UNIVERSIDADE FEDERAL DO PARANÁ

TATIANE KLINGELFUS

TOXICIDADE DE NANOMATERIAIS EM PEIXES UTILIZANDO MÉTODOS *in vivo* E *in vitro*

CURITIBA

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TATIANE KLINGELFUS

TOXICIDADE DE NANOMATERIAIS EM PEIXES UTILIZANDO MÉTODOS *in vivo* E *in vitro*

Tese apresentada como requisito parcial à obtenção do grau de Doutora em Genética, no Curso de Pós-Graduação em Genética, Setor de Ciências Biológicas, da Universidade Federal do Paraná.

Orientadora: Profa. Dra. Marta Margarete Cestari

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Curitiba, 31 de Julho de 2017.

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Não posso deixar de citar alguns recursos tecnológicos que fizeram parte deste trabalho como: Google, Sci-hub, Skell, Thesaurus, Mendeley, Spotfy e Netflix (os dois últimos para estudar e entretenimento, respectivamente). Juntamente a esses recursos posso incluir os chineses, agradecendo ao montante de artigos publicados na área de estudo.

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O Pensador (Augusto Rodin)

“A arte é a ideia da obra, a ideia que existe sem matéria.”

(Aristóteles)

RESUMO

A nanotecnologia tem importante interesse social, devido sua ampla aplicação em diferentes produtos industriais. A atual preocupação com nanomateriais (NMs) é o perigo para a saúde humana e para o ambiente. Considerando que os NMs podem chegar a corpos d'água, há necessidade de estudar os efeitos tóxicos dos NMs em organismos aquáticos. Neste contexto, métodos *in vivo* utilizando peixes podem fornecer informações sistêmicas sobre efeitos de NMs no organismo e por meio de métodos *in vitro*, utilizando linhagens de células de peixes, é possível corroborar os efeitos observados nos métodos *in vivo*, sendo relevante em estudos ecotoxicológicos, uma vez que é possível ter tanto uma abordagem dos efeitos em um animal inteiro quanto aos modos de ação de NMs em moléculas específicas, como o DNA. Assim sendo, os objetivos do presente trabalho foram: 1) avaliar os efeitos de nanoesferas de prata (AgNS) na espécie de peixe *Hoplias intermedius*, contaminada a curto e a longo prazo por exposição via trófica e comparar estes com os efeitos causados por íons de prata (Ag^{+}) — material não particulado e de mesma composição química —, por meio de biomarcadores morfológicos (retina), genéticos (sangue, cérebro, fígado e rim) e bioquímicos (fígado); 2) avaliar o modo de ação da nano-titânio (TiO_2NP), da nano-prata sem estabilizante (AgNP) e da nanoesfera de prata com estabilizante polivinilpirrolidona (AgNS) no DNA, por meio de método *in vitro* com a linhagem de células de peixe RTG-2. Na retina, na esclera e no nervo óptico de *H. intermedius* foram observadas deposições de AgNS após 40 dias de exposição, além de danos como: alterações morfológicas em bastonetes, melanossomos aderidos a cones, hemorragia e ruptura do epitélio pigmentado. A citotoxicidade foi observada pela alteração da atividade das enzimas antioxidantes (superóxido-dismutase, catalase e glutationa S-transferase) no fígado após exposição aguda, assim como alteração na concentração de hidroperóxidos lipídicos e de metalotioneínas, indicando estresse oxidativo. As AgNS foram genotóxicas em todos os tecidos de *H. intermedius*, tanto na exposição aguda quanto na subcrônica, em que foi observada diminuição do escore do ensaio Cometa alcalino padrão dos tratamentos em relação ao controle negativo, sugerindo o dano no DNA do tipo *crosslink* DNA-DNA ou DNA-proteína. O fígado foi o principal tecido alvo na exposição aguda, apresentando maiores efeitos genotóxicos nos tratamentos com AgNS e Ag^{+}, em comparação com os outros tecidos; e na exposição subcrônica, os efeitos genotóxicos por AgNS foram maiores no rim e no cérebro, enquanto que o Ag^{+} causou danos principalmente no fígado e no rim. Com o método *in vitro* e modificações em etapas do ensaio Cometa alcalino foi possível dois modos de ação de TiO_2NP, AgNP a AgNS no DNA: o dano oxidativo e o *crosslink* DNA-DNA ou DNA-proteína. Assim, confirmando o dano *crosslink* DNA-DNA ou DNA-proteína encontrado no DNA dos tecidos da *H. intermedius*. Portanto, o presente trabalho confirmou o potencial tóxico dos NMs estudados, além de demonstrar que o uso de peixes em métodos *in vivo* e *in vitro*, conjuntamente, pode avaliar de forma mais precisa os efeitos causados por NMs, predizendo o perigo para o ambiente aquático.

Palavras-chave: contaminantes emergentes; nanoecotoxicologia; titânio; prata; retina; estresse oxidativo; genotoxicidade.

ABSTRACT

Nanotechnologies are at the center of societal interest, due to their broad spectrum of application in different industrial products. The current concern about nanomaterials (NMs) is the hazard to the human health and the environment. Considering that NMs can reach bodies of water, there is a need for studying the toxic effects of NMs on aquatic organisms. Within this context, the use of *in vivo* method with fish as model can provide systemic information of the NMs effects, and the *in vitro* method based on fish cell lines can be able to corroborate the effects in the *in vivo* methods found. In addition, the *in vivo* and *in vitro* methods together are important to the ecotoxicology, due to the possibility of a higher spectrum of results with the use of both whole animal and individual cells in the assessment toxic of NMs. For this reason, the goals were: 1) evaluate the effects of silver nanospheres (AgNS) in *Hoplias intermedius* fish after acute and subchronic trophic exposition and compare with the silver ion (Ag^{+}) — not particle material with same chemical composition —, through of morphology (retina), genetic (blood, brain, liver and kidney) and biochemical (liver) biomarkers; 2) confirm the mode of action of nanoparticle of titanium dioxide (NPTiO2), silver nanoparticle no functionalized (AgNP) and silver nanosphere polyvinylpirrolidone functionalized (AgNS) in the DNA through *in vitro* method with RTG-2 fish cells line. AgNS deposits in retina, sclera and optic nerve region were found after exposition for 40 days. In addition, damage were observed, such as: morphologic changes in rods, melanosomes aderhered on cones, hemorrhage and deterioration of the pigment epithelium. Cytotoxic effects, such as oxidative stress, were observed through activity alteration in antioxidant enzymes (superoxide dismutase, catalase and glutathione S-tranferase) in the liver after acute exposition, besides the lipid hydroperoxide and metalothioneins concentration were changed. AgNS were genotoxic in all *H. intermedius*' tissues, both acute and subchronic exposition. The alkaline Comet assay scores of the treatments were smaller than negative control, suggesting crosslink DNA-DNA or DNA-protein damage type. In addition, the liver was the main target tissue in the acute exposition with higher genotoxic effects in both AgNS and Ag^{+} treatments, in relation to the other tissues. In the subchronic exposition, AgNS caused higher genotoxic effects in kidney and brain, whereas the Ag+ caused effect specific tissue in liver and kidney. In order to investigate de mode of action of NMs, *in vitro* method with modifications in the alkaline Comet assay was performed, and two modes of actions were found, such as: oxidative DNA damage and crosslink DNA-DNA or DNA-protein. Thus, the crosslink DNA damage type found in *H. intermedius* could be confirmed. In conclusion, the present work confirmed the toxicity of NMs studied with *in vivo* and *in vitro* methods together that herein demonstrated an accurately evaluation of the NMs effects, with the possibility to predict the hazard of them to the aquatic environment.

Keywords: emerging contaminants; nanoecotoxicology; titanium; silver; retina; oxidative stress; DNA damage.

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1 INTRODUÇÃO

A definição mais atual para nanomateriais (NMs) é um material natural ou manufaturado contendo partículas isoladas ou em forma de agregados, assim como aglomerados, sendo que ao menos 50%, ou mais, da distribuição das partículas esteja entre 1 e 100 nm de tamanho. Em casos específicos relacionados com o ambiente, a saúde ou a segurança, considera-se também como nanomaterial aquele que conter em sua maioria — mais do que 50% — partículas agregadas ou aglomeradas, ou seja, maiores que 100 nm (RAUSCHER et al., 2014). Logo, nanoecotoxicologia seria a avaliação de propriedades toxicológica de NMs para designar um possível impacto ambiental.

A utilização de NMs aumentou exponencialmente na última década em diversos setores, como: indústria, ciência farmacêutica e médica, eletrônica e fabricação de produtos de comunicação, tendo como destaque os NMs metálicos e de carbono que apresentaram o crescimento mais rápido em sua produção, com relatos de um aumento de 30 vezes entre 2011 e 2015 (VANCE et al., 2015).

Estruturas nanométricas acarretam benefícios na nanotecnologia devida maior área de superfície, reatividade química e resistência mecânica, em comparação com material não particulado de mesma composição química (RAJAS et al., 2017). Os autores citados também mencionam que NMs podem ser sintetizados para uma finalidade específica, como por exemplo, manufaturados à base de nanotubos de carbono, ou podem ser gerados a partir de algum processo químico como emissão de partículas de carbono por automóveis ou fulerenos em cinzas vulcânicas. Além disso, mencionam a ampla diversidade morfológica de NMs, como em forma de tubo, placa, esfera, fibra, partícula ou polímero.

No presente trabalho foram estudados os NMs compostos por dióxido de titânio (nano-TiO_2) ou prata (nano-Ag), que segundo a literatura, são utilizados principalmente em produtos de consumo humano, como pasta de dente, desodorante, xampu, roupas, brinquedos, máquinas de lavar roupa, purificadores de água, produtos alimentares e agrícolas, protetor solar, produto de autolimpeza de vidros, entre outros. Ainda, os mesmo autores relatam que a estimativa de produção de nano-TiO_2 seja de 1.000 t/ano e de nano-Ag 100 t/ano (MARAMBIO-JONES & HOEK, 2010, CERKEZ et al., 2012, PETERS et al., 2014, STARK et al., 2015).

Produtos contendo NMs são listados em um inventário *online* (*The Project on Emerging Nanotechnologies*, 2017) — atualizado constantemente —, e há registro de 14 produtos composto por nano-TiO_2 e 442 por nano-Ag.

Assim sendo, a utilização de modelos experimentais de ambiente aquático tem relevante importância para nanoecotoxicologia, uma vez que, especialmente, os corpos d'água são o destino final desses compostos. Além disso, a nanotecnologia cresce extraordinariamente mesmo sem o conhecimento sobre o perigo de materiais nanoestruturados a organismos, ademais, os resultados apresentados na literatura científica sobre a toxicidade de NMs (citotoxicidade, genotoxicidade, mutagenicidade e mecanismos de ação) são ainda muito divergentes.

Com a utilização de diferentes métodos para avaliação de toxicidade, pode-se chegar a conclusões mais precisas em relação ao perigo da exposição à NMs. Tanto métodos *in vivo* quanto métodos *in vitro* têm vantagens exclusivas.

Os testes com abordagem ecotoxicológica ainda utilizam modelos *in vivo*, devido à possibilidade do estudo da distribuição e efeitos tóxicos de partículas de tamanhos e formas diferentes, além de diferentes condições de exposição e em diferentes concentrações. Porém métodos alternativos, como os testes *in vitro* utilizando células de peixe apresentam a vantagem de uma abordagem mecanística de contaminantes (LILLICRAP et al., 2016). Outra vantagem seria a redução do uso de animais em experimentos, aplicando o princípio dos 3Rs (em inglês: *Reduction*, *Refinement* and *Replacement*; em português: Redução, Refinamento e Substituição), conceituado por Russel e Burch (1959), sendo a utilização de qualquer técnica experimental que considere a redução do número de animais, o aperfeiçoamento dos procedimentos a fim de minimizar o sofrimento dos animais ou substituição do uso de animais sempre que possível.

Com estudo *in vitro* é possível obter resultados de forma rápida, e, além disso, a possibilidade de conhecer mecanismos de ação dos NMs, uma vez que estão em contato direto com células individualizadas. No entanto, métodos *in vivo* são importantes no sentido de saber como os NMs se comportam em organismos inteiros, sendo possível estudar a distribuição nos diferentes tecido e acessar a toxicidade por meio de diversos biomarcadores.

Biomarcadores de citotoxicidade a nível bioquímico são considerados como biomarcadores precoces de contaminação ambiental, visto que a detecção de

alterações bioquímicas precedem as alterações em outros níveis de funções biológicas (PAYNE et al., 1987; STEGEMAN et al., 1992; BUCHELI & FENT, 1995).

O estresse oxidativo pode ser detectado por meio da análise da atividade de enzimas ou concentração de moléculas específicas relacionadas ao sistema antioxidante da célula. Estas impedem a atuação de espécies reativas de oxigênio [por exemplo, radical superóxido ($O2^{-\bullet}$), peróxido de hidrogênio (H_2O_2), hidroperóxidos, etc.] ou a formação de outros radicais livres, como radical hidroxil ($OH^{-\bullet}$), que estão relacionadas à oxidação de macromoléculas, como lipídios e proteínas (HAYES et al., 1997; GUTTERIDGE & HALLIWELL, 2000).

Algumas reações enzimáticas na célula formam, após a redução de oxigênio, o radical $O2^{-\bullet}$. Este é convertido em H_2O_2 pela metalenzima superóxido dismutase (SOD) e, posteriormente, em água e oxigênio por meio da atuação da catalase (CAT) e/ou glutationa peroxidase (GPx) (GUTTERIDGE & HALLIWELL, 2000).

Outra enzima com importância na condição de estresse oxidativo ou na metabolização de toxicantes é a glutationa S-tranferase (GST). Esta se conjuga com a glutationa reduzida (GSH) para eliminar compostos potencialmente reativos da célula, promovendo estabilização de um xenobióticos ou agindo como peroxidase na transformação de radicais hidroperóxidos em monohidróxi-álcool (GEORGE, 1993; GUTTERIDGE & HALLIWELL, 2000; DIXON & LAPTHORN, 2002; MARIONNET et al., 2006).

Além disso, quando um organismo é exposto a contaminantes que contém metais, a síntese de metalotioneínas (MTs) pode ser induzida (VALLEE, 1991; KIME, 1999; ANDREWS, 2000). Esta atua como metalochaperona no controle da concentração e detoxicação de metais traço, sendo importante para o metabolismo intracelular de cobre e zinco, assim como em condições de estresse oxidativo causado pela exposição à xenobióticos (MUTO et al., 1999).

Técnicas histológicas podem ser utilizadas como biomarcadores toxicológicos, como a autometalografia (AMG), que tem como objetivo localizar elementos metálicos no tecido, por meio da amplificação destes por prata. O método consiste na formação de grânulos de prata ao redor do contaminante, evidenciando sua deposição no tecido (DANSCHER, 1984). A aplicação de histologia para estudo de potenciais agentes neurotóxicos é muito efetiva, uma vez que alterações morfológicas provocadas por um toxicante no sistema nervoso central (SNC), seja por deposição com ação direta ou por

seus metabólitos, podem levar a uma perturbação na percepção do meio pelo organismo (MELA, 2010). Sendo assim, a retina pode servir para estudar isoladamente uma rede neural, uma vez que se localiza separada do SNC (BILOTTA & SASZIK, 2001).

Em 1893, Cajal descreveu a morfologia e a transmissão do impulso nervoso da retina em vertebrados. Em seus diagramas, Cajal mostra a cadeia formada por fotorreceptores, células bipolares e células ganglionares (PICCOLINO, STRETTOLI & LAURENZI, 1983 apud RAMÓN & CAJAL 1888). Com este estudo, diversos outros, posteriormente, dividiram a retina em 10 camadas: 1) camada do epitélio pigmentado; 2) camada de fotorreceptores; 3) membrana limitante externa; 4) camada nuclear externa; 5) camada plexiforme externa; 6) camada nuclear interna; 7) camada plexiforme externa; 8) camada de células ganglionares; 9) camada de fibras nervosas; e 10) camada limitante interna (VELERI et al., 2015).

Uma das possíveis maneiras que um contaminante atinge a retina é através da camada do epitélio pigmentado, pois é uma única camada de células que estão posicionadas entre o coriocapilar e a retina neural, sendo responsável por nutrir as células adjacentes (BRIDGES et al., 2007). Outra maneira seria através do sistema complexo de vasos sanguíneos presentes na camada limitante interna que tem contato direto com as células de Müller e a camada de células ganglionares, e assim o contaminante seria transportado para outras células (ALVAREZ et al., 2007).

Os dados na literatura em relação aos danos causados por NMs ainda são muito controversos. Diversos estudos mostram o estresse oxidativo como principal dano (VEVERS & JHA 2008, HUDECOVÁ et al., 2012, RINNA et al., 2015, ARMAND et al., 2016, IGLESIAS et al., 2017), e consequentemente a genotoxicidade dos NMs é associada ao estresse oxidativo. Porém, modificações no método do ensaio Cometa podem identificar mecanismos de danos ao DNA com maior exatidão.

O ensaio Cometa avalia danos no DNA em células individualizadas, por meio da medida da migração do DNA de células em gel, após corrida de eletroforese (SINGH et al., 1988). O nome cometa refere-se à formação de uma longa cauda com os fragmentos de DNA carreados após a passagem da corrente elétrica (BOMBAIL et al., 2001).

A técnica baseia-se no fato de que fragmentos menores tendem a migrar com mais velocidade que fragmentos maiores, sendo que as formações de muitos fragmentos de tamanhos variados migrarão com velocidades diferentes, formando

então, a figura típica de um cometa (OLIVE et al., 1990). Em sua versão alcalina (pH > 13) é possível identificar diversos tipos de danos, como quebras de fita-simples, sítios alcali-lábeis, *crosslink* DNA-DNA ou DNA-proteína (FAIRBAIRN et al., 1995). Quando há aumento na migração dos fragmentos de DNA no gel, ou seja, o aumento da cauda do cometa, pode-se inferir que houve quebras no DNA. Porém, é possível identificar o dano do tipo *crosslink* DNA-DNA ou DNA-proteína quando há uma diminuição na migração dos fragmentos de DNA no gel (TICE et al., 2000). Neste tipo de dano um agente químico ou físico, chamado de alquilante bifuncional, apresenta dois radicais que podem produzir aductos covalentes com DNA ou proteínas. Estes aductos impedem a separação das fitas de DNA, podendo bloquear os processos de replicação e/ou transcrição do DNA (NOLL et al., 2006), além de alterar estruturalmente a dupla-hélice do DNA (RAJSKI & WILLIAMS, 1998). Logo, no ensaio Cometa alcalino não há a desnaturação do DNA (quebra de pontes de hidrogênio que ligam as fitas de DNA) para a formação de fragmentos, uma vez que as fitas de DNA estão ligadas entre si, ou com proteínas, de forma covalente.

Modificações no método do ensaio Cometa alcalino podem identificar mecanismos de danos ao DNA com maior exatidão, na qual versões utilizando enzimas específicas para identificação de dano oxidativo no DNA ou a coexposição de NMs a um agente clastogênico conhecido para observação de possível dano do tipo *crosslink* DNA-DNA ou DNA-proteína. Esses métodos (citados a seguir) têm validação na *Organization for Economic Co-operation and Development* (OECD) com o *Guideline for the testing of chemicals in vivo mammalian alkaline Comet assay* 489 (2014).

