflammation by long carbon nanotubes: Interaction of long fibres with macrophages stimulates them to amplify pro-inflammatory responses in mesothelial cells. *Particle and Fibre Toxicology*, *9*(1), 8-23. doi: 10.1186/1743-8977-9-8

Oliveira, S. E., Costa, P. M., Nascimento, S. B., Castro, W. V., Ribeiro, R. I. M. A., Santos, H. B., & Thomé, R. G. (2018). Atrazine promotes immunomodulation by melanomacrophage centre alterations in spleen and vascular disorders in gills from *Oreochromis niloticus*. *Aquatic Toxicology*, *202*(1), 57-64. doi: 10.1016/j.aquatox.2018.06.018

Palomäki, J., Välimäki, E., Sund, J., Vippola, M., Clausen, P. A., Jensen, K. A., ... Alenius, H. (2011). Long, needle-like carbon nanotubes and asbestos activate the NLRP3 inflammasome through a similar mechanism. *ACS Nano*, *5*(9), 6861-6870. doi: 10.1021/nn200595c

Qiu, W., Liu, S., Chen, J., Hu, L., Wu, M., & Yang, M. (2016). The primary culture of carp (*Cyprinus carpio*) macrophages and the verification of its phagocytosis activity. *In Vitro Cellular and Developmental Biology - Animal*, *52*(1), 10-19. doi: 10.1007/s11626-015-9942-7

Rao, A. M., Jorio, A., Pimenta, M. A., Dantas, M. S. S., Saito, R., Dresselhaus, G., & Dresselhaus, M. S. (2000). Polarized raman study of aligned multiwalled carbon nanotubes. *Physical Review Letters*, *84*(8), 1820-1823. doi: 10.1103/PhysRevLett.84.1820

Rastgar, S., Ardeshir, R. A., Zabihi, E., Movahedinia, A., & Salati, A. P. (2019). Immunotoxicity of estrogen and nonylphenol on apoptosis and expression of ERs in goldfish macrophage: Opening new avenue for discovering the role of experimental model systems and sexes. *Aquatic Toxicology*, *209*(1), 159-167. doi: 10.1016/j.aquatox.2019.02.007

Ribeiro, H. J., Procópio, M. S., Gomes, J. M. M., Vieira, F. O., Russo, R. C., Balzuweit, K., ... Corrêa, J. D. (2011). Functional dissimilarity of melanomacrophage centres in the liver and spleen from females of the teleost fish *Prochilodus argenteus*. *Cell and Tissue Research*, *346*(3), 417-425. doi: 10.1007/s00441-011-1286-3

Sales, C. F., Silva, R. F., Amaral, M. G. C., Domingos, F. F. T., Ribeiro, R. I. M. A., Thomé, R. G., & Santos, H. B. (2017). Comparative histology in the liver and spleen of three species of freshwater teleost. *Neotropical Ichthyology*, *15*(1), 1-12. doi: 10.1590/1982-0224-20160041

Steinel, N. C., & Bolnick, D. I. (2017). Melanomacrophage centers as a histological indicator of immune function in fish and other poikilotherms. *Frontiers in Immunology*, *8*(1), 827. doi: 10.3389/fimmu.2017.00827

Vigliano, F. A., Bermúdez, R., Quiroga, M. I., & Nieto, J. M. (2006). Evidence for melano-macrophage centres of teleost as evolutionary precursors of germinal centres of higher vertebrates: An immunohistochemical study. *Fish and Shellfish Immunology*, *21*(4), 467-471. doi: 10.1016/j.fsi.2005.12.012

Wang, J., Sun, P., Bao, Y., Liu, J., & An, L. (2011). Cytotoxicity of single-walled carbon nanotubes on PC12 cells. *Toxicology in Vitro*, *25*(1), 242-250. doi: 10.1016/j.tiv.2010.11.010

Wang, X., Qu, R., Huang, Q., Wei, Z., & Wang, Z. (2015). Hepatic oxidative stress and catalyst metals accumulation in goldfish exposed to carbon nanotubes under different pH levels. *Aquatic Toxicology*, *160*(1), 142-150. doi: 10.1016/j.aquatox.2015.01.015

Wick, P., Manser, P., Limbach, L. K., Dettlaff-Weglikowska, U., Krumeich, F., Roth, S., ... Bruinink, A. (2007). The degree and kind of agglomeration affect carbon nanotube cytotoxicity. *Toxicology Letters*, *168*(2), 121-131. doi: 10.1016/j.toxlet.2006.08.019

Wolke, R. E. (1992). Piscine macrophage aggregates: a review. *Annual Review of Fish Diseases, 1*(2), 91-108. doi: 10.1016/0959-8030(92)90058-6

Zarbin, A. J. G., & Oliveira, M. M. (2013). Nanoestruturas de carbono (nanotubos, grafeno): Quo vadis? *Quimica Nova*, *36*(10), 1533-1539. doi: 10.1590/S0100-40422013001000009