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Modulation of immune genes mRNA levels in mucosal tissues and DNA damage in red blood cells of *Sparus aurata* by gold nanoparticles

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ABSTRACT

Gold nanoparticles (AuNP) effects on *Sparus aurata* were evaluated on skin, gills and intestine by assessing the expression of immune genes and in peripheral blood evaluating genetic damage. Fish were exposed to 0.5 and 50 μg/L AuNP for 96 h. Results showed that exposure to 50 μg/L AuNP induced an upregulation in the expression of innate immune genes in gills (*c3*, *lys*, *il1β*, *tnfa*, *il6*, *il10 and tgfβ*) and intestine (*il1β*, *tnfa* and *il6*). Furthermore, mRNA levels of *hsp70* and *hsp90* were increased in gills after exposure to 0.5 μg/L AuNP, when compared to 50 μg/L. Present data demonstrated the sensitivity of gills and intestines to AuNP exposure supporting their use in the study of fish responses to other nanoparticles. Genotoxic potential of AuNP was demonstrated by increased DNA strand breaks in red blood cells of fish exposed to AuNP, suggesting that AuNP represent a potential hazard to fish.

1. Introduction

In aquatic ecotoxicology, genomic tools have been increasingly used, alone or in combination with classic biomarkers, as they provide valuable information to help to understand the mechanisms that underlie environmental adaptations and organisms' condition (Kim et al., 2015). The exposure to a given xenobiotic/environmental alteration may alter the expression pattern of selected genes that are often unique signatures of a specific stressor. Moreover, the analysis of gene expression has the potential to anticipate early signs of injury and predict adverse outcomes. Reverse transcriptase quantitative real-time PCR (RT-qPCR) is the most commonly used technique in gene expression studies, since it is sensitive, specific, fast, easy to use, and reproducible (Bustin and Nolan, 2004). However, an accurate normalization of data is required to reduce errors arising from variation in the quantity and integrity of the RNA template and efficiency of cDNA synthesis (Taylor et al., 2010). Relative quantification using reference genes is one of the most commonly used strategies to analyze gene expression data in RT-qPCR. However, it is known that the transcript levels of reference genes can vary under different experimental conditions (Pfaffl, 2001). Thus, it is important to validate the expression stability of candidate reference genes in any experimental system prior to its use for posterior normalization of data. For this, specific statistical algorithms, such as geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) have been developed and have demonstrated its utility (Lacroix et al., 2014).

Gold nanoparticles (AuNP) are among the most used nanoparticles, with proposed applications in several activities such as diagnosis of cancer and HIV (Kumar et al., 2011), drug delivery (Dreaden et al., 2012), water remediation (Ojea-Jimenez et al., 2012), and aquaculture practices (Saleh et al., 2016). Estimates for environmental levels of AuNP were derived for risk assessment using environmental exposure models and global information on consumer products in the UK, assuming 10% market penetration. According to these, 0.14 μg/L and 5.99 μg/kg could be expected for water and soil, respectively. Other studies make reference of levels ranging from 1 to 20 μg/L as environmentally relevant (Baalousha et al., 2016). Thus, their widespread use placed them as an emerging environmental threat and has led to their inclusion in OECD list of representative manufactured nanomaterials. Furthermore, the increasing number of potential applications of these particles in different fields raises the importance of understanding how fish may react to waterborne nanoparticles. Thus, investigating fish responses to AuNP will have a major impact on future nanosafety research and regulatory frameworks, with reflex on the success of nanotechnology application.

The response of fish to external stressors, such as pathogens or

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contaminants, is carried out by mucosal tissue located in the portals of entry of fish (gills, skin and intestine), which are important routes of xenobiotics and nanoparticles uptake. Thus, external stimuli may produce alteration in mucosal tissues that can be sensed by specific receptors located at these tissues. This recognition may activate a cellular signaling cascade leading to the production of messenger substances that are responsible to initiate a local response which depends on the magnitude and duration of the stimuli (Parra et al., 2015). This local response is mainly based in innate immunity and both cellular and humoral immunity (Parra et al., 2015). These mechanisms help to protect the mucosal tissues but can also activate the overall physiological mechanisms to mount a systemic response. Therefore, both local and systemic responses could be activated against potentially external hazardous stimuli. For example, the exposure of *Mytilus galloprovincialis* to silver nanoparticles through water induced a local inflammatory response in gills (Bouallegui et al., 2017), whereas *Cyprinus carpio* exposed to zinc oxide nanoparticles through diet presented increased levels of complement proteins in the serum (Chupani et al., 2017). Although the local response can be able to mount a primary response at mucosal level by itself without the intervention of the central organs classically associated to systemic response. In this context, and considering the previous evidence of the immunotoxic potential of AuNP in mammals (Devanabanda et al., 2016) we decided to evaluate the effects of AuNP in the molecular expression of immune genes in the portals of entry of *Sparus aurata*. These marker genes included complement component c3 (*c3*), lysozyme (*lys*), immunoglobulin M (*igm*), interleukin 1β (*il1*), interleukin 6 (*il6*), tumour necrosis factor-α (*tnfα*), interleukin 10 (*il10*), transforming growth factor-β (*tgfβ*). Moreover, genes representative of the general namely heat-shock protein 70 and 90 (*hsp70*, *hsp90*) and metallothionein (*mt*). The choice of these marker genes was made taking into account previous studies of the group with the same fish species (Khansari et al., 2018), demonstrating that mRNA levels of these genes are altered in the mucosal tissues of fish after immune challenges, as well the knowledge that rats exposed to AuNP presented an increased gene expression of *il1β*, *tnfα* and *il6* genes in the liver (Khan et al., 2013).