Para identificar danos oxidativos no DNA, adiciona-se alguma endonuclease (FPG, ENDOIII e hOGG1) antes da etapa de corrida eletroforética. Estas promovem quebras na cadeia de DNA em aductos e/ou nucleotídeos oxidados, aumentando assim, a quantidade de fragmentos de DNA na cauda do cometa. Mais especificamente, a endonuclease hOGG1 identifica guaninas oxidadas [8-oxo-7,8-di-hidroguanina (8-oxoGua) e 2,6-diamino-4-hidroxi-5-formamidopirimidina (FaPyGua)], promovendo quebras nessas regiões do DNA (SMITH et al., 2006).

O *crosslink* DNA-DNA ou DNA-proteína pode ser confirmado por coexposição entre o contaminante de estudo com um agente clastogênico conhecido, como por exemplo, o metil metano sulfonato (MMS) (PFUHLER & WOLF, 1996). Como já citado acima, o agente *crosslink* forma uma ligação covalente com

DNA ou proteínas (como as histonas ou proteínas maiores) e esta ligação impede que os fragmentos causados pelo agente clastogênico migrem no gel do ensaio Cometa alcalino, e, consequentemente, observa-se uma diminuição na cauda do cometa (COSTA, 1991).

2 JUSTIFICATIVA

O ambiente aquático é o destino final da maioria das substâncias químicas, incluindo os nanomateriais. Diversos setores industriais estão utilizando nanomateriais por apresentarem propriedades físico-químicas vantajosas, substituindo assim, o uso do material não particulado de mesma composição química. Nano-TiO_2 e nano-Ag estão sendo produzidas em grande escala e são principalmente utilizadas em produtos de consumo humano, chegando mais facilmente a corpos d'água pelo esgoto doméstico. Além disso, os dados na literatura sobre a toxicidade destes nanomateriais ainda são muito contraditórios. Sendo assim, peixes podem representar um bom modelo para nanoecotoxicologia, uma vez que é possível elucidar o perigo de nanomateriais no ambiente aquático, utilizando tanto o animal inteiro em ensaios *in vivo* como linhagens de células em ensaios *in vitro*. Com este é possível realizar ensaios com diferentes nanomateriais, em diversas concentrações e com diferentes métodos para avaliação da toxicidade, ocupando pouco espaço físico e gerando dados mais rapidamente. E ainda, com a combinação destas duas metodologias, é possível obter informação sobre os efeitos dos nanomateriais em diversos tecidos do peixe, assim como corroborar um efeito observado e elucidar o mecanismo de ação em uma molécula específica, como o DNA.

3 OBJETIVOS

O presente estudo teve como objetivos: avaliar os efeitos citotóxicos e genotóxicos causados em *Hoplias intermedius* pela exposição via trófica a nanoesferas de prata; observar se as nanoesferas de prata foram mais tóxicas que o nitrato de prata, que é o material não particulado; observar se os efeitos da exposição aguda às nanoesferas de prata ou aos íons de prata foram diferentes da exposição subcrônica; e com a linhagem de células de peixe RTG-2, identificar os mecanismos de ação de nano-TiO_2 e outros dois tipos de nano-Ag no DNA, a fim de confirmar o efeito genotóxico observado nos tecidos de *H. intermedius*.

4 DESENVOLVIMENTO

As sessões a seguir, com os resultados obtidos dos experimentos *in vivo* com *H. intermedius* e *in vitro* com a linhagem de células RTG-2, apresentam-se no formato de artigos científicos.

4.1 Acute and long-term effects of trophic exposure to silver nanospheres in the central nervous system of freshwater fish

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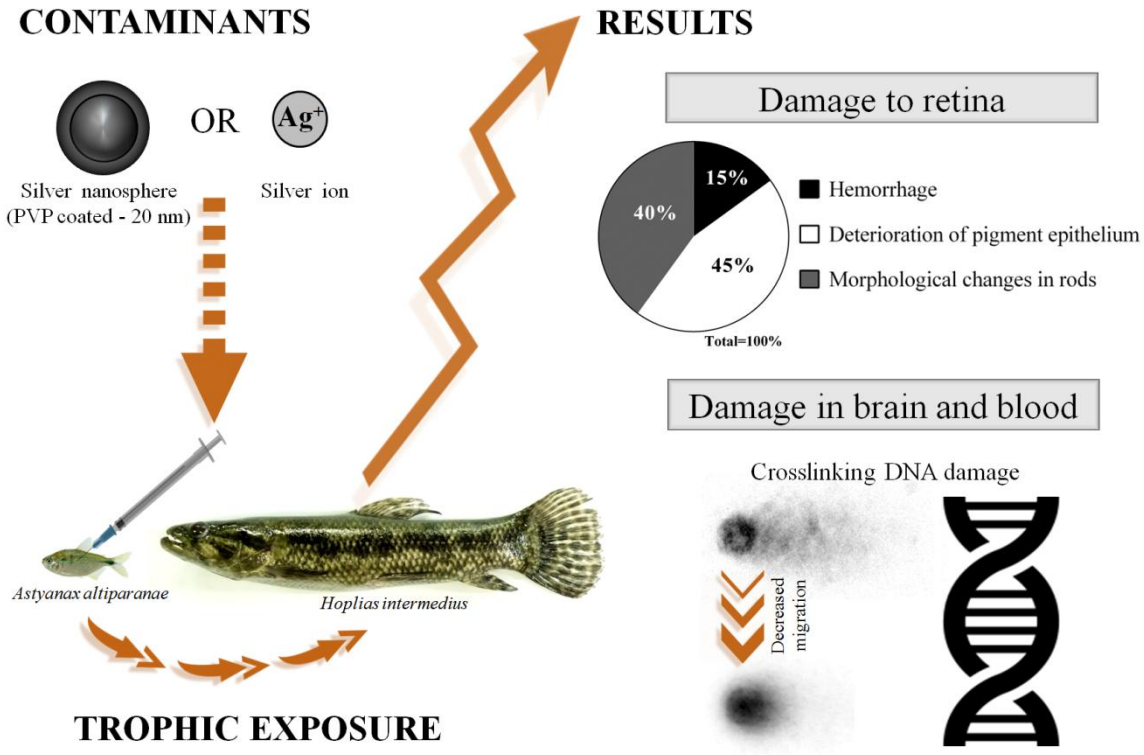
Figure 1 – Graphical Abstract. Author: Tatiane Klingelfus.



Abstract

Nanotechnologies are at the center of societal interest, due to their broad spectrum of application in different industrial products. The current concern about nanomaterials (NMs) is the hazard they carry for human health and the environment. Considering that NMs can reach bodies of water, there is a need for studying the toxic effects of NMs on aquatic organisms. Among the NMs' toxic effects on fish, the interactions between NMs and the nervous system are yet to be understood. For this reason, our goal was to assess the neurotoxicity of polyvinylpyrrolidone coated silver nanospheres [AgNS (PVP coated)] and compare their effects in relation to silver ions (Ag^+) in carnivorous *Hoplias intermedius* fish after acute and subchronic trophic exposure through the analysis of morphological (retina), biochemical (brain) and genetic biomarkers (brain and blood). For morphological biomarkers, damage by AgNS (PVP coated) in retina were found, including morphological changes in rods, cones, hemorrhage and epithelium rupture, and also deposition of AgNS (PVP coated) in retina and sclera. In the brain biomarkers, AgNS (PVP coated) did not disturb acetylcholinesterase activity. However, lowered migration of the DNA tail in the Comet Assay of blood and brain cells was observed for all doses of AgNS (PVP coated), for both acute and subchronic bioassays, and in a dose-dependent manner in acute exposure. Ag^+ also reduced the level of DNA damage only under subchronic conditions in the brain cells. In general, the results demonstrated that AgNS (PVP coated) do not cause a similar effects in relation to Ag^+. Moreover, the lowered level of DNA damage detected by Comet Assay suggests that AgNS (PVP coated) directly interacts with DNA of brain and blood cells, inducing DNA-DNA or DNA-protein crosslinks. Therefore, the AgNS (PVP coated) accumulating, particularly in the retina, can lead to a competitive disadvantage for fish, compromising their survival.

Keywords: emerging contaminants, nanomaterial, neurotoxicity, retina, DNA damage, *Hoplias intermedius*.

4.1.1 Introduction

The aquatic environment has been a target of continuous disposal of toxic substances, both natural and manmade, with an increasing concern around the emerging contaminants known as nanomaterials — with effects that remain to be elucidated. Although there are many data about the effects of nanomaterials, there are still more questions than answers (SAUVÉ & DESROSIERS, 2014) including with respect to their hazard to human health and ecological impacts.

Nanoparticles (NPs) range in size from 1 to 100 nanometers in diameter – a feature that ascribes specific properties or behaviors to these materials (NEL et al., 2013). Among the different types of nanomaterials, the silver ones present antimicrobial properties and have been used in several consumer products, such as personal care products (e.g. toothpaste, deodorants, shampoo), apparel, footwear, toys, washing machines, water purifiers, among others (MARAMBIO-JONES & HOEK, 2010). There are more than 400 nanosilver products on the market today (The Project on Emerging Nanotechnologies, 2017), most of which eventually find their way to our water systems. However, NPs aquatic concentrations were calculated only by mathematical modeling in the European Union (EU) and Switzerland, where estimated average 0.66 µg/L and 0.45 ng/L for nanosilver, respectively (SUN et al., 2014). Put simply, the environment has been reached since the humans are consuming a considerable amount of nanosilver every day, with little understanding regarding their effects and discard.

Adverse effects (e.g. liver toxicity, genotoxicity, neurotoxicity, etc) of nanosilver have been pointed out in the literature using animal models and *in vitro* test systems (HACKENBERG et al., 2010; PARK et al., 2010; GHOSH et al., 2012; HADRUP et al., 2012, LANKOFF et al., 2012; SARDARI et al., 2012; SHAHARE et al., 2013). Nanoparticles are 100 times smaller than normal red blood cells, which increases the potential for interaction with cells (SEEMAN, 2006). Hence, understanding the interaction of nanoparticles with living cells and other biological systems is critical, especially as the potential and exploitation of such technologies is rapidly gathering pace, requiring more studies with ecotoxicological approach.

In order to investigate the potential harmful effects of silver nanomaterials, this study assessed the neurotoxicity of polyvinylpyrrolidone coated silver nanospheres [AgNS (PVP coated)] and compared their effects in relation to silver

ions (Ag^+) in carnivorous *Hoplias intermedius* fish after acute and subchronic trophic exposure through the analysis of morphological (retina), biochemical (brain) and genetic biomarkers (blood and brain).

To the best of our knowledge, the current study is the first comprehensive investigation aiming to determine the morphological changes in the retina and DNA in the brain of a vertebrate after exposure to coated silver nanospheres.

4.1.2 Material and Methods

Chemicals and characterization of the silver nanospheres suspensions

AgNS (PVP coated) suspension from Sigma-Aldrich® [supplier information: 20 nm average primary particle size, 20 µg/mL in water and PVP (polyvinylpyrrolidone) functionalized] and diluted in ultrapure water. The three working suspensions (20, 2 and 0.2 µg/mL) were analyzed by Zetasizer® Nano Series ZS90 (Malvern Instruments, Worcestershire, UK) for hydrodynamic size, zeta potential (ζ) and polydispersion index (PdI) for calculation of % of polidispersity (%PD), according to the equation:

$$\%PD = \sqrt{PdI} \times 100.$$

The silver ions solution (Ag^+) was prepared from $AgNO_3$ (Merck®) which was diluted in ultrapure water. First, we performed a balanced stoichiometric calculation to obtain only Ag^+ at 20 µg/mL concentration. After this, it was diluted in ultrapure water to obtain three working solutions (20, 2 and 0.2 µg/mL).

Animals

Hoplias intermedius (GÜNTHER, 1864) fish are on top of the food chain in the aquatic environment and *Astyanax* sp fish are their natural prey. In their native Brazil, these fish are attractive to economic interests because of the quality of their meat and sport fishing (OYAKAWA & MATTOX, 2009). Despite its prominence and importance in South American waters, to our knowledge this is the first ecotoxicological study with *H. intermedius*.

Predator fish specimens were obtained as donation from the Eletrobras Furnas (Fish Farming and Hydrobiology Station of Furnas, Minas Gerais, Brazil) and were acclimatized for about six months in 2000 L tanks. They were first fed with

commercial fish food, and during the bioassay with *Astyanax altiparanae* (GARUTTI & BRITSKI, 2000) species. The *A. altiparanae* prey fish specimens were obtained from fish farms and were acclimatized for about one week in 250 L tanks before the experiment. The predators had about 11.27 cm ± 1.21 of size and 16.54 g ± 4.86 of weight (mean ± standard deviation), and the prey had 4.13 cm ± 0.38 and 1.10 g ± 0.34 (mean ± standard deviation).

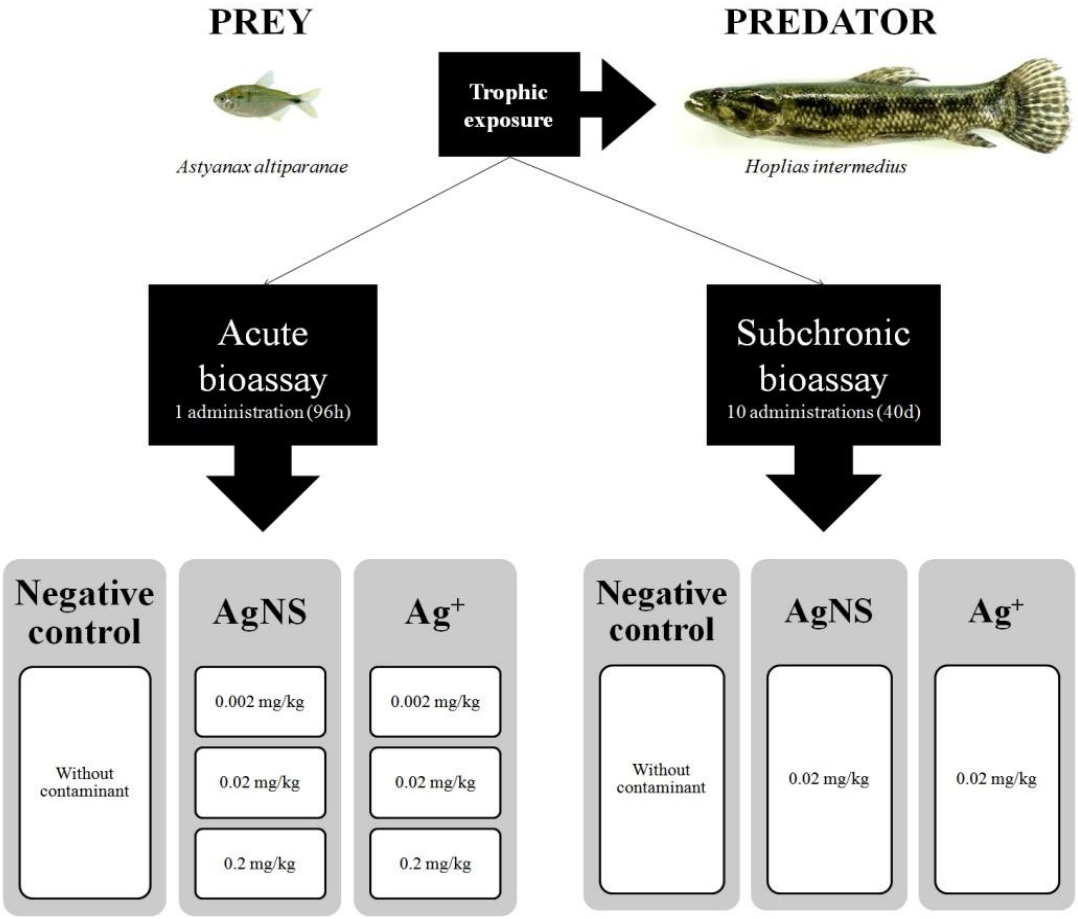
Experimental design

The doses used in this study were approximated to the value of silver ion (CASRN 7440-22-4) LOAEL (lowest-observed-adverse-effect level) determined by the Environmental Protection Agency (EPA) on Integrated Risk Information System (IRIS, 2017) for humans, which is 0.014 mg/kg/day. However, as yet there are no established values for NOAEL (no observed-adverse-effect level) and LOAEL for silver nanoparticles in organisms and there are only a few studies regarding nanosilver exposure in fish, most of concerning hydric exposure (ASHARANI et al., 2008; SHAHBAZZADEH et al., 2009; BILBERG et al., 2010; WU et al., 2010).

As a contamination vehicle, we used the *A. altiparanae* (prey) that received an intraperitoneal injection with one of the AgNS (PVP coated) suspensions (20, 2 or 0.2 µg/mL) or Ag^+ solutions (20, 2 or 0.2 µg/mL), and afterwards were offered as food to the *H. intermedius* (predator) (**Figure 2**). The administrated dose was calculated according to the predator's weight individually. Two trophic bioassays were performed. In the acute bioassay, each predator received only one prey contaminated with doses of 0.2 mg/kg, 0.02 mg/kg or 0.002 mg/kg of Ag^+ or AgNS (PVP coated) (both with n = 15 for each treatment), and the fish that received prey without contaminants were used as negative control (n = 30) (**Figure 3**). After 96 hours, the animals were anaesthetized with 150 mg of benzocaine per L of water to collect the samples of blood, brain and eyes (Gontijo et al., 2003). In the subchronic bioassay, the predators received one prey every 4 days (and a total of 10 prey) contaminated with a dose of 0.02 mg/kg of Ag^+ (n = 15) or AgNS (PVP coated) (n = 13, one of them refused the prey and other one jumped out of the water - events were not linked to the contamination), and the fish that received prey without contaminants were used as negative control (n = 15). After 40 days, the same sampling procedures used in the acute bioassay were carried out.

Figure 2 – *Hoplias intermedius* predating an *Astyanax altiparanae*. Author: Tatiane Klingelfus.

Figure 3 – Experimental design. Author: Tatiane Klingelfus.



Biochemical and genetic biomarkers

In this study, we used biochemical and genetic biomarkers to compare the different responses between AgNS (PVP coated) and Ag^{+}. All the animals were analyzed.

Biochemical - acetylcholinesterase activity in brain

A brain sample of each fish was homogenized in potassium phosphate buffer (0.1 M, pH 7.5), in a proportion of 1:10 and then centrifuged to 10,000 x*g* for 20 minutes (4 ºC). Afterwards, the supernatant was stored in the -80 ºC freezer. The methodology adopted to AChE assay was from Ellman et al. (1961), modified by Silva de Assis (1998), using acetylcholine iodide (10 mM) and DTNB (*5,5'-dithiobis-2-nitrobenzoic acid*) (0,75 mM). The method by Bradford (1976) was used to quantify the tissue protein.

Genetic - comet assay of blood and brain cells

The Comet Assay technique used in this study was previously described by Speit and Hartmann (1995) and modified by Ramsdorf et al. (2009). This technique was used to detect DNA damage, characterized by any difference in DNA score (increase or decrease) relative to the control. Both brain homogenization and blood were maintained in fetal bovine serum. The brain tissue was homogenized at 1500 rpm (homogenizer Potter type) in fetal bovine serum to obtain a cell suspension. 15 µL of blood sample or 40 µL of brain cell suspension were mixed with 120 µL of agarose with a low melting point (LMP) and the samples were placed on slides previously covered with a normal agarose layer. The slides were submerged in a lysis solution [Lysis stock solution: NaCl (2.5 M), EDTA (100 mM), Tris (10 mM), NaOH (0.8%), N-lauryl-sarcocinate (1%); Lysis reaction solution - Triton X100 (1%), DMSO (10%), Lysis stock (89%)] for 72h at 4 °C. Afterwards, the slides were immersed in an alkaline buffer [NaOH (10 N) and EDTA (200 mM), pH 13], for 25 minutes to induce DNA denaturation. The samples were then submitted to electrophoresis at 300 mA, 1V.cm^{-1} for 25 minutes. The reaction was neutralized using Tris-HCl (0.4 M, pH 7.5, 4 °C), and the samples were fixed in absolute ethanol for 10 minutes. The slides were stained with 2 µg.ml^{-1} of ethidium bromide. For each fish we counted 100 nucleoids visually categorized (Leica® epifluorescence microscope) according to damage classes from 0 to 4 (Collins et al., 1997), and a score was calculated with the sum of nucleoids number of each class multiplied by its respective class.

Histology

To analyze the uptake of contaminants and morphology damage in the retina, eight animals of each treatment — AgNS (PVP coated), Ag^{+} or negative control of both acute and subchronic bioassay — were selected.

Uptake of AgNS (PVP coated) and Ag+ on retina from autometallography

Retina samples were fixed with Bouin fluid for 24 hours, dehydrated in a graded series of ethanol baths and embedded in Paraplast Plus (Sigma®). For the autometallography development, tissue sections (5 µm) were coated with a thin film

of gelatin by dipping the slides in 0.5% of gelatin and then AMG developed for 60 minutes. The process was stopped by replacing the AMG developer with thiosulphate solution for 10 minutes and rinsing the slides in 40 ºC running tap water to remove the gelatin. Then, the slides were dipped in a 2% Farmer's solution as described by Danscher and Nörgaard (1983). The sections were stained with Hematoxylin and Eosin (WOODS & ELLIS, 1994), dehydrated with ethanol and xylene series, mounted with Entellan (Merck®), and observed under the Leica DME Light Microscope. Before every experiment the emulsion was checked to test uniformity of silver grains, by covering a slide without sections. Metal deposits [AgNS (PVP coated) or Ag^{+}] appear as black silver deposits (BSD) indicating the presence of silver shells around the metals (DANSCHER, 1984; SOTO et al., 1998).

Morphology and nuclear layer measures

For scanning electron microscopy, the retinas were fixed overnight in 3% glutaraldehyde (0.1 M phosphate buffer, pH 7.4) at 4 ºC. Tissue samples were dehydrated using a graded ethanol series until absolute ethanol was reached, and a graded ethanol/acetone series until pure acetone was reached, after which they were dried to the critical point using CO_2 as a transition liquid. Filament pairs (part of the thermionic electron gun) were glued with silver paint onto the specimen stub, coated with gold in a vacuum sputter, and examined in a JEOL JSM- 6360LV scanning electron microscope.

Morphological damage in photoreceptors were measured according to the injury index described by Bernet et al. (1999) and modified by Mela et al. (2012), where cell alterations were classified in three factors (minimal, moderate and severe pathological importance). The injury indexes were obtained after the application of a mathematical equation established for each group of lesions:

$$LI = \sum_{rp} \sum_{alt} (a \times w),$$

where *LI* is the lesion index; *rp* is the reaction pattern; *alt* is the alteration; *a* is the score value; *w* is the importance factor. The lesions considered were hemorrhage, deterioration of pigment epithelium and morphological changes in rods.

To evaluate possible signs of toxicity, the thickness, the cell count and the nucleus cell diameter in the central and peripheral regions of the outer (ONL) and

inner (INL) nuclear layers were measured by ImageJ®. The values of central and peripheral regions of the ONL and INL were joined together to statistical analysis.

Statistical analysis

The values of biochemical and genetic biomarkers were expressed as mean and standard deviation of each treatment and ANOVA was applied with a *t* test (LSD) *post hoc* to compare the differences among treatment and negative control groups. For the morphological biomarkers, treatments were compared to negative control using *t* test and data were expressed as means and standard errors. The significance level was set at $p < 0.05$.

4.1.3 Results

Silver nanospheres size on suspensions

The hydrodynamic size of AgNS (PVP coated) suspensions at 2 µg/mL and 20 µg/mL were lower than 100 nm: 52.41 nm and 46.19 nm, respectively. The potential zeta indicated that all the suspensions were unstable (+30 mV > ζ > -30 mV), because when the ζ is next to zero, the particles do not have power to repulse themselves and may flocculate. Also, the % of PD was higher than 20%, which demonstrates that the suspensions were heterogeneous; presenting several sizes of nanosphere agglomerates (**Table 1** – Characterization of the silver nanosphere (PVP coated) suspensions that were injected in prey and offered them, subsequently, to *H. intermedius*.).

Table 1 – Characterization of the silver nanosphere (PVP coated) suspensions that were injected in prey and offered them, subsequently, to *H. intermedius*.

AgNS (PVP coated) suspension (µg/ml)	Hydrodynamic size mean (nm)	Distribution		Zeta potential (mV)	% of PD
		d.nm (min-max)	Intensity (%)		
0.2	237.02	487.3 (0.7-974.1) 92.4 (8.0-176.8)	68 32	-10.1	100.0
2	52.41	94.1 (82.7-136.4) 152.0 (15.4-388.5) 16.2	75 22 3	-22.6	61.7
20	46.19	85.6 (61.7-116.2) 199.0 (21.4-336.6) 318.1 (11.3-930.1)	54 32 14	-21.6	86.1

Suspensions: Silver nanosphere polyvinylpyrrolidone (PVP) coated (AgNS) at 0.2, 2 and 20 µg/mL exposure concentrations. Measures: hydrodynamic size mean (nm), distribution of particle size (mean of diameter in nm with minimum and maximum values) for intensity (%); Zeta potential (mV); and % of polydispersity. The values of each suspension were measure on Zetasizer® Nano Series ZS90 (Malvern Instruments, Worcestershire, UK).

Blood biomarker

The Comet Assay in blood cells in the three AgNS (PVP coated) doses (0.002; 0.02; and 0.2 mg/kg) in the acute experiment presented a lower score than the control (**Figure 4A**), and the same score decrease happened in the subchronic AgNS (PVP coated), thus confirming DNA damage (**Figure 4B**). In contrast, the animals treated by Ag^+ in both acute (**Figure 5A**) and subchronic bioassays (**Figure 4B**) had no significant change of DNA score.

Brain biomarkers

The Comet Assay in brain cells in the three AgNS (PVP coated) doses (0.002; 0.02; and 0.2 mg/kg) in the acute experiment presented a lower score than the control (**Figure 4C**). Additionally, the Comet Assay score was the lowest in the 0.2 mg/kg of AgNS (PVP coated) when compared to 0.02 mg/kg and 0.002 mg/kg of AgNS (PVP coated) (**Figure 4C**). The treatments of Ag^+ had no significant change of DNA score in the acute bioassay (**Figure 5B**). In addition, both 0.02 mg/kg AgNS (PVP coated) and 0.02 mg/kg Ag^+ had lower scores than the control only in the

subchronic bioassay, and the 0.02 mg/kg AgNS (PVP coated) had a lower score than 0.02 mg/kg of Ag^+ (**Figure 4D**).

AChE activity increased at 0.002 mg/kg and 0.2 mg/kg Ag^+ acute treatments only (**Figure 6**), and alterations were not observed in the AChE activity in the groups exposed to AgNS (PVP coated) (**Figure 7**), when compared to the control.

Figure 4 – DNA damage in the blood and brain cells of *H. intermedius* after trophic exposure with AgNS (PVP coated) or Ag^+. **[A]** Blood cells DNA damage after acute bioassay in three different doses (0.002; 0.02 and 0.2 mg/kg) of AgNS (PVP coated) (n = 30 for control; n = 15 for each treatment); **[B]** Blood cells DNA damage after subchronic bioassay in one dose (0.02 mg/kg) of AgNS (PVP coated) or Ag^+ [n = 15 for control; n = 13 for AgNS (PVP coated) and n = 15 for Ag^+]; **[C]** Brain cells DNA damage after acute bioassay in three different doses of AgNS (PVP coated) (n = 30 for control; n = 15 for each treatment); **[D]** Brain cells DNA damage after subchronic bioassay in one dose of AgNS (PVP coated) or Ag^+ [n = 15 for control; n = 13 for AgNS (PVP coated) and n = 15 for Ag^+]. Comparison among treatments by ANOVA *post hoc t* test (LSD). Different letters indicate statistical differences, considering $p < 0.05$.

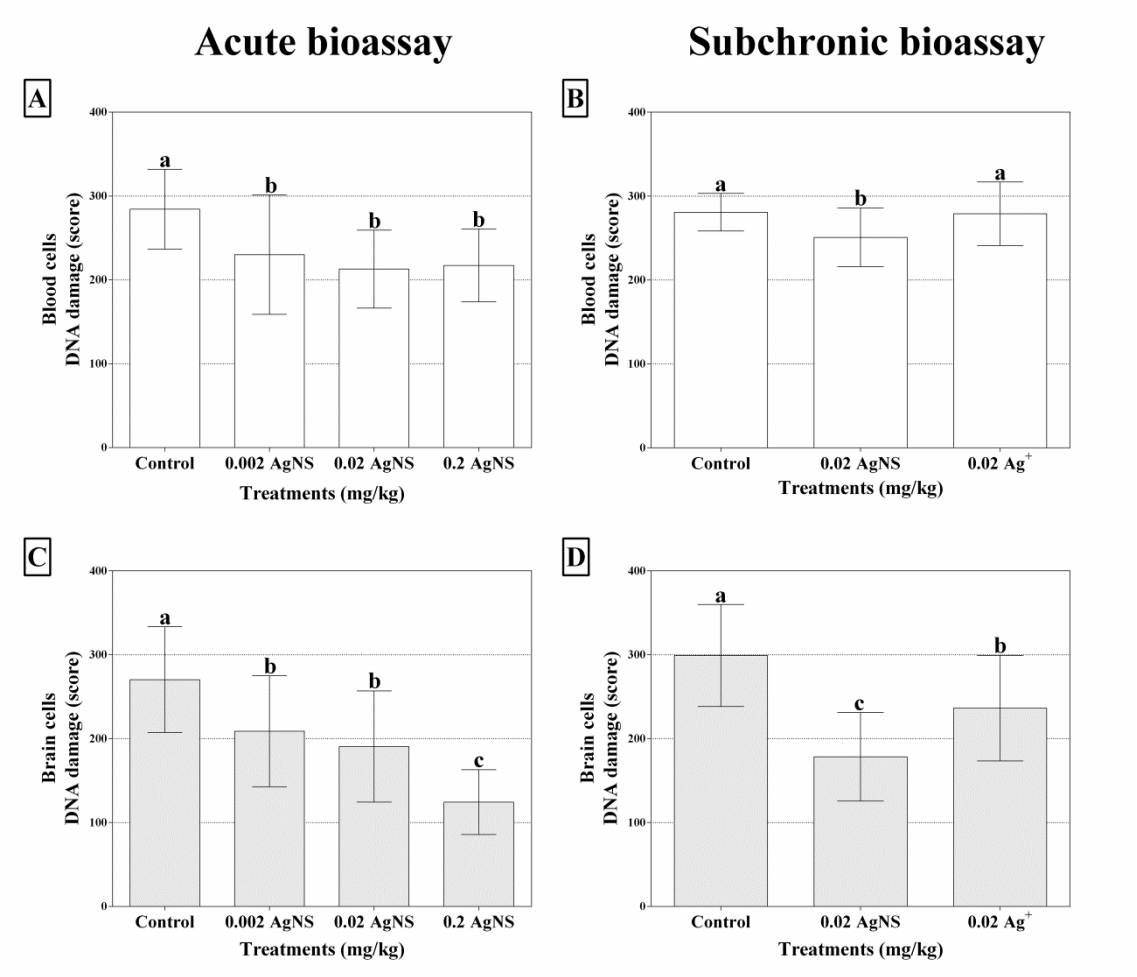


Figure 5 – DNA damage in the blood and brain cells of *H. intermedius* after acute exposure with Ag^{+}. [A] Blood and [B] brain cells DNA damage after acute bioassay with three doses of Ag^{+} (n = 30 for control; n = 15 for each treatment). Comparison among treatments by ANOVA *post hoc t* test (LSD). Different letters indicate statistical differences, considering $p < 0.05$.

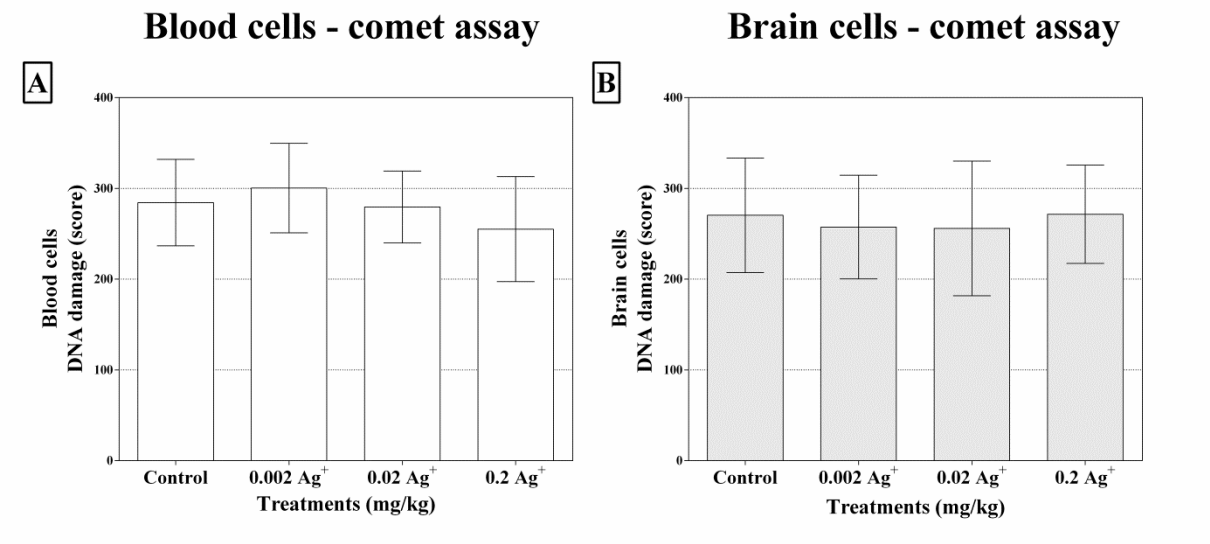


Figure 6 – AChE activity in the brain of *H. intermedius* after acute trophic exposure with three different doses of Ag^{+} (0.002; 0.02 and 0.2 mg/kg). Comparison among treatments by ANOVA *post hoc t* test (LSD). Different letters indicate statistical differences, considering $p < 0.05$ (n = 30 for control; n = 15 for each treatment).

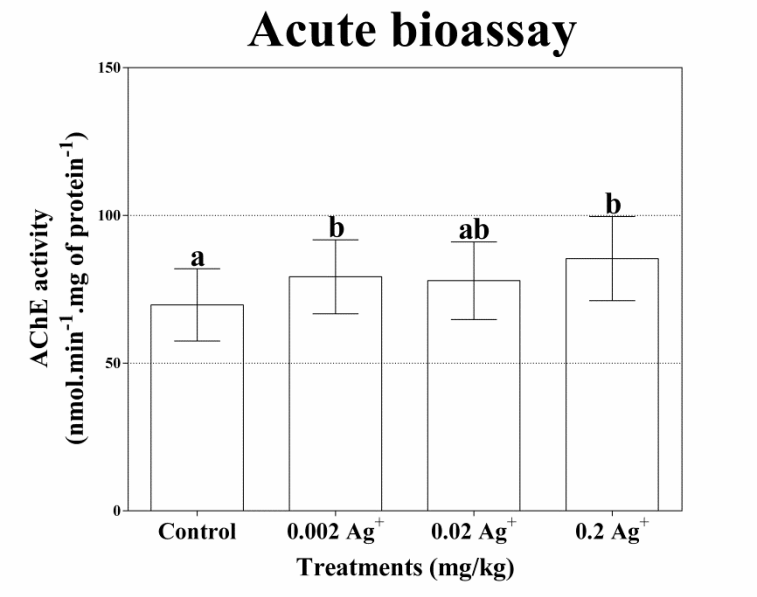
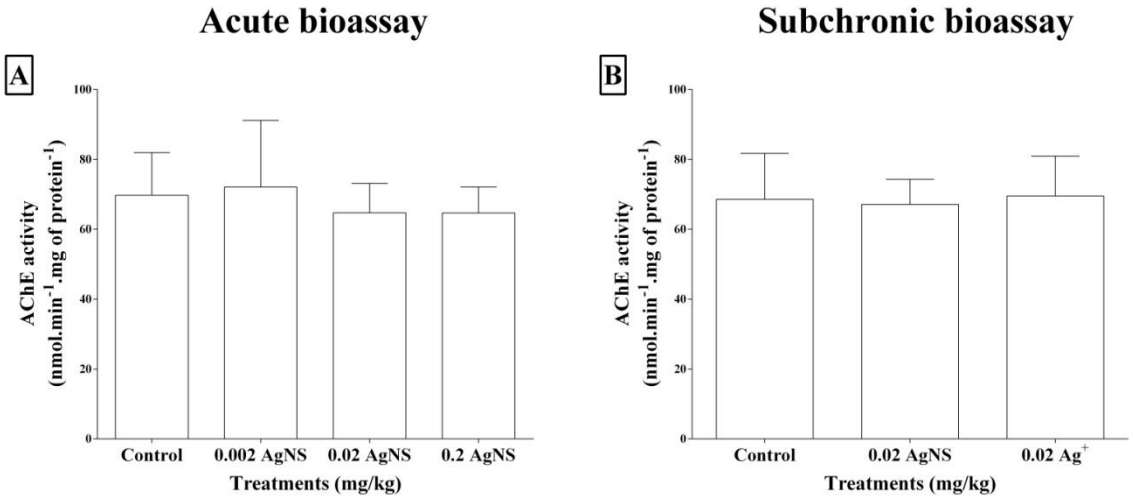


Figure 7 – AChE activity in the brain of *H. intermedius* after acute trophic exposure with AgNS (PVP coated) or Ag^{+}. [A] Acute bioassay in three different doses of AgNS (PVP coated) (n = 30 for control; n = 15 for each treatment); [B] Subchronic bioassay with one dose of AgNS (PVP coated) or Ag^{+}(n = 15 for control; n = 13 for AgNS (PVP coated) and n = 15 for Ag^{+}). Comparison among treatments by ANOVA *post hoc t* test (LSD). Different letters indicate statistical differences, considering $p < 0.05$.