Considering the important role of blood, in the transfer of xenobiotics absorbed through gills, skin and gut to other tissues, blood cells are a potential target for their toxic effects (Oliveira et al., 2010). Genetic lesions in red blood cell have proven its value as tools to assess acute and chronic exposure to genotoxic substances, able to detect exposure to low concentrations of contaminants in a wide range of species (de Lapuente et al., 2015; Valavanidis et al., 2009). Previous studies demonstrated that, phenanthrene exposure induced genotoxicity measured as a significant increase in erythrocytic nuclear abnormalities (ENA) in blood of *Liza aurata* (Oliveira et al., 2007), and that acute and chronic exposures to erythromycin induced an increase in DNA strand breaks and ENA in the blood of *Oncorhynchus mykiss* (Rodrigues et al., 2016). In this sense, different techniques have been employed to study genotoxicity in fish, including the comet assay and the scoring of total nuclear abnormalities in erythrocytes (ENA assay). The comet assay evaluates the damaged DNA caused by a clastogenic agent by assessing DNA strand breaks (de Lapuente et al., 2015) and is considered a biomarker of exposure. This type of damage may be reparable by the cellular DNA repairing machinery. The ENA assay, on the other hand evaluates chromosome clastogenesis by quantifying morphological alterations in the nuclei of erythrocytes, and is considered a biomarker of effect. This type of damage is irreparable and can compromise cell viability (Costa et al., 2011). Studies assessing genotoxic effects of AuNP reported that AuNP-citrate coated is able to induce DNA damage, measured by the comet assay, in human hepatocellular carcinoma cells and in human peripheral blood mononuclear cells (Paino et al., 2012). In fish, AuNP-citrate genotoxicity to *Danio rerio* (zebrafish) was shown by random amplified polymorphic DNA (RAPD)-based methodology. To the authors' knowledge, so far, no study is available concerning AuNP genotoxic potential in marine fish. *S. aurata* (gilthead sea bream) was chosen as a model marine fish to assess the effects of AuNP, after waterborne exposure, because this fish is a top predator, widespread in Atlantic and Mediterranean waters and has a high commercial importance for fishery and aquaculture. As one of the most consumed fish in Europe, it may represent an important route of possible entry of contaminants into humans. The concentrations of AuNP tested in this study include one, 0.5 μg/L, below a predicted environmental value (including water and soil of 6.13 g/L) (García-Negrete et al., 2013) and higher concentration. 50 μg/L, that is within the same order of magnitude of the lowest concentrations tested in studies with bivalves (García-Negrete et al., 2013; Tiede et al., 2009). The purposes of the current research work were i) to evaluate the molecular effects of AuNP in *S. aurata*, by analyzing changes in the expression of immune- and stress-related genes in the mucosal tissues and ii) to assess the genotoxic potential of AuNP in fish peripheral blood by means of the comet and ENA assays. Considering that the reliability of molecular results is directly influenced by the housekeeping genes chosen for normalization, the stability of five potential reference genes was evaluated, to determine the most suitable genes to be used in RT-PCR analysis. These genes included β-actin (*act*), elongation factor 1α (*ef1α*), glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), 18s ribosomal RNA (*18s*) and α-tubulin (*tub*).

2. Material and methods

2.1. Fish husbandry and experimental design

S. aurata (57.7 ± 9.4 g) were purchased from an aquaculture in Spain, transported to the aquarium facility and immediately transferred to 250 L aquaria containing aerated saltwater (salinity 30, Ocean Fish, Prodac) at room temperature (RT, 20 ± 1 °C) and 12:12 light-dark cycle. Fish were fed every day (Sorgal, Portugal) and water temperature and salinity was recorded twice a day. No fish mortality was documented during the acclimatization period (10 days) or during the experimental assay. After a 48 h fasting period, fish were randomly divided into six similar 80 L tanks and the experimental groups (duplicated) were: (1) control group (0 μg/L AuNP); (2) 0.5 μg/L AuNP group and (3) 50 μg/L AuNP group. The AuNP used in this study were synthesized by our research group as described by Barreto et al. (2015) (Barreto et al., 2015) and present the following characteristics: 37 nm diameter; 0.3 polydispersity index and −44.5 zeta potential, which were determined by UV–vis spectrophotometry (Cintra 303, GBC Scientific), dynamic light scattering (Zetasizer Nano ZS, Malvern) and transmission electron microscopy (Hitachi, H9000 NAR) (Supplementary material Fig. S1). After 48 h of the beginning of the exposure, 80% of the exposure media was replaced with new containing the same amount of AuNP. After 96 h of exposure, 10 fish from each group (n = 10) were captured, anesthetized with MS222 and blood collected. Gills, intestine and skin were collected, frozen in liquid nitrogen and kept at −80 °C until analysis. Experimental protocols were approved by Portuguese National Authorities, which agree with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63).

2.2. RNA isolation, retrotranscription and real-time quantitative PCR

Total RNA was extracted from the selected tissues of control and exposed fish using Tri Reagent® (Sigma-Aldrich T9424) and following manufacturer's recommendations. RNA quantification was done using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA) and RNA quality checked with Experion, using the Experion Standard Sens RNA chip (Bio-Rad Laboratories, USA). Reverse transcription was performed using 1 μg of the total RNA using the iScript™ cDNA synthesis kit (Bio-Rad, USA) according to the manufacturer's instructions. Efficiency of the amplification was determined for each primer pair using serial 5-fold dilutions of pooled cDNA and calculated as $E = 10(-1/s)$, where s