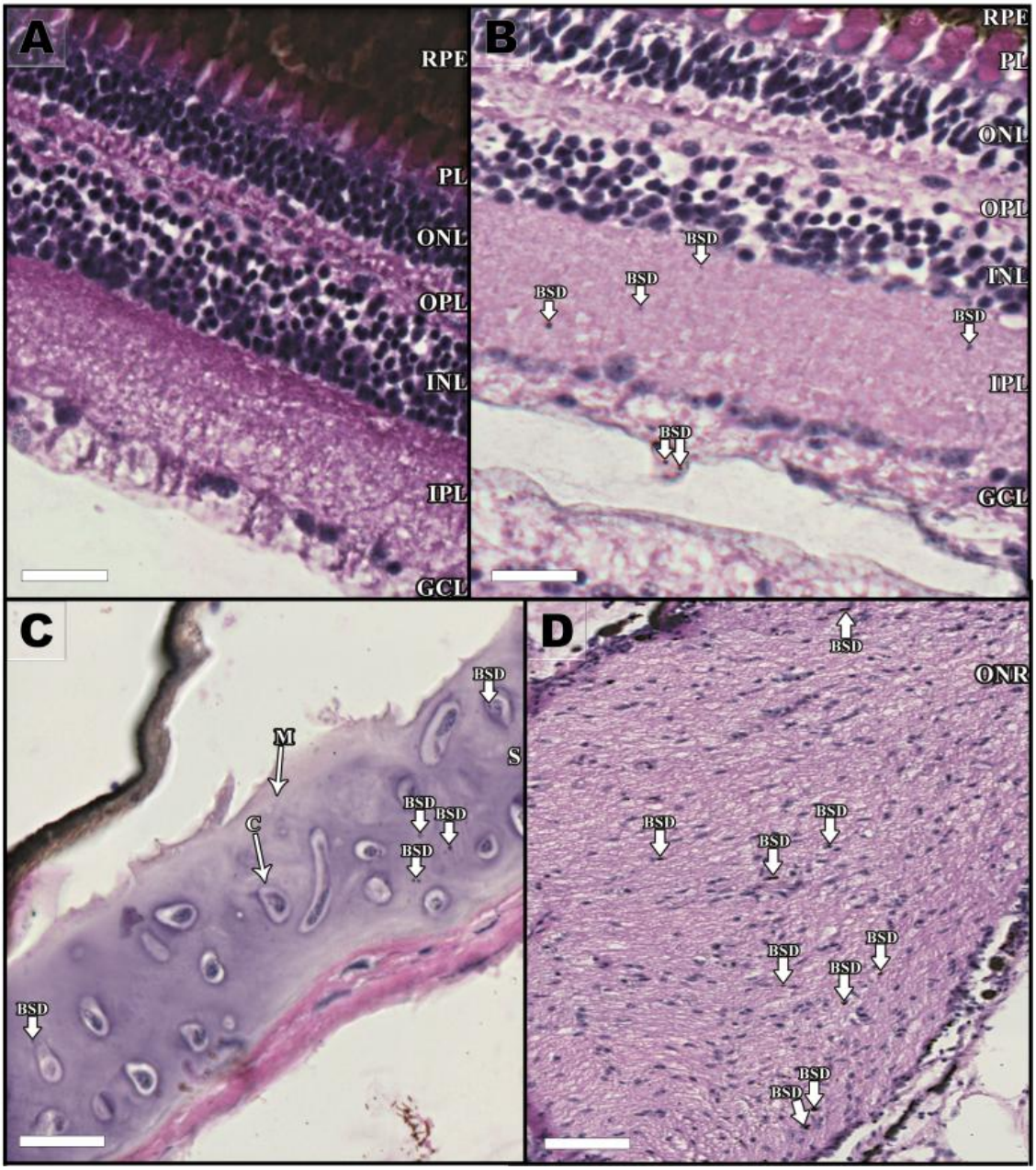
Retina biomarkers

Deposits of AgNS (PVP coated) and Ag^{+}

The retinas from control animals (**Figure 8A**) did not reveal the presence of Ag^{+} and AgNS (PVP coated) deposits by AMG analysis. In the acute bioassay, the presence of AgNS (PVP coated) in the retina at a dose of 0.2 mg/kg was observed (figure not shown); and in the subchronic bioassay the presence of both AgNS (PVP coated) (**Figure 8B, Figure 8C** and **Figure 8D**) and Ag^{+} (figures not shown) at a dose of 0.02 mg/kg was observed.

In relation to retina regions, deposits of AgNS (PVP coated) or Ag^{+} were found both outer and inner nuclear cells (figures not shown), as well as in the inner plexiform layer (**Figure 8B**), in the ganglion cell layer (**Figure 8B**), in the matrix and chondrocytes of sclera (**Figure 8C**), and occasionally in the optic nerve region (**Figure 8D**).

Figure 8 – Autometallographical demonstration of AgNS (PVP coated) in cross section of retina from *H. intermedius*. [A] Cross section of retina from negative control group counterstained with Hematoxylin–Eosin. Retinal pigment epithelium (RPE); photoreceptor layer (PL); outer nuclear layer (ONL); outer plexiform layer (OPL); inner nuclear layer (INL); inner plexiform layer (IPL); ganglion cell layer (GCL). [B, C and D] Cross section of retina after subchronic exposure to 0.02 mg/kg of AgNS (PVP coated). Section counterstained with Hematoxylin–Eosin, wherein showing black silver deposits (BSD –arrow): [B] in IPL and GCL; [C] in the sclera (S), both matrix (M) and cells-chondrocytes (C); [D] in optic nerve region (ONR). For all groups n = 8. Optical microscopy; Scale bar = 20 µm.



Inner and outer nuclear layer measures

The measure of both outer and inner nuclear layer thickness, the cell count and the measure of nucleus cell diameter is shown in **Table 2**. Only in the retina of individuals trophically exposed to 0.02 mg/kg of AgNS (PVP coated) in the

subchronic bioassay demonstrated difference in relation to control. There were a decrease in thickness and cell count in ONL. In contrast, an increase in thickness and cell count and also a decrease in nucleus cell diameter in INL were found.

Table 2 – Measure of nuclear layers thickness, the cell count and the measure of cell nucleus diameter of the retina of *Hoplias intermedius* in the subchronic bioassay. Comparison between negative control (control) and treatment (AgNS or Ag^{+}) by *t* test. ***** indicate statistical difference, considering $p < 0.05$. Values expressed by mean ± standard deviation, n = 8 animals in each treatment.

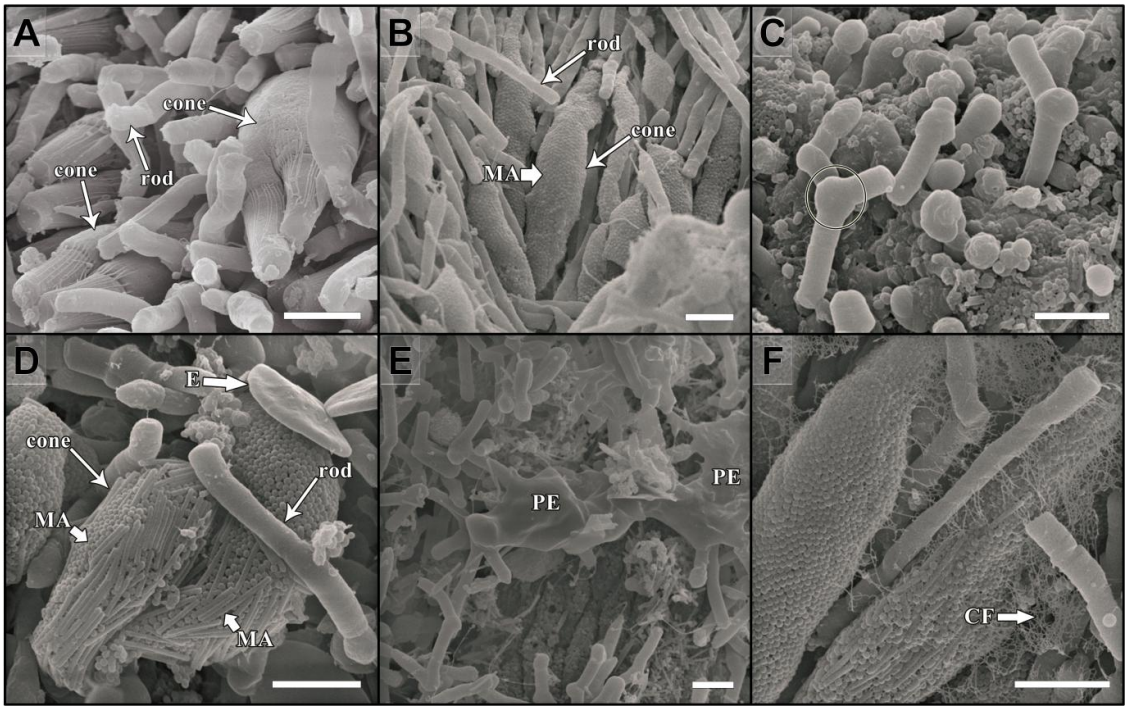
		Subchronic bioassay		
		Control	**AgNS (PVP coated)**	**Ag^{+}**
Thickness (µm)	Central ONL	15.42 ± 1.43	12.48 ± 0.07*****	15.23 ± 0.03
	Peripheral ONL	13.42 ± 0.59	9.49 ± 0.71*****	13.41 ± 0.48
	Central INL	20.76 ± 0.63	25.92 ± 0.43*****	20.65 ± 0.21
	Peripheral INL	18.23 ± 0.54	22.78 ± 0.16*****	18.12 ± 0.39
Cell count (cell/mm^2)	Central ONL	32.51 ± 0.31	21.47 ± 0.50*****	32.13 ± 0.58
	Peripheral ONL	30.40 ± 0.38	20.43 ± 0.48*****	29.90 ± 0.25
	Central INL	20.71 ± 0.77	35.92 ± 0.52*****	21.33 ± 0.65
	Peripheral INL	18.23 ± 0.35	33.72 ± 0.44*****	17.90 ± 0.35
Nucleus cell diameter (µm)	Central ONL	8.16 ± 0.19	7.51 ± 0.51	7.18 ± 0.24
	Peripheral ONL	7.63 ± 0.65	6.27 ± 0.34	7.12 ± 0.37
	Central INL	10.36 ± 0.20	5.51 ± 0.70*****	9.80 ± 0.45
	Peripheral INL	9.07 ± 0.90	4.07 ± 0.08	9.12 ± 0.58

Morphological damage

The morphological analysis in photoreceptor cells, through of ultrastructural analysis, revealed that 90% of retinas presented damage only in the 0.02 mg/kg of AgNS (PVP coated) treatment of the subchronic experiment. The damage observed was: morphological changes in the outer segment of rods (**Figure 9C**); hemorrhage (Fig. 9D); many melanosomes adhered in the plasmatic cones membrane (**Figure 9B**

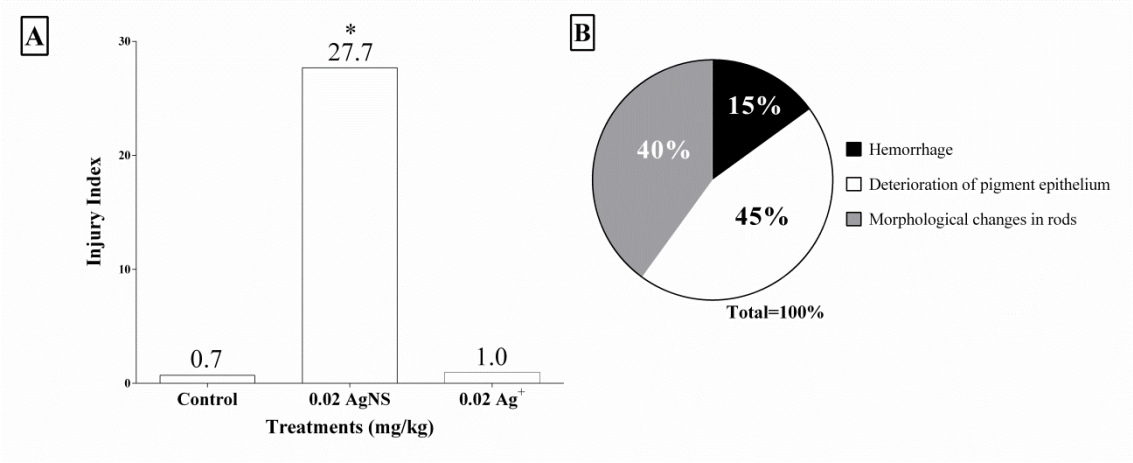
and **Figure 9D**); deterioration of pigment epithelium (**Figure 9E**) with cytoplasmic fragments of the pigment epithelium (**Figure 9F**).

Figure 9 – Scanning electron microscopy (SEM) showing the morphological damage in retina from *H. intermedius*. [A] View of retina (rods and cones) of negative control fish without damage. [B, C; D; E and F] Retina from *H. intermedius* after subchronic exposure to 0.02 mg/kg of AgNS (PVP coated) showing: [B] the melanosomes adhered in the plasmatic cones membrane (MA). [C] Morphological changes in the outer segment of rods (circle). [D] Hemorrhage with the presence of erythrocyte (E), and two types of melanosomes (granule and stick) adhered in the plasmatic cones membrane (MA). [E] Deterioration of pigment epithelium (PE). [F] Cytoplasmic fragments (CF) of the pigment epithelium. For all groups n = 8. SEM; Scale bar = 5 µm.



The lesion index increased significantly in individuals exposed to AgNS (PVP coated) in the subchronic bioassay, both in relation to the control and to Ag^{+} (**Figure 10A**). This shows that AgNS (PVP coated) caused more morphological damage than Ag^{+}. The deterioration of pigment epithelium and the morphological changes in rods together was 85% (45% and 40%, respectively) of the damage types found in the 0.02 mg/kg treatment of AgNS (PVP coated) (**Figure 10B**).

Figure 10 – Injury index in the retina of *H. intermedius* after 40 days of AgNS (PVP coated) trophic exposure. [A] Graphic comparing the control with the 0.02 mg/kg treatment of AgNS (PVP coated) or Ag^{+}; [B] Percentage of damage types found in the 0.02 mg/kg treatment of AgNS (PVP coated). Comparison between control and treatment by *t* test. * indicate statistical difference, considering $p < 0.05$. For all groups $n = 8$.



4.1.4 Discussion

Ingestion is considered the main route of uptake of nanosilver into several organism types (GAISER et al., 2009). Studies show endocytosis as the main internalization mechanism of nanoparticles and an average of 50 nm particles are internalized faster than other particles (REJMAN et al., 2004; CHITHRANI et al., 2006). In the present work, there is evidence of cell uptake since the suspensions of AgNS (PVP coated) presented very small particles (average 50 nm at 2 and 20 µg/ml) and additionally, AgNS (PVP coated) deposits in the retina through the autometallography were found (for example, in chondrocytes).

Moreover, the DNA damage in blood and brain cells revealed in our study might be seen as additional evidence of this internalization since a direct mode of action in the DNA was found through alkaline Comet Assay, which is able to detect single-strand breaks (SSB), alkali-labile sites (ALS), DNA-DNA and DNA-protein crosslinks (FAIRBAIRN et al., 1995). In electrophoresis gel there is an increase in migration of DNA fragments in SSB and ALS types of DNA damage. In contrast, both DNA-DNA and DNA-protein crosslink cause a decrease in migration of DNA fragments (TICE et al., 2000). Chemical or physical agents called bifunctional alkylanting present two radicals that can produce covalent adducts with DNA or proteins. These adducts prevent DNA strand separation, blocking DNA replication and/or transcription (NOLL et al., 2006) and could alter the structure of the DNA helix (RAJSKI & WILLIAMS, 1998).

The results of this work thus suggest that this AgNS (PVP coated) could be a crosslinking agent, which led to a decrease in DNA migration in electrophoresis gel. Additionally, this result suggests a direct interaction with the DNA, in line with the hypothesis forwarded by McShan et al. (2014) showing the toxic mechanism of silver nanoparticles and both direct and indirect damage to DNA. In fact, recent *in vitro* experiments with RTG-2 fish line cells to test if this AgNS (PVP coated) is indeed a crosslinking agent further support this hypothesis (Klingelfus et al., in preparation).

No definitive explanation has yet been determined in relation to AChE activity. Studies indicate the inhibition of AChE activity, as in erythrocytes of zebrafish (KATULI et al., 2014) and in an *in vitro* enzyme assay with specific subtract (ŠINKO et al., 2013). However, in our study the AChE activity was not affected by AgNS (PVP coated). Experiments with different fish species should be performed to find an accurate statement in relation to cholinesterase activity.

Our study also examined the retina. With respect to those results, we can see evidence of other types of important damage.

The deposition of AgNS (PVP coated) to the retina can be explained by two pathways. First, nanosilvers could access photoreceptor cells via the retinal pigment epithelium (RPE), which is involved in the transportation of essential nutrients between the choriocapillaris and the neural retina. Zucker et al. (2013) detected the uptake of silver nanoparticles (also coated with PVP) in human derived retinal pigment epithelial cell line (ARPE-19), whereon silver nanoparticles agglomerates were arranged around the nucleus cell in the environs of the endoplasmic reticulum. Another possibility could be through the complex system of blood vessels, located at the inner limiting membrane in direct contact with the Müller cells and the ganglion cell layer nutrients (KANDEL & SCHWARTZ, 1985).

After AgNS (PVP coated) crossed these membranes, damage in other layers could be observed, due to distribution among cells, including decreased nuclear layer thickness and cell count in ONL, which in turn may be related to apoptotic process of cells. In the adult organism, a fine-tuned balance between cell proliferation and cell death is required to maintain tissue homeostasis and to remove cells with functional or structural deficits, which otherwise might affect the whole organ and eventually endanger the viability of the entire organism. However, if exogenous stimuli are present, apoptotic cell death may drastically affect the proper functioning of organs. In the retina, apoptosis plays a major role in the loss of visual cells in blinding

disorders (DUNAIEF et al., 2002). It is possible that the INL reacted in reverse order (increasing the number of cells and therefore increasing thickness) trying to compensate for the damage observed in the ONL. Furthermore the result of diameter nucleus cell decreased in INL suggests impact in chromatin. As mentioned previously, the possible DNA damage caused by AgNS (PVP coated) is crosslink DNA-DNA or DNA-protein, and there is evidence of crosslinking agent can increase the chromatin condensation, consequently, decrease the nucleus diameter (GKOTZAMANIDOU et al., 2015). Perhaps the alteration in the ONL and INL is a reflection of damage observed in the photoreceptor layer.

In addition, critical damage was also found in the form of the rupture of the RPE. As will be described in greater detail below, damage — in particular the rupture of the RPE — may have caused hemorrhaging and the release of melanin granules adhered to the outer surface of the cone. The RPE is essential for the maintenance and survival of the overlying photoreceptor cells and is thought, in addition, to regulate the integrity of the choroidal capillaries. Moreover, in its quest for optimal retinal function, the RPE performs a number of critical functions, i.e. formation of the outer blood-retinal barrier, transepithelial transport of nutrients and waste products, transport and storage of retinoids, phagocytosis and degradation of spent outer segments, protection against light and free radicals and the production of growth factors (BOULTON & DAYHAW-BARKER, 2001).

Specifically, the rupture of the RPE may have caused hemorrhaging and, more importantly, the release of melanin granules adhered to the outer surface of the cone. Greene and Su (1987) report that deposition of silver after chronic exposure increases the melanin production in connective tissue surrounding sebaceous glands, in perineural tissue and in arteriolar walls, causing a discoloration of skin. Another study demonstrated the increase of epidermic melanization and dermal melanophages after silver colloidal ingestion (WADHERA & FUNG, 2005). In relation to the adherence of melanin granules to the surface of cones, it is possible that AgNS (PVP coated) may caused an increase of melanin production, and the rupture of the RPE promoted the melanin granules liberation to photoreceptors region.

In addition to all the aforementioned damage, the rod mediated vision may have been impaired in this species due to the outer segment alteration of them. Structural changes in the cells interfere in important intracellular processes and also,

change the phototransduction efficiency due to a decreased probability that a photon will be absorbed by a cone or rod (MELA et al., 2012).

Finally, judging by the deposition of AgNS (PVP coated) in several retina regions, the present work provides evidence that AgNS (PVP coated) is able to cross blood-retinal barrier (BRB). Chen and Liu (2012) propose that cell-mediated transport of nanomaterials, called Trojan Horse mechanism, is the way to cross the blood-brain barrier (e.g. monocytes can cross blood-brain barrier with high turnover). It is possible that the Trojan Horse mechanism occurred in the retina. However, specifically in the matrix of sclera, a cartilaginous tissue without vascularization, the possible uptake mechanism of AgNS (PVP coated) could be diffusion.

4.1.5 Conclusions

In conclusion, the findings of this study indicate that silver nanospheres (PVP coated) may be a neurotoxicant for fish and present a different behavior in relation to silver ion due to the finding of differential responses in uptake and in other biomarkers. The lower levels of DNA damage observed in AgNS (PVP coated) treatments indicate that silver nanospheres might be a potential crosslinking agent. Moreover, AgNS (PVP coated) deposits were observed in different retina regions, which consequently triggered photoreceptor morphological changes, hemorrhage and epithelium damage. In addition to AgNS (PVP coated) found in the eyes, the deposition demonstrates that AgNS (PVP coated) can cross the blood-retina barrier. Thus, altogether, all these harmful effects can lead to a competitive disadvantage for fish, compromising their survival.

Acknowledgments

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Furnas (Fish Farming and Hydrobiology Station of Furnas, Minas Gerais, Brazil) for fish donation.

Ethical issues

The experiments conducted in this study were approved by the Ethics Committee for Animal Experimentation (CEEA) of the Federal University of Paraná certificate number 973.

4.2 Trophic exposure to silver nanosphere in *Hoplias intermedius*: short and long term toxic effects

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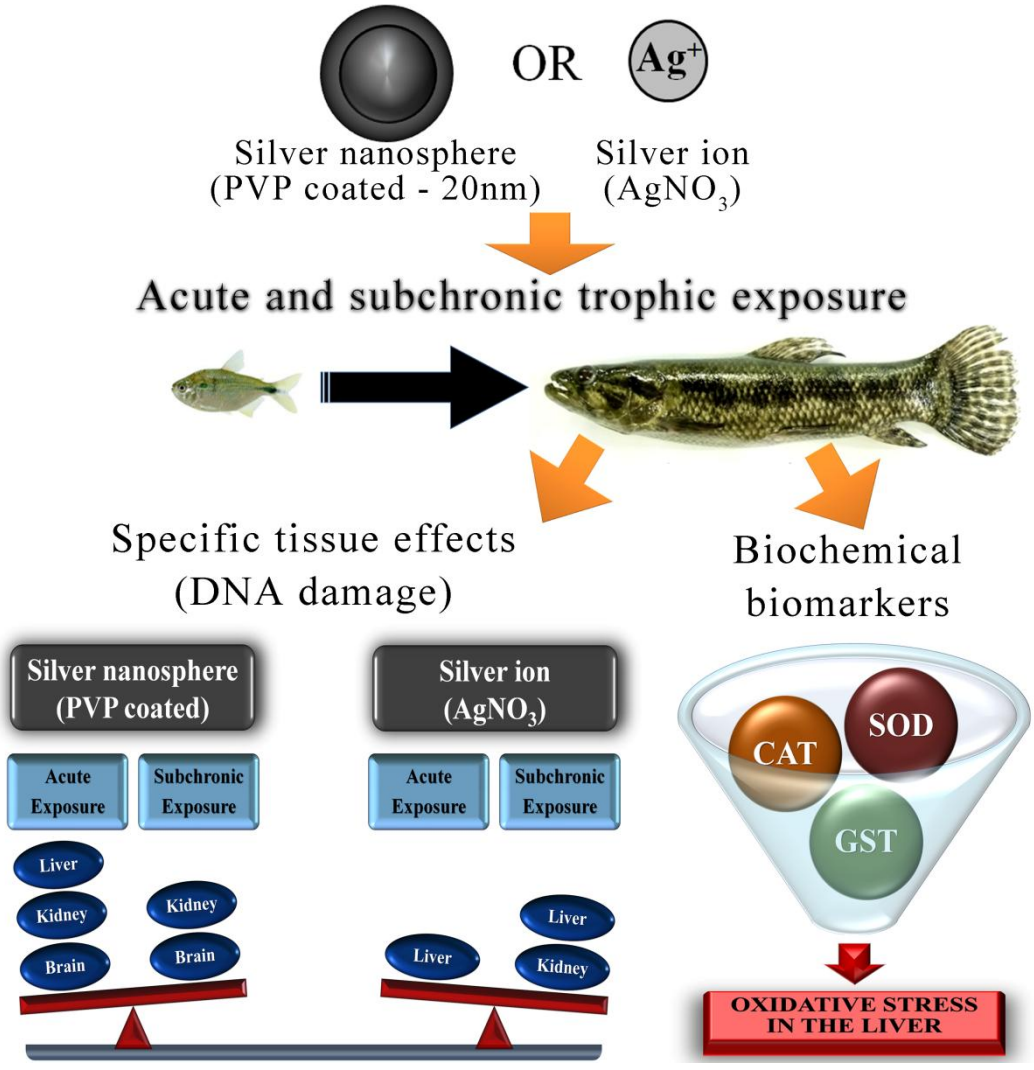
Figure 11 – Graphical abstract. Author: Tatiane Klingelfus.



Abstract

Nanomaterials (NMs) production has grown exponentially in several industries, medical and technological sectors and the discard in water bodies may be happening with no careful. Consequently, there is hazard for both environment and human safety, and ecotoxicological approach is required to access the toxic effects of NMs. With trophic exposure in short term of 96 hours (only one dose administration of: 0.002, 0.02 and 0.2 mg/kg) and long term of 40 days (ten doses of 0.02 mg/kg), the present study aimed to find the specific tissue effects of a silver nanosphere coated by polyvinylpyrrolidone [(AgNS (PVP coated)] and the silver ion (Ag^{+}) in a carnivorous fish *Hoplias intermedius* through alkaline Comet assay in blood, liver kidney and brain. Moreover, the detoxication and oxidative stress were accessed by concentration of metallothioneins (MTs), lipoperoxidation (LPO), activities of superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST). The lowered migration of the DNA tail in all fish tissues of AgNS (PVP coated) treatments, both in acute and subchronic exposure, demonstrated the crosslink DNA-DNA or DNA-protein damage type. In acute exposure, the liver, kidney and brain were the main affected tissues, while the kidney and brain had the greater DNA damage level in the subchronic exposure. In contrast, the liver was the main tissue with DNA damage in Ag^{+} treatments; in addition, the kidney also presented a high DNA damage in subchronic exposure. In relation to biochemical biomarkers, oxidative stressed was evidenced in AgNS treatment at 0.002 mg/kg of the acute exposure, with LPO, SOD and CAT increased, while GST and MTs decreased. Certainly, the damage caused by nano-silver was evident in essential tissues, as kidney and brain, moreover, the toxic effects in both short and long term exposure were found, demonstrating the hazard of nano-silver in aquatic environment.

Keywords: emerging contaminants; nanomaterial; silver; specific tissue; DNA damage; oxidative stress; *Hoplias intermedius*.

4.2.1 Introduction

Nanometric structures have benefits to the nanotechnology due to specific chemical-physic properties, such as greater superficie area, both high chemical reactivity and mechanical resistance. Nanomaterials (NMs) can be synthesized in order to a specific product (e.g. clothes with silver nanoparticles, due to antimicrobial activity of them) or can be generated from chemical reaction (e.g. carbon particles emission from automotives or nanoparticles from volcanic ashes). Moreover, there is a morphological structure diversity of NMs, such as tube, plaque, sphere, fiber, particle or polymer (RAJAS et al., 2017).

More importantly, the global production of NMs has exponentially increased in several industrial, pharmaceutical, medical and electronical sectors. Additionally, the metallic NMs have had a faster growing production (VANCE et al., 2015). Production of silver nanomaterials (nano-silver) is about 100 t/year with application in human consume products (e.g. . toothpaste, deodorants, shampoo, apparel, footwear, toys, washing machines, water purifiers, among others) (MARAMBIO-Jones & HOEK 2010, CERKEZ et al., 2012, PETERS et al., 2014, STARK et al., 2015). The Project on Emerging Nanotechnologies (2017) marks 442 nano-silver products. Then it can be expected to find NMs in water bodies, eventually. However, there are no data about real environment concentration of nano-silver; only Sun et al. (2014) estimated average 0.66 µg/L and 0.45 ng/L for nano-silver in the European Union (EU) and Switzerland, respectively.

In relation to the difficulties to measure the environment concentration or the hazard of NMs, it has been a concern their discard for the safety to the environment and human health (PETERS et al., 2014). For these reason cited, ecotoxicological studies are needed and the trophic exposure in fish may represent a more realistic model. Gaiser et al. (2009) report the ingestion route as main uptake of nano-silver in several organisms and, single or repeated trophic exposure can have differences in relation to the tissue distribution and, moreover, it provides insight of bioaccumulation (LANKVELD et al., 2010).

In the present study, the aim were to examine — through genetic and biochemical biomarkers — 1) the effects caused in a carnivorous fish *Hoplias intermedius* after acute and subchronic trophic exposure by a nano-silver [silver nanosphere (AgNS) with polyvinylpyrrolidone (PVP) coating] and the silver ion (Ag^{+});

2) the specific tissue effects of AgNS versus Ag^+ and 3) the difference between acute versus subchronic exposure.

4.2.2 Material and Methods

Contaminants and characterization of the AgNS (PVP coated) suspensions

Silver nanosphere from Sigma-Aldrich® with the supplier information: 20 nm average primary particle size, 20 µg/mL in water and PVP (polyvinylpyrrolidone) functionalized]. The working suspensions were at 20, 2 and 0.2 µg/mL (in ultrapure water) and through of Zetasizer® Nano Series ZS90 (Malvern Instruments, Worcestershire, UK) analyzed the hydrodynamic size, the distribution of particles size per % intensity, zeta potential (ζ) and polydispersion index (PdI). The latter was modified to % of polidispersity (%PD), according to the equation:

$$\%PD = \sqrt{PdI} \times 100.$$

The silver ions solution (Ag^+) from $AgNO_3$ (Merck®, CAS number 7761-88-8) was diluted in ultrapure water to obtain the working suspensions at 20, 2 and 0.2 µg/mL (in ultrapure water) concentrations. In order to obtain only Ag^+, a balanced stoichiometric calculation was performed for each concentration.

Native fish species and acclimating conditions

Hoplias intermedius (GÜNTHER, 1864) was used as experimental model and *Astyanax altiparanae* (GARUTTI & BRITSKI, 2000) as vehicle of contamination. Both species are native Brazil fish, and especially *H. intermedius* have presented attractive to economic interests due to quality of their meat and sport fishing (OYAKAWA & MATTOX, 2009). Moreover, *H. intermedius* are on top of the food chain in the aquatic environment, thus they are an important model specie to ecotoxicological studies, with the possibility to several modes of exposure, as herein, the trophic exposure using their natural prey *A. altiparanae*.

Predator fish (*H. intermedius*) from the Eletrobras Furnas (Fish Farming and Hydrobiology Station of Furnas, Minas Gerais, Brazil) as donation and for about six months were acclimatized in 2000 L tanks. The specimens, in the acclimatizing period, were fed with commercial fish food and in the bioassays with *A. altiparanae*

(prey fish) live specimens. The prey fish from fish farms and for about one week were acclimatized in 250 L tanks previously bioassays.

Size and weight of both the predators and preys were measured. Predators had about 11.27 cm ± 1.21 cm of size and 16.54 g ± 4.86 g of weight (mean ± standard deviation), and the preys 4.13 cm ± 0.38 cm and 1.10 g ± 0.34 g (mean ± standard deviation).

Contaminant doses and experimental design

Since there are no established values for LOAEL (lowest-observed-adverse-effect level) or NOAEL (no-observed-adverse-effect level) of silver nanomaterials in organisms and there are only a few studies regarding nanosilver exposure in fish — most of studies concerning hydric exposure (ASHARANI et al., 2008; SHAHBAZZADEH et al., 2009; BILBERG et al., 2010; WU et al., 2010) — the doses used in present study were approximated to the value determined by the Environmental Protection Agency (EPA) on Integrated Risk Information System (IRIS, 2017) of silver ion (CASRN 7440-22-4) LOAEL at 0.014 mg/kg/day for humans.

The administrated dose in the prey was calculated according to the predator's weight individually.

Two bioassays were performed at followed procedure:

- Acute bioassay (96 hours): prey fish received an intraperitoneal injection with one of the AgNS (PVP coated) suspensions or Ag^+ solutions (20, 2 or 0.2 µg/mL), and immediately afterwards were offered as food to the predator fish (Fig. 1 and Video 1 in the next chapter — click here to access them). Each predator received a single prey contaminated with doses at 0.2 mg/kg, 0.02 mg/kg or 0.002 mg/kg of AgNS (PVP coated) or Ag^+ (both with n = 15 for each treatment). In the same conditions, predators received prey without contaminants, wherein were used as negative control (n = 30). Fish were kept for up 4 days in freshwater (constant aeration, 28.3ºC ± 0.9, 12/12 h photoperiod), and afterward were killed to collected samples of blood, liver, anterior kidney and brain were collected.
- Subchronic bioassay (40 days): each predator received a single prey every 4 days, a total of 10 preys contaminated over 40 days, with a dose of 0.02 mg/kg of Ag^+ (n = 15) or AgNS (PVP coated) (n = 13). Two specimens of AgNS

treatments, one of them refused the prey and other one jumped out of the water were drawn of the analysis — events were not linked to the contamination. Over 40 days the fish group of the negative control (n = 15) kept in same treated fish conditions and preys without contaminants fed the predators. Fish were kept for up 40 days in freshwater in the same experimental conditions of the acute bioassay, likewise the sample collecting procedures aforementioned.

DNA damage in blood, liver, kidney and brain cells

The alkaline Comet assay was previously described by Singh et al. (1988) and modified by Ferraro et al. (2004) and Cestari et al. (2004). This method in alkaline condition (pH > 13) was used to detect any difference in DNA score (increase or decrease) in relation to the control, which can considered DNA damage. After sampling collect, blood and homogenized tissues (liver, anterior kidney and brain) were maintained in fetal bovine serum. Tissues were homogenized at 1500 rpm (homogenizer Potter type) in fetal bovine serum to obtain a cell suspension. 15 µL of each cell suspension mixed with 120 µL of agarose with a low melting point (LMP) and placed immediately on slides previously covered with a normal agarose layer. The slides were submerged in a lysis solution [Lysis stock solution: NaCl (2.5 M), EDTA (100 mM), Tris (10 mM), NaOH (0.8%), N-lauryl-sarcocinate (1%); Lysis reaction solution — Triton X100 (1%), DMSO (10%), Lysis stock (89%)] for 72h at 4°C. The following step was immersed the slides in an alkaline buffer [NaOH (10 N) and EDTA (200 mM), pH 13], for 25 minutes in order to denature the DNA strands. Slides followed to electrophoresis at 300 mA, 1V.cm^{-1} for 25 minutes; neutralized by Tris-HCl (0.4 M, pH 7.5, 4°C), and fixed in absolute ethanol for 10 minutes. Stained slides by 2 µg.ml^{-1} of Ethidium bromide were analyzed on Leica® epifluorescence microscope and were counted 100 nucleoids in each one. Nucleoid categorization accorded to damage classes from 0 to 4 (Collins et al., 1997), and score of each animal was calculated by the multiplication of the classified nucleoid number with its particular class, the followed of sum of them.

Liver biochemical biomarkers

The liver samples were stored frozen at -80ºC until analyzed.

LPO was measure according to Jiang, Hunt e Wolff (1992) method through ferrous oxidation xylenol orange (FOX) reaction solution (xylenol orange 100 µM, sulfuric acid 25 mM, butylated hydroxytoluene (BHT) 4 µM, ferrous ammonium sulphate 250 µM, methanol 90%).

SOD activity was measure according to Gao et al. (1998), with Tris/EDTA buffer (1 M/5 mM, pH 8), pyrogallic acid (15 mM) and HCl 1N.

CAT activity was measure according to Aebi (1984), with reaction solution of Tris/EDTA buffer (1M/5mM, pH 8), hydrogen peroxide 30% and water.

GST activity was measure according Keen et al. (1976), with 1-chloro-2,4-dinitrobenzene (CDNB- 3 mM).

Metallothioneins concentration was measure according to Viarengo et al. (1997), using 35 mg of the homogenized liver tissue in Tris-HCl/sacarose buffer (20mM/500 mM, pH 8.6) in a proportion of 1:10 and then centrifuged to 15,000 xg for 30 minutes (4ºC).

Statistical analysis

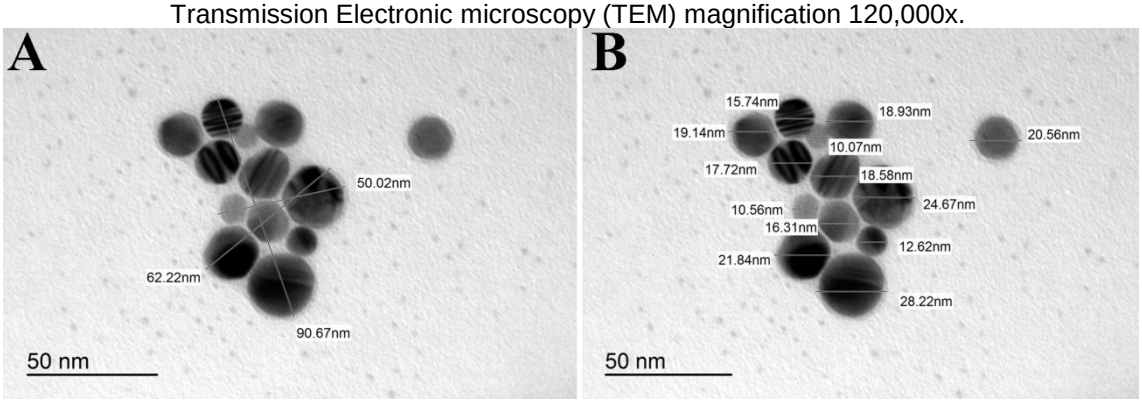
A normal distribution of all data was found, thus the ANOVA was applied with a *t* test (LSD) *post-hoc*, and the values of biomarkers as mean and standard deviation of each treatment were expressed. Firstly, the raw data of the treatments were compared to the negative control for each biomarker and each bioassay, individually. The data were subsequently modified to relative values (%) and the specific tissue effects and the acute condition in relation to subchronic condition could be compared, thereafter. The significance level was set at $p < 0.05$.

4.2.3 Results

Characterization of the AgNS suspensions

The characterization analysis demonstrated both agglomerated (**Figure 12A**) and individualized states of the AgNS, with average size of 20 nm in relation to the primary particle (nanosphere isolated) (**Figure 12B**). The suspensions at 2 and 20 µg/mL had -22.6 and -21.6 mV zeta potential and 52.41 and 46.19 nm particle mean sizes, respectively. The zeta potential next to -30 mV (at 2 and 20 µg/mL) may indicate greater stability than the suspension at 0.2 µg/mL, which the particle mean size was 237.02 nm and -10.1 mV zeta potential. In other words, the particles are bigger when the zeta potential is next to zero. The size distribution varieties of each suspension may be due to the % of polydispersity greater than 20%, indicating heterogeneous suspensions with variation in particle size ranges (**Table 1**, 20).

Figure 12 – Eletromicrography illustrating the agglomerated and isolated states of the silver nanosphere PVP coated [AgNS (PVP coated)]. (A) Diameter measures of the agglomerated and (B) of the each isolated nanosphere of the suspension at 20 µg/ml. Transmission Electronic microscopy (TEM) magnification 120,000x.

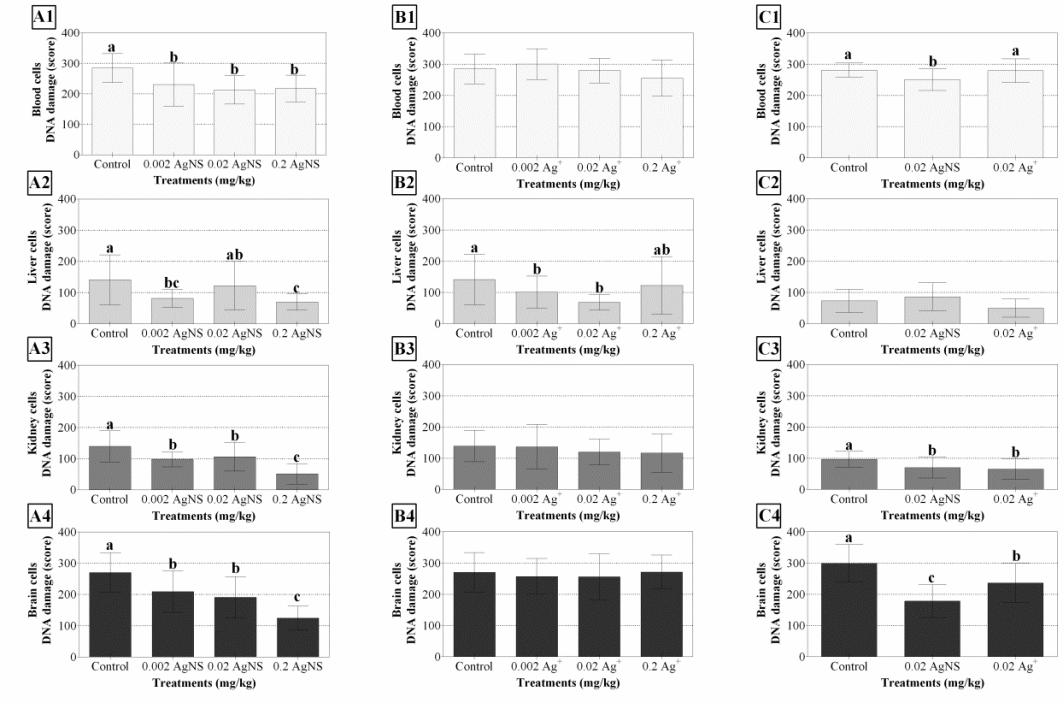


DNA damage and specific tissue effects

All the tissues had a decreasing in their DNA scores in relation to control, therewith, the crosslink DNA-DNA or DNA-protein can be suggested thus as mode of action of AgNS in the DNA, as mentioned before in the 2.1 section.

In acute bioassay, AgNS was be able to decrease of DNA score in relation to negative control both all doses and all analyzed tissues, such as blood (**Figure 13A1**), liver (**Figure 13A2**), kidney (**Figure 13A3**) and brain (**Figure 13A4**). Only in the liver at 0.02 mg/kg the DNA score had no difference in relation to control (**Figure 13A2**). The liver of the fish in the Ag^+ acute treatments had the DNA score decreased only at 0.002 and 0.02 mg/kg (**Figure 13B2**) in relation to negative control. The AgNS treatments in subchronic condition had the decreased DNA score in blood (**Figure 13C1**), kidney (**Figure 13C3**) and brain (**Figure 13C4**), while only the kidney (**Figure 13C3**) and brain (**Figure 13C4**) of the Ag^+ treatments had the decreased DNA scores in relation to negative control.

Figure 13 – Alkaline Comet assay showing crosslink DNA-DNA or DNA-protein damage in blood, liver, kidney and brain cells of *H. intermedius* after acute and subchronic trophic exposure by silver nanosphere PVP coated or by silver ions. (A1-A4) Acute bioassay with AgNS trophic exposure at 0.002, 0.02 and 0.2 mg/kg (blood, liver, kidney and brain, respectively); (B1-B4) acute bioassay by Ag^+ trophic exposure at 0.002, 0.02 and 0.2 mg/kg (blood, liver, kidney and brain, respectively); and (C1-C4) subchronic bioassay by AgNS or Ag^+ trophic exposure at 0.02 mg/kg (blood, liver, kidney and brain, respectively). The y-axis shows the mean with standard deviation of the DNA damage (score). Different letters indicate statistical differences, $p < 0.05$ statistical level, ANOVA with *t* test (LSD) *post-hoc.*



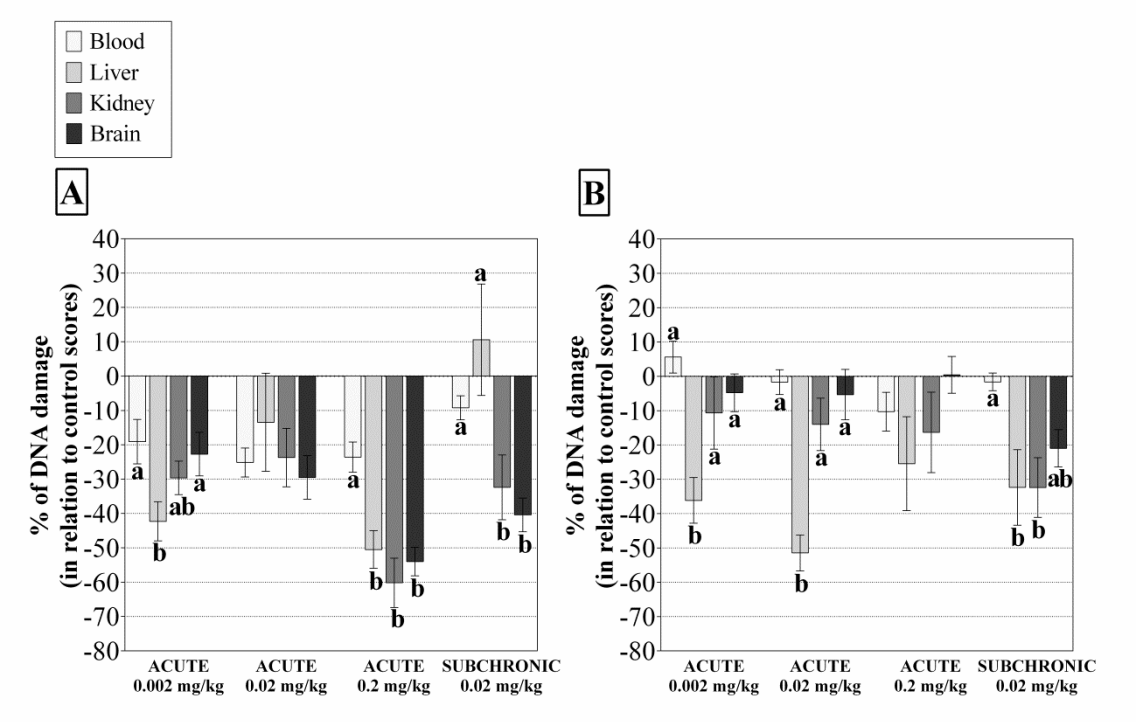
Specific tissue effects of AgNS in *H. intermedius* in acute and subchronic conditions had some differences, in the same way the effects of AgNS and Ag^+.

In order to demonstrate the specific tissue effects in the fish treated by AgNS or Ag^+, the % relative values of the treatments (% of difference in relation to negative control) were used.

In AgNS exposure (**Figure 14A**), both kidney and brain in acute condition at 0.2 mg/kg were the main tissues with DNA damage, decreasing 60.18% and 57.02% of score in relation to negative control, respectively. In addition, the subchronic exposure showed effects only in two tissues, both kidney and brain with decreased score of 32.41% and 40.41% in relation to negative control, respectively. In the liver, AgNS only in acute exposure at 0.002 mg/kg and 0.2 mg/kg caused effects, comparing to other tissues, with 42.28% and 50.53% of score decreased in relation to negative control, respectively.

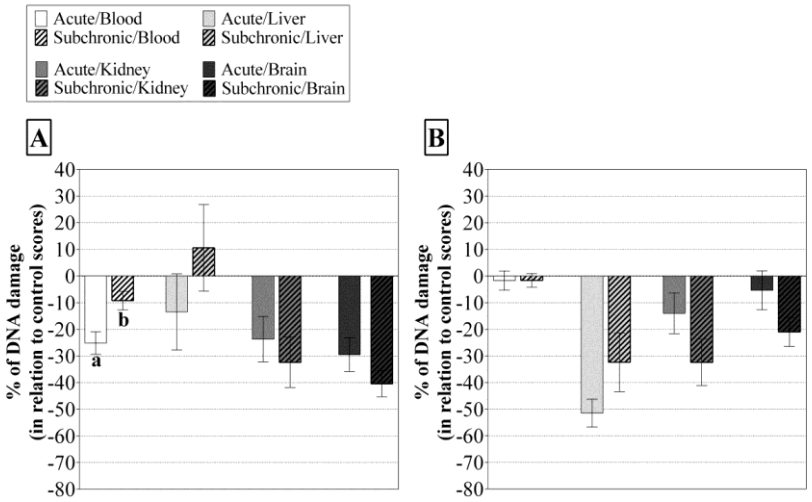
In contrast, the Ag^+ acute exposure caused a score decreased in the comet assay only in the liver cells (**Figure 14B**), with 36.17% and 51.46% scores smaller than negative control at 0.002 and 0.02 mg/kg, respectively. Moreover, Ag^+ showed the decrease of 32.36% of the DNA score in the liver in the subchronic condition in relation to negative control, and also in the kidney a decrease of 32.42%.

Figure 14 – Specific tissue effects of silver nanosphere PVP coated and silver ion in *H. intermedius* after acute and subchronic trophic exposure. The y-axis shows the percentage of DNA damage difference of the (A) AgNS or (B) Ag^{+} treatments in relation to negative control mean in acute (0.002, 0.02 and 0.2 mg/kg) and subchronic (0.02 mg/kg) conditions. Expressed values for mean (%) with standard deviation of the DNA damage of blood, liver, kidney and brain cells. Different letters indicate the significant difference among tissues (blood, kidney, liver and brain), $p < 0.05$ statistical level, ANOVA with *t* test (LSD) *post-hoc.*



In relation to the comparison of DNA damage in acute and subchronic conditions, there was no difference between them in tissues analyzed, both AgNS (**Figure 15**A) and Ag^{+} (**Figure 15**B). A few % of difference in relation to negative control of blood in relation to the other tissues was found. In addition, there was no difference among tissues in acute exposure at 0.02 mg/kg. However, the blood cells evidenced difference between acute and subchronic exposure to AgNS, wherein acute condition had decreased score in 9.23%, while in subchronic, 25.15% was decreased in relation to negative control.

Figure 15 – DNA damage difference between acute and subchronic trophic exposure by silver nanosphere PVP coated and silver ion in *H. intermedius*. The y-axis shows the percentage of DNA damage difference of the (A) AgNS or (B) Ag^{+} treatments in relation to negative control mean in subchronic (0.02 mg/kg) condition. Expressed values for mean (%) with standard deviation of the DNA damage of blood, liver, kidney and brain cells. Different letters indicate the significant difference among tissues (blood, kidney, liver and brain), $p < 0.05$ statistical level, *t* test (for independent samples).



Biochemical biomarker alterations in the liver

Table 2 shows the biochemical biomarker responses, wherein some alterations were found. The lipid hydroperoxides concentration increased only in AgNS treatment at 0.002 mg/kg, in the acute condition, when compared to negative control. In the same treatment, the activity of SOD and CAT were increased, while GST has had a decreased activity, as well as the metallothionein concentration. These alterations, previously cited, indicate that a possible oxidative stress and membrane damage in liver cells were caused by AgNS.

In relation to the other treatments in the acute condition, the exposure to 0.2 mg/kg AgNS caused increase in SOD and CAT activity, and decrease in GST activity and metallothioneins concentration. In contrast, the animals treated by Ag^{+} have had increase only of CAT at 0.02 and 0.2 mg/kg, and decrease of both GST activity and metallothioneins concentration at 0.002 and 0.2 mg/kg.

In another way, the SOD activity decreased in subchronic exposure to AgNS as well as the GST activity and metallothioneins concentration. Biochemical biomarkers in Ag^{+} subchronic treatment were not altered response.

Table 2 – Biochemical biomarkers in the liver of *H. intermedius* after acute and subchronic trophic exposure by silver nanosphere (PVP coated) or by silver ions.

Treatments			LPO nmol.mg protein^{-1}	SOD U.min^{-1}.mg protein^{-1}	CAT µmol.min^{-1}.mg protein^{-1}	GST nmol.min^{-1}.mg protein^{-1}	MTs µg.mg protein^{-1}
Acute	Control		11.9 ± 2.58	192.5 ± 26.57	162.2 ± 32.23	294.3 ± 77.87	27.2 ± 10.38
	AgNS (mg/kg)	**0.002**	14.6 ± 4.48*	221.2 ± 42.39*	193.3 ± 27.07*	216.3 ± 44.09*	16.4 ± 5.45*
		0.02	13.8 ± 4.84	195.6 ± 29.95	175.0 ± 25.02	197.4 ± 35.39*	25.6 ± 7.78
		0.2	10.3 ± 3.73	223.1 ± 53.50*	185.9 ± 52.79*	213.8 ± 71.81*	21.0 ± 10.42*
	Ag^+ (mg/kg)	**0.002**	10.1 ± 3.43	176.5 ± 8.72	166.4 ± 24.37	253.3 ± 64.40*	17.5 ± 7.63*
		0.02	13.2 ± 3.59	195.6 ± 21.99	189.5 ± 33.36*	262.1 ± 49.49	23.0 ± 9.92
		0.2	14.2 ± 5.50	198.3 ± 42.06	195.7 ± 31.34*	186.1 ± 29.97*	20.2 ± 9.24*
Subchronic	Control		12.2 ± 3.62	244.5 ± 19.79	256.7 ± 39.12	270.7 ± 35.57	20.5 ± 6.27
	AgNS (mg/kg)	**0.02**	12.1 ± 5.35	215.0 ± 11.31*	246.7 ± 46.61	219.4 ± 33.30*	14.4 ± 6.82*
	Ag^+ (mg/kg)	**0.02**	10.2 ± 2.15	248.2 ± 41.21	258.8 ± 48.44	276.4 ± 52.20	16.9 ± 5.40

Doses of acute condition: 0.002, 0.02 and 0.2 mg/kg of AGNS or Ag^+; doses of subchronic condition: 0.02 mg/kg of AGNS or Ag^+. (LPO) Lipid-peroxidation measured by lipid hydroperoxides concentration (nmol.mg of protein^{-1}); (SOD) Superoxide-dismutase enzyme activity (U of SOD.min^{-1}.mg of protein^{-1}); (SOD) Superoxide-dismutase enzyme activity (U of SOD.min^{-1}.mg of protein^{-1}); (CAT) Catalase enzyme activity (µmol of CAT.min^{-1}.mg of protein^{-1}); (GST) Glutathione S-transferase enzyme activity (nmol of GST.min^{-1}.mg of protein^{-1}); (MTs) Metallothioneins concentration (µmol.mg of protein^{-1}). *indicate the statistical difference related to control (without contamination), $p < 0.05$ statistical level, ANOVA with *t* test (LSD) *post-hoc.*

4.2.4 Discussion

The main use of the nano-silver is in human consumer products and probably they are reach out to bodies of water and hence study of the interaction between nano-silver and aquatic organism is very important. Moreover, the oral ingestion of nano-silver and their fate during metabolic process is still unexplained (GAILLET & ROUANET, 2015).

In general, no consensus in literature has yet emerged in relation to specific tissue effects caused by nano-silver in several exposure conditions. The most of studies show accumulation, inflammation and oxidative stress biomarkers, and different damage levels are found. Inflammation in heart, gallbladder, spleen and kidney of zebrafish adults contaminated via intramuscular inoculation by AgNP (PVP coated) at 1 mg/kg an 5mg/kg were found in different levels, causing a generalized toxicity (SPESHOCK et al., 2016). Acute oral exposure in mice with AgNP in different size and coating, in addition with relevant doses to human exposure, demonstrated fecal elimination and no toxicity associated (BERGIN et al., 2015). In contrast, a study with AgNP (PVP coated) at 0.01 mg/kg intranasal instillation in neonatal rats showed a high accumulation of silver in brain after long term-exposure and a declined silver contents in the liver (WEN et al., 2015).

Our findings as concerns specific tissue effects in *H. intermedius* fish after trophic exposure to a nanosphere PVP coated showed that AgNS (PVP coated) caused DNA damage in the kidney and brain mainly and in both acute and subchronic conditions. In addition, the DNA damage in the liver was found only in acute condition by AgNS (PVP coated) exposure. In contrast, the non-particle chemical, as Ag^{+}, demonstrated a different behavior, wherein the liver was the main tissue with DNA damage in both acute and subchronic conditions. Dziendzikowska et al., (2012) found time- and size-dependent accumulation in liver of rats, firstly, and silver content was redistributed to the other tissues, such as kidney and brain — after silver nanoparticles intravenous administration at 5 mg/kg (20 nm) —, corroborating the damage in the tissues found.

Additionally to the DNA damage results related to the specific tissue effects, the decrease in DNA migration suggests that AgNS (PVP coated) can be a crosslinking agent, since both DNA-DNA and DNA-protein crosslink cause a decrease in migration of DNA fragments in the alkaline Comet assay (TICE et al.,

2000). We found herein that the DNA migration was decreased in blood, liver, kidney and brain cells, considerably. AgNS as potential bifunctional alkylanting may produce covalent adducts with DNA or proteins, preventing DNA strand separation, blocking DNA replication and/or transcription (NOLL et al., 2006), or also, altering the structure of the DNA helix (RAJSKI & WILLIAMS, 1998). The crosslink DNA-DNA or DNA-protein suggests a direct interaction with the DNA, since Alber et al. (2007) reported the vertebrates nuclear pore or nuclear pore complex (NPC) present about 80 to 120 nm in diameter and the suspensions of AgNS (PVP coated) presented very small particles (average 50 nm at 2 and 20 µg/ml), thus, the AgNS (PVP) nuclear internalization could be possible.

Our study also examined the biochemical biomarkers in the liver of *H. intermedius*.

With respect to those results, other types of damage could be evidenced, such as oxidative stress and lipid peroxidation in the liver after AgNS acute exposure, with the SOD and CAT activity increased and GST activity decreased. The decreasing of antioxidant enzyme activity is related, probably, with the excessive production of reactive oxygen species (such as superoxide anion, hydrogen peroxide and other ones), that may imply the disruption of antioxidant defense (VALERIO-GARCÍA et al., 2017). Rajkumar et al. (2016) detected a decrease of GST activity after oral administration of silver nanoparticles in *Labeo rohita* fish, reporting that the alteration is related to a disruption in homeostasis. However, both increasing and decreasing indicate oxidative stress in the liver.

Other important finding, in present study, was the alteration of metallothionein concentrations in several treatments. Metallothioneins are chelating proteins of metals and other free radicals, and for these reason, the ones are involved in homeostasis and detoxification of the organism (VIARENGO et al., 1997).

In relation to aforementioned evidences, there is possibility NMs can be translocated across the cell membrane reaching cytosol by diversified pathways, depending of heterogeneity of particle size — both NMs isolate or aggregates smaller the 120 nm — in the organism (ZHU et al., 2012). The % of polydispersity indicates if a colloidal suspension is homogeneous (% PD < 20) or heterogeneous (% PD > 20) in relation to the particle sizes (MALVERN, 2005). High % of PD (> 20%) were found in the AgNS (PVP coated) suspensions used to trophic administration, consequently,

a high range of size particle distribution (16.2 nm to 487.3 nm), with the internalization to cytosol could be possible.

4.2.5 Conclusions

The present study investigated the toxic effects of nano-silver, specifically a silver nanosphere (PVP coated), and our findings were DNA damage in blood, liver, kidney and brain, moreover oxidative stress and lipid peroxidation.

In relation to specific tissue effects of AgNS, the liver appeared to be the first target tissue, demonstrating effects in acute conditions, and subsequently, after subchronic exposure, the kidney and brain have had higher effects. Differently, the silver ion caused effects mainly in liver. Therefore, the nano-silver can be a genotoxic and cytotoxic agent, indicating the hazard of discarding nanomaterials in the aquatic environment.

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Ethical issues

The experiments conducted in this study were approved by the Ethics Committee for Animal Experimentation (CEEA) of the Federal University of Paraná certificate number 973.

4.3 Nanomaterials like a DNA crosslinkers: an approach for assessing the genotoxic mode of action using *in vitro* Comet assay

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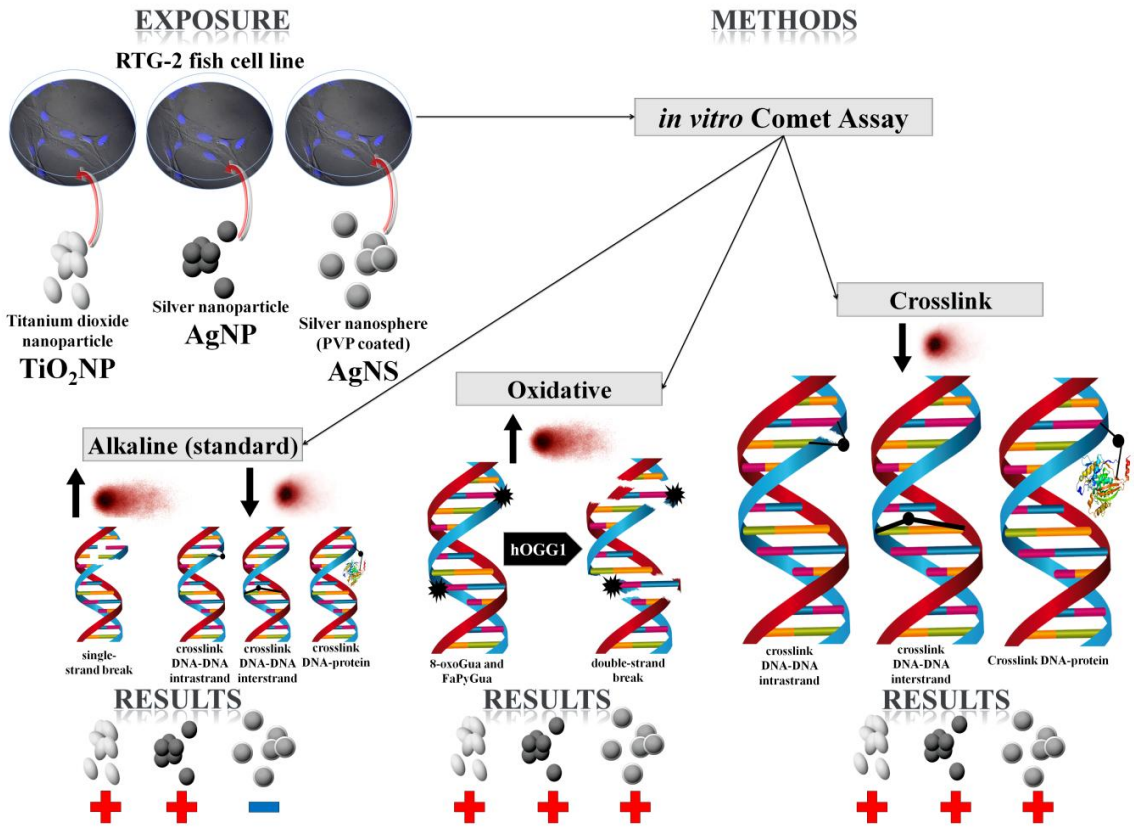
Figure 16 – Graphical abstract. Author: Tatiane Klingelfus.



Abstract

Nanotechnology has increased the production of nanomaterials (NMs), and therefore the prediction of their hazard potential for living organisms, such as the aquatic ones, is a challenge to science. Within this context, the use of *in vitro* models based on fish cell lines has had ecotoxicological relevance, since the primary interaction between chemicals and life-forms starts at cellular levels. Thus, aimed at predicting the genotoxic modes of action (MoA) of NMs, RTG-2 cells (rainbow trout gonadal) were exposed to nano-titanium and -silver (uncoated and PVP-coated) and types/levels of DNA damages were assessed by the Comet assay (standard alkaline, hOGG1-modified alkaline and crosslink-modified alkaline versions). The present data shown that the use of standard alkaline Comet assay alone can lead to false-negative results in the hazard estimation of NMs, since hOGG1-modified alkaline Comet assay demonstrated the oxidative DNA damage in RTG-2 fish cells [indentifying 8-oxo-7,8-dihydroguanine (8-oxoGua) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPyGua), specifically] at lower concentrations than studies with human cells. In addition, crosslink-modified alkaline Comet assay was be able to detect that both nano-titanium and -silver (uncoated and PVP-coated) caused the crosslink DNA-DNA or DNA-protein in RTG-2 fish cells. This latter finding still have not reported in other researches, due to the use standard Comet assay as the main method into hazard test batteries of NMs. Therefore, in order to achieve an accurate hazard identification of NMs, other testing strategies which detect different types of DNA damage should be used.

Keywords: nanoparticles, titanium, silver, fish, hOGG1, crosslink, DNA damage.

4.3.1 Introduction

Nanotechnology offers several advantages to industries, due to the unique functional properties of nanometric materials, which differ of the bulk ones. Consequently, the development of new products on a large scale has increased. However, their special properties, such as the small size and large specific surface area, provide different behavior in the absorption, distribution, metabolism and excretion on organisms (GAILLET & ROUANET 2015) and have also been a concern with respect to their safety for the environment and human health (PETERS et al., 2014).

The nano-titanium (TiO_2) and -silver (Ag) are the most produced and used among nanomaterials (NMs). In general, these have been increasing exponentially (PICCINNO et al., 2012). The global production of nano-TiO_2 has the most quantity with more than 1,000 t/year and of nano-Ag is produced moderately with more than 100 t/year. They are commonly used in consumer products, such as toothpaste, deodorants, shampoo, apparel, footwear, toys, washing machines, water purifiers, food and agricultural products, sunscreen, self cleaning window, among others (MARAMBIO-JONES & HOEK 2010, CERKEZ et al., 2012, PETERS et al., 2014, STARK et al., 2015). It has been listed in The Project on Emerging Nanotechnologies (2017) 14 nano-TiO_2 and 442 nano-Ag consumer products. For these reasons, the environment, specially the aquatic, ends up being the final destination of them.

Up to now, however, the nano-TiO_2 and nano-Ag aquatic concentrations have only been calculated by mathematical modeling in the European Union (EU) and Switzerland, estimating 0.53 µg/L (EU) and 0.67 µg/L (Switzerland) for nano-TiO_2 and 0.66 µg/L (EU) and 0.45 ng/L (Switzerland) for nano-Ag (SUN et al., 2014).

One of the main difficulties of ecotoxicology in predicting NMs effects in organisms is the lack of data about environmental concentration of them. For this reason, other ecological approaches can be used to determinate the hazard of NMs. The conventional fish tests are still the most common way in ecotoxicological studies, and a little mode of action (MoA) data are generated. In contrast, alternatives methods as *in vitro* tests with cells fish have been used to ecotoxicogical approaches with the greatest benefit to mechanistic studies (LILLICRAP et al., 2016). Moreover, the use of *in vitro* test as alternative method promotes the reduction of whole-fish in

experiments, following the 3Rs' principle (Reduction, Refinement, and Replacement), an widespread goal of both scientists and the population.

In ECVAM Workshop 47, Castaño et al. (2003) reported the use of fish cells to ecotoxicological approaches. The authors commented that the focus of ecotoxicology is the effects evaluation of chemical both population and ecosystem and *in vitro* studies with animal cells provide information in supraindividual and ecological levels. They mentioned some reasons to use *in vitro* test in ecotoxicology, such as: cells present key features of all living creatures to comprehend unique and common mechanisms of toxicity; they are maintained in controlled environment and for this why the toxic mechanisms in molecular or cellular levels are assessed more easily; their toxicity evaluation is more fast and inexpensive, and at the same time many chemicals can be studied.

In the present study, RTG-2 line cell – established for the first time by Wolf and Quinby (1962) from rainbow trout gonads – was used and evidenced two MoA of NMs in the DNA through modified methods of Comet assay, important data to ecological hazard assessment.

The Comet assay — also called single-cell gel electrophoresis assay — can identify DNA damages and DNA repair through modifications in the method (KOPPEN et al., 2017).

The first published method was the neutral Comet assay that identifies double-strand breaks (OSTLING & JOHANSON 1984). In 1988, Singh and collaborators published the alkaline version, which unwound the DNA helix through alkaline treatment (pH > 13). Thus, several damages are detected by alkaline Comet assay, such as single-strand breaks, alkaline-labile sites, DNA-DNA and DNA-protein crosslinks (FAIRBAIRN et al., 1995, TICE et al., 2000).

The crosslink DNA-DNA or DNA-protein can be confirmed by co-exposition of chemical studied with a known clastogenic agent, as the Methyl methanesulfonate (MMS) (PFUHLER & WOLF 1996). The crosslink agent presents radicals that form a covalent link with DNA or proteins (as the histones or larger proteins). This link prevents that fragments – caused by clastogenic agents – migrate in the gel of the alkaline Comet assay (COSTA, 1991). In relation to the consequences for the cell, the crosslink damage can prevent the separation of the strands to the replication and/or transcription processes (NOLL et al., 2006).

Other modified method is the addiction of endonuclease (FPG, ENDOIII and hOGG1) before the electrophoresis step to identify adducts and nucleotide oxidized that may promote breaks in the DNA strand. The hOGG1, used herein, identify 8-oxo-7,8-dihydroguanine (8-oxoGua), 2,6- diamino-4-hydroxy-5-formamidopyrimidine (FaPyGua), specifically (SMITH et al., 2006).

These methods have validation in the Guideline for the testing of chemicals *in vivo* mammalian alkaline Comet assay of the OECD 489 (2014).

In nanogenotoxicology, the most of the studies report only one MoA of NMs, the oxidative damage in the DNA (VEVERS & JHA, 2008; HUDECOVÁ et al., 2012; RINNA et al., 2015; ARMAND et al., 2016; IGLESIAS et al., 2017). However, since NMs genotoxicity has still conflicting results, the hypothesis that there is other NMs MoA in the DNA requires knowledge.

Therefore, the present study aimed to verify if NMs have other genotoxic MoAs. In order to confirm DNA oxidative and crosslink DNA-DNA (or DNA-protein) damage, standard alkaline Comet assay, hOGG1- and crosslink-modified alkaline versions were applied in the contaminated RTG-2 fish cell by three different NMs: Titanium dioxide nanoparticle (TiO_2NP), Silver nanoparticle (AgNP) and Silver nanosphere PVP coated [AgNS (PVP coated)].

4.3.2 Material and methods

Chemical characterization of the nanomaterial suspensions

The TiO_2NP (supplier information: anatase, nanopowder, < 25 nm particle size, 99.7% trace metals basis), AgNP (supplier information: nanopowder, <100 nm particle size, 99.5% trace metals basis) and AgNS (PVP coated) [supplier information: 20 nm average particle size, 20 µg/mL in water and polyvinylpyrrolidone (PVP) functionalized] were all from Sigma-Aldrich®. The choice of using commercial NMs allows the reproducibility of this study.

The concentration ranges of the tested NMs for the *in vitro* Comet assay were determined based upon cytotoxicity assays, aiming to avoid false genotoxic results due to cytotoxic effects (data not shown). Thus, the final concentration ranges for each tested NMs were as follows: TiO_2NP at 0.1, 1 and 10 µg/mL; AgNP at 0.01, 0.1, 1 and 10 µg/mL and AgNS (PVP coated) at 0.01, 0.1 and 1 µg/mL. Leibovitz's

medium (L-15, Cultilab®) was used as the vehicle to prepare the NMs' test suspensions. TiO_2NP and AgNP were sonicated in the ultrasonic bath (60 Hz) for 30 minutes immediately before the exposure for dispersing particles and avoid aggregation. The AgNS (PVP coated) suspension was not sonicated to not have interference in the PVP coating.

The NM suspensions were analyzed by Zetasizer® Nano Series ZS90 (Malvern Instruments, Worcestershire, UK) — under the same conditions of cell exposure to the tested NMs (in L-15 medium and within of time period 3 hours) — to measure hydrodynamic size, zeta potential (ζ) and polydispersion index (PdI). To arrive at the % of polydispersity (%PD), the following equation was used:

$$\%PD = \sqrt{PdI} \times 100.$$

Cell culture and treatment conditions

RTG-2 cells (obtained from European Collection of Cell Cultures UK, ECACC 90102529) were culture in L-15 medium supplemented with 10% fetal bovine serum (FBS, Gibco®), 2 mM L-glutamine (Gibco®) and 1% Antibiotic-Antimycotic (penicillin, streptomycin and amphotericin B, Gibco®) at 22°C in a B.O.D. incubator. Subcultures were performed when the cells reached ~ 80% of confluence.

RTG-2 cells were seeded into 24-well plates ($5x10^4$ cells/well) and incubated at 22°C for 24 hours. Thereafter, the cells were exposed to the test NMs' suspensions, excepted in the crosslink-modified alkaline version wherein the cells were co-exposed to the NMs with a known-genotoxic or -clastogenic agent (Pfuhler and Wolf, 1996) [Methyl methanesulfonate (MMS) — CAS nº: 66-27-3, Sigma-Aldrich® — at 0.5 mM]. After 3 hours of exposure of RTG-2 to the test articles, the cells were harvest and the pellet obtained was carefully resuspended in the remain supernatant (~ 50 µL). 10 µL of these single cell suspensions were used to Trypan Blue Dye Exclusion Test, in order to verify the cell viability. The remaining cell suspensions were processed for the *in vitro* Comet assay.

Ultrapure water at 10%-v/v final concentration was used as negative control, whereas MMS at 0.5 mM, hydrogen peroxide (H_2O_2) (Sigma-Aldrich®) at 50 µM (on the slide for 10 min) and formaldehyde (FA) — CAS nº: 50-00-0, Vetec® — at 3.3 mM were used as positive controls of standard alkaline Comet assay, hOGG1- and crosslink-modified alkaline versions, respectively.

The experiments were performed using a single well/treatment and repeated three times.

In vitro Comet assay

The standard alkaline Comet assay, hOGG1- and crosslink-modified alkaline versions were carried out according to Tice et al. (2000) and Leme et al. (2014); Reeves et al., (2008) and the manufacturer's protocol of hOGG1 enzyme (New England Biolabs); Pfuhler and Wolf (1996) and OECD 489 (2014), respectively.

The single cell suspensions (50 µL) were resuspended in 120 µL of low-melting point agarose (LMP) [0.5% (w/v) in PBS] and immediately spread on slides that were previously coated with agarose at 1.5% and were placed by coverslips. To agarose solidification, the slides were kept at 4ºC for 5 min and were transferred (without coverslip) into a lysis solution (2.5 M NaCl; 100 mM EDTA; 10 mM Tris-HCl, pH 10; 1% Triton X-100 and 10% DMSO). After 2 hours, the slides were maintained on horizontal electrophoresis system (4ºC) with a buffer solution (200 mM EDTA; 10 M NaOH; pH > 13) for 25 min (DNA denaturation). Then, the electrophoresis runs for 25 minutes at 300 mA, 1 $V.cm^{-1}$. Afterward, the slides were neutralized with 4.85% Tris-HCl buffer (pH 7.5) for 20 minutes and dehydrated in 100% ethanol.

With respect to oxidative damage detection, the hOGG1-modified alkaline version was conducted. After lysis, an additional step was carried out, wherein the comet slides were washed — 3 times for 5 minutes — with enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 Mm EDTA, 0.2 mg/mL BSA, pH 8). Then, in a moistened chamber, incubated slides with hOGG1 (0.08 U/slide, Biolabs®, New England) were kept 30 minutes at 37°C. The slides were immersed in distilled water and placed into electrophoresis thereafter, as above described method.

There were not additional steps to the crosslink-modified alkaline version slides.

Ethidium bromide solution (20 µg/mL, Sigma-Aldrich®) was used to stain the slides and then examined under a fluorescence microscope (Axio Imager Z2, Carl Zeiss, Jena, DE), equipped with Metafer 4 / VSlide automated capture software (Metasystems, Altlussheim, DE) and model of the Camera: CoolCube 1 – Metasystems. DNA lesions were quantified as DNA tail intensity (% DNA in Tail)

(KUMARAVEL et al., 2009) using the computer-based image analysis Metafer CometScan v.2.8.0® (Metasystems, Germany) on 100 randomly selected cells.

Statistical analysis

The percentage of difference in relation to controls and the Tail Intensity were expressed as median with interquartile range. Relative values (%) were calculated through the difference of treatments in relation to the median of the specific control of each alkaline Comet assay versions (negative control without contamination, control with hOGG1 and clastogenic agent to: standard, hOGG1- and crosslink-modified comet assay, respectively). NMs types were compared with Kruskal-Wallis test thereafter. The Mann-Whitney test was applied to compare the Tail Intensity of each treatment in relation to the specific control in the alkaline Comet assay versions. The statistical level was set at $p < 0.05$.

4.3.3 Results

The DNA oxidation (8-oxo-gua and FaPyGua) and the crosslinking DNA-DNA or DNA-protein MoA were confirmed through hOGG1- and crosslink-modified alkaline Comet assay versions, respectively.

The analysis of zeta potential and polydispersity indicated instability in all nanomaterials in the culture medium of the cell, in general $-30\ mV < \zeta < +30\ mV$ and % PD > 20%. Consequently, a size distribution of particles was very diverse (Table 1). TiO_2, AgNP and AgNS (PVP coated) had a size range (particle diameter) of 5.92 nm to 316.90 nm; 7.40 nm to 217.90 nm; and 9.25 nm to 4,675.00 nm; respectively (see distribution in the **Table 1**).

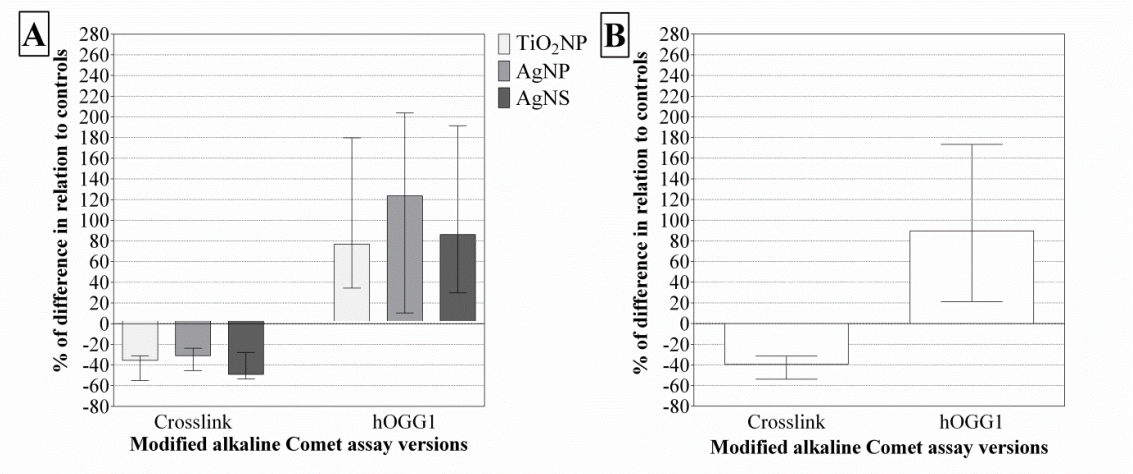
Table 1 – Characterization of the nanomaterials' (NMs) suspensions in relation to: distribution of particle size (diameter in nm) for intensity (%); Z-average size (diameter in nm); Zeta potential (mV); and % of polydispersity.

NMs	Suspension (µg/mL)	Distribution		Z-average size (d.nm)	Zeta potential (mV)	Polydispersity (%)
		Size (d.nm)	Intensity (%)			
TiO_2NP	Stock - 50	474.10	100.0	785.8	-4.42	82.04
	0.1	13.72 58.32 316.90	55.0 31.6 8.4	21.22	-1.03	66.60
	1	14.60 100.30 5.92	42.1 39.5 18.4	504.8	-7.82	93.86
	10	299.80 12.37	84.7 15.3	816.6	-6.70	93.86
AgNP	Stock-100	258.10 5331.00	96.5 3.5	247.30	-21.20	65.65
	0.01	45.94 10.35 269.60	47.1 43.1 6.4	18.58	-0.07	72.80
	0.1	11.40 42.62 144.80	51.7 36.3 12.0	49.44	-4.93	41.11
	1	212.70 34.86 9.15	36.3 33.4 30.3	103.70	-66.60	53.57
	10	217.90 21.44 7.40	87.8 7.8 4.4	249.00	-0.99	66.85
AgNS	Stock-20	104.80 14.80	91.8 8.2	74.94	-5.53	65.88
	0.01	10.68 50.88 206.50	44.1 42.9 9.9	23.67	-6.56	62.27
	0.1	35.96 196.50 9.25	36.3 32.1 29.1	35.77	-7.46	66.93
	1	99.29 12.55 4,675.00	59.9 36.4 3.7	24.10	-7.46	91.54

The values of each suspension were measure on Zetasizer® Nano Series ZS90 (Malvern Instruments, Worcestershire, UK) in the same exposure conditions. Suspensions: Titanium dioxide nanoparticle (TiO_2NP) at 50 µg/mL in L-15 culture medium (Stock suspension) and at 0.1, 1 and 10 µg/mL exposure concentrations; Silver nanoparticle (AgNP) at 100 µg/mL in water (Stock suspension) and at 0.01, 0.1, 1 and 10 µg/mL exposure concentrations; and Silver nanosphere polyvinylpyrrolidone (PVP) coated (AgNS) at 20 µg/mL in water (Stock suspension) and at 0.01, 0.1 and 1 µg/mL exposure concentrations.

With respect to the aforementioned MoAs, the Tail Intensity differences of NMs treatments in relation to negative control (with hOGG1 enzyme) in the hOGG1-modified alkaline Comet assay version were increased in 74.35% (31.87% – 177.30%), 121.1% (7.80% – 201.20%) and 83.58% (24.22% – 188.90%) [median% (first% – third% quartiles)] for TiO_2NP, AgNP and AgNS (PVP coated), respectively. In contrast, the Tail Intensity differences of NMs treatments in relation to clastogenic agent (MMS) in the crosslink-modified alkaline Comet assay version were decreased in 38.05% (57.53% – 33.80%), 33.49% (47.85% – 26.30%) and 51.59% (56.15% – 30.31%) for TiO_2NP, AgNP and AgNS (PVP coated), respectively. Whereas the NMs types showed no difference between them (**Figure 17A**), the joined NMs data demonstrated an increase of 89.71% (21.25% – 173.30%) in the hOGG1-modified alkaline Comet assay version, while the crosslink-modified alkaline Comet assay version caused a decrease of 39.39% (31.48% – 53.75%) of the Tail Intensity in relation to clastogenic agent (MMS) (**Figure 17B**). Cell viability was greater than 80-90% for all treatments by Trypan Blue Dye Exclusion Test (data not shown).

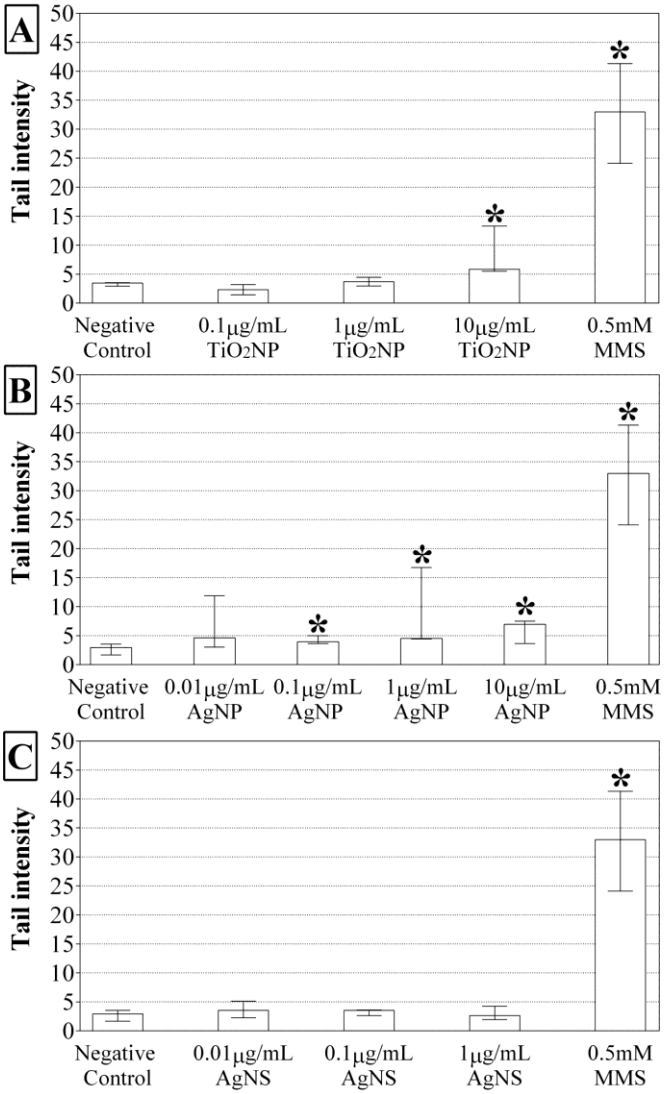
Figure 17 – Percentage of damage difference of the treatment in relation to control of crosslink-modified alkaline Comet assay (control was a clastogenic agent - Methyl methanesulfonate) and hOGG1-modified alkaline Comet assay (negative control without contamination and treated with hOGG1 enzyme). [A] % of difference in relation to control of each nanomaterial (NMs) in each method: Titanium dioxide nanoparticle (TiO_2NP); Silver nanoparticle (AgNP) and Silver nanosphere polyvinylpyrrolidone (PVP) coated (AgNS). In each NMs the concentration was joined to represent the relative values in order to compare the NMs types. There was no difference among NMs ($p < 0.05$ statistical level, Kruskal-Wallis test). [B] Descriptive graphical of the % of difference of all NMs treatments joined in relation to respective controls showing the damage decreased in crosslink-modified alkaline Comet assay and increased in hOGG1-modified alkaline Comet assay. The y-axis shows the median (%) with interquartile ranges in both graphical.



Both hOGG1- and crosslink-modified alkaline Comet assay could confirm the NMs genotoxicity, since false negative results of the standard alkaline Comet assay

were found, wherein TiO_2NP (**Figure 18**A) caused an increase of Tail Intensity only in the 10 µg/mL concentration; AgNP (**Figure 18**B) in the 0.1, 1 and 10 µg/mL; and AgNS (PVP coated) (PVP coated) (**Figure 18**C) treatments did not show difference in relation to the negative control.

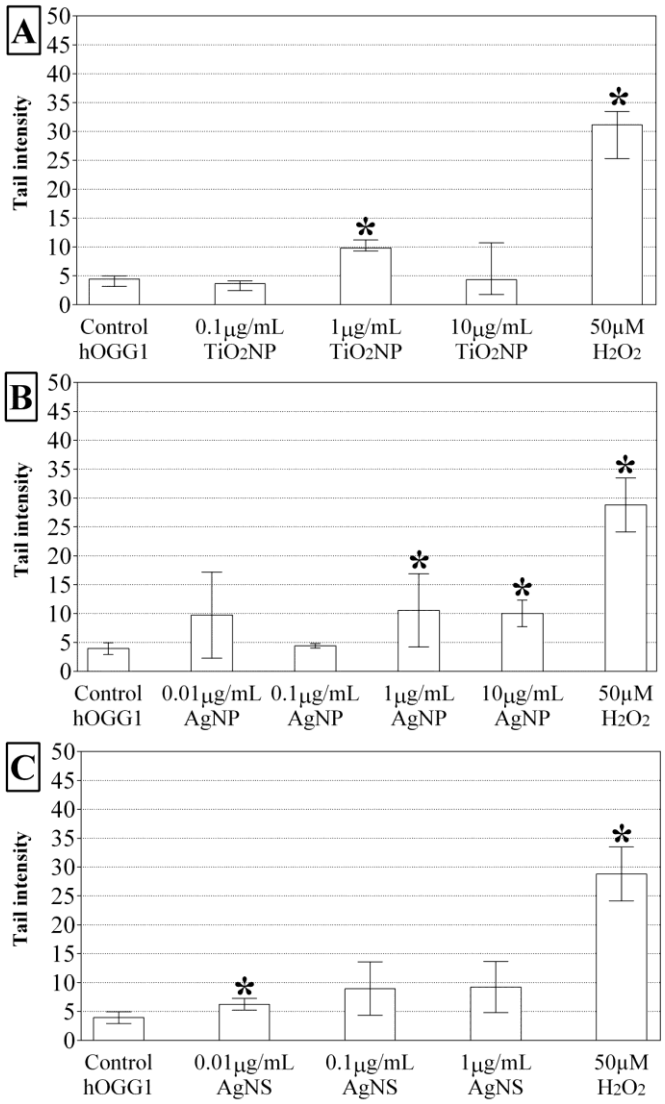
Figure 18 – Standard alkaline Comet assay in RTG-2 cells exposed to three different nanomaterials (NMs). [A] Titanium dioxide nanoparticle (TiO_2NP) at 0.1, 1 and 10 µg/mL; [B] Silver nanoparticle (AgNP) at 0.01, 0.1, 1 and 10 µg/; and [C] Silver nanosphere polyvinylpyrrolidone (PVP) coated (AgNS) at 0.01, 0.1 and 1 µg/mL. The y-axis shows the median with interquartile ranges of the Tail Intensity (% of DNA in the tail). *indicate the statistical difference related to control (without contamination), $p < 0.05$ statistical level, Mann-Whitney test. MMS: Methyl methanesulfonate at 0.05mM – positive control.



The results obtained into hOGG1-modified alkaline Comet assay version pointed out that the DNA oxidation by tested NMs was induced, as observed by the increased levels of Tail Intensity compared to negative control (with hOGG1 enzyme). Significant levels of Tail Intensity were observed for TiO_2NP at 1 µg/mL

(**Figure 19A**); AgNP (**Figure 19B**) at 1 and 10 µg/mL; and AgNS (PVP coated) (**Figure 19C**) at 0.01 µg/mL.

Figure 19 – Oxidative alkaline Comet assay version with hOGG1 enzyme in RTG-2 cells exposed to three different nanomaterials (NMs). [A] Titanium dioxide nanoparticle (TiO_2NP) at 0.1, 1 and 10 µg/mL; [B] Silver nanoparticle (AgNP) at 0.01, 0.1, 1 and 10 µg/; and [C] Silver nanosphere polyvinylpyrrolidone (PVP) coated (AgNS) at 0.01, 0.1 and 1 µg/mL. The y-axis shows the median with interquartile ranges of the Tail Intensity (% of DNA in the tail). *indicate the statistical difference related to control (with hOGG1), $p < 0.05$ statistical level, Mann-Whitney test. H_2O_2: Hydrogen peroxide 50 µM – positive control



Method validation of the crosslink-modified Comet assay alkaline version with co-exposure between known-crosslinking (FA) and -clastogenic agent (MMS) was conducted. FA decreased the Tail Intensity of the MMS at 330, 1000, 3300 and 4000 µmol/mL (**Figure 20**). Then, the crosslink-modified Comet assay alkaline version shown that all NMs [TiO_2NP (**Figure 21A**), AgNP (**Figure 21B**) and AgNS (PVP coated) (**Figure 21C**)] and in all tested concentrations were able to reduce the

migration of clastogenic agent DNA fragments (decreased Tail Intensity related to MMS treatment alone), indicating the crosslinking (DNA-DNA or DNA-protein) — only the AgNP (**Figure 21B**) at 0.1 µg/mL did not show difference in relation to control, due to the high variance among experiment repetitions.

Figure 20 – Method validation of crosslink alkaline Comet assay version in RTG-2 cells co-exposed to known-crosslinker Formaldehyde (FA) and known-clastogenic agent [Methyl methanesulfonate (MMS)]. FA + MMS: co-exposure of Formaldehyde 100, 330, 1000, 3300 and 4000 µmol/mL and Methyl methanesulfonate (MMS) at 0.5 mM. The y-axis shows the median with interquartile ranges of the Tail Intensity (% of DNA in the tail). *indicate the statistical difference related to clastogenic agent alone [Methyl methanesulfonate (MMS) at 0.5 mM], $p < 0.05$ statistical level, Mann-Whitney test.

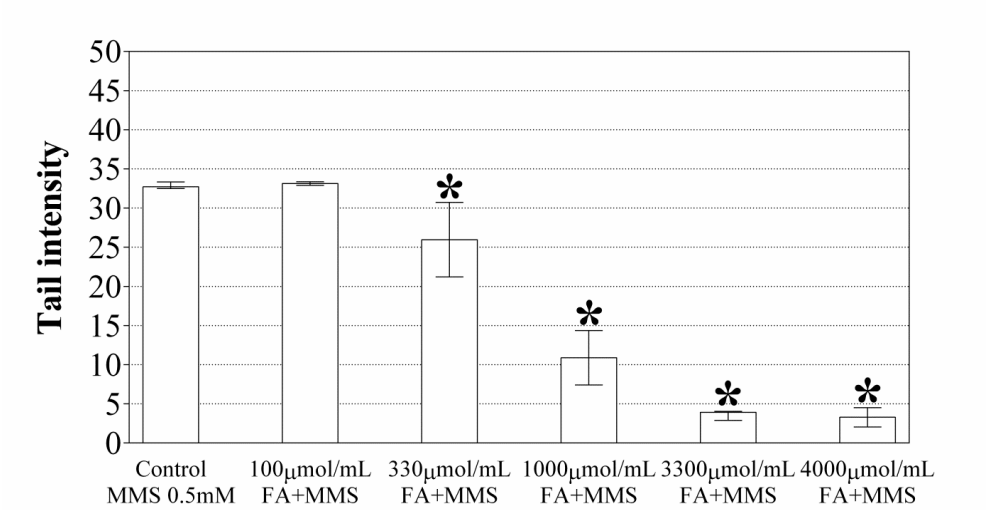
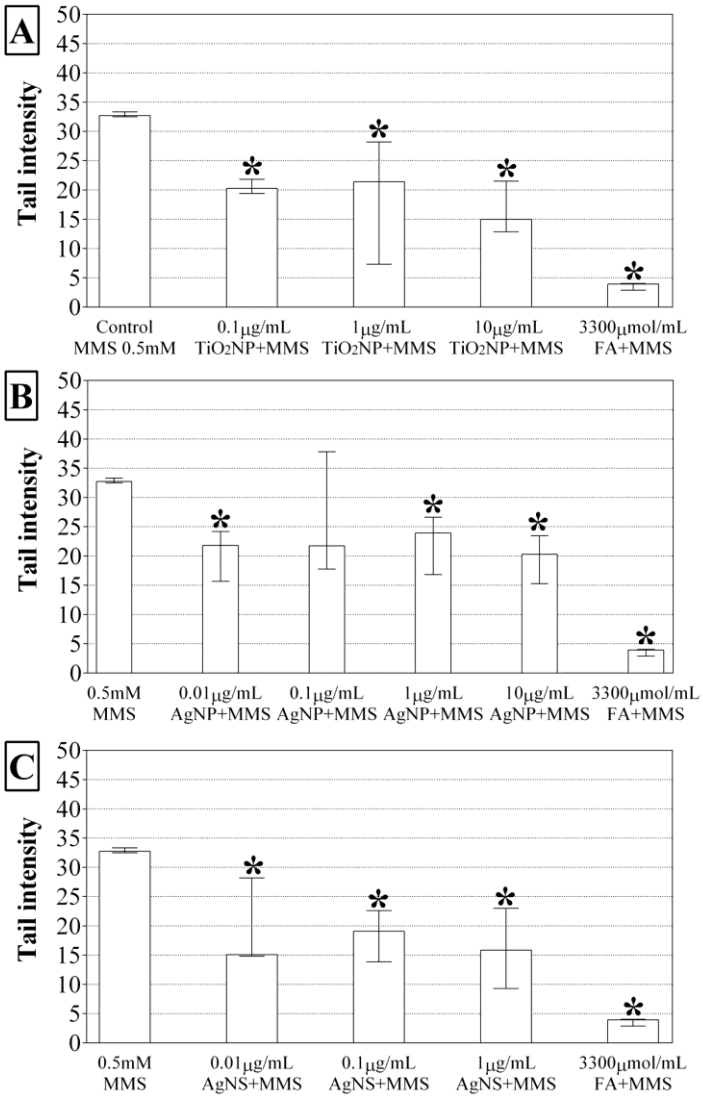


Figure 21 – Crosslink alkaline Comet assay version co-exposed to three different nanomaterials (NMs) and known-clastogenic agent [Methyl methanesulfonate (MMS)]. [A] Co-exposure of Titanium dioxide nanoparticle (TiO_2NP) at 0.1, 1 and 10 µg/mL and Methyl methanesulfonate (MMS) at 0.5 mM; [B] Co-exposure of Silver nanoparticle (AgNP) at 0.01, 0.1, 1 and 10 µg/mL and Methyl methanesulfonate (MMS) at 0.5 mM; and [C] Co-exposure of Silver nanosphere polyvinylpyrrolidone (PVP) coated (AgNS) at 0.01, 0.1 and 1 µg/mL and Methyl methanesulfonate (MMS) at 0.5 mM. The y-axis shows the median with interquartile ranges of the Tail Intensity (% of DNA in the tail). *indicate the statistical difference related to clastogenic agent alone [Methyl methanesulfonate (MMS) at 0.5 mM], $p < 0.05$ statistical level, Mann-Whitney test. FA + MMS: co-exposure of Formaldehyde 3300 µmol/mL and Methyl methanesulfonate (MMS) at 0.5 mM – positive control.



4.3.4 Discussion

The main goal of this study was to elucidate the genotoxic MoA of the most used NMs in industries, such as the TiO_2- and Ag-NMs. The findings obtained from the standard alkaline Comet assay was not enough to estimate their genotoxicity,

due to the tested NMs showed both an indirectly (oxidative damage) and a directly (crosslinking DNA-DNA or DNA-protein) genotoxic actions in the DNA, which may not caused strand-break as primary effects, observing the other ones by the different Comet assay protocols [hOGG1-modified alkaline version (REEVES et al., 2008) and crosslink-modified alkaline version (PFUHLER & WOLF 1996). Thus, the standard alkaline Comet assay version — which is commonly used to assessment into hazard test batteries of potential genotoxicant — can lead to false-negative results in the hazard estimation of NMs.

There are many pathways in which NMs can disrupt the plasmic membrane for further being uptake by the cell and therefore reaching the cytosol. In non-phagocytic cells, such as gonadal cells, the main pathways are pinocytosis or direct penetration. NMs or NMs aggregates smaller than 120 nm can release clathrin-mediated, caveolin-mediated or clathrin/caveolin independent endocytose. Duo to the heterogeneity of particles size, they may be targeting the cytosol by pathways diversified (ZHU et al., 2012). Furthermore, the NMs internalization into cell nucleus is also possible since the vertebrates nuclear pore or nuclear pore complex (NPC) present about 80 to 120 nm in diameter and its proteins modulate the molecules input or output into nucleus (ALBER et al., 2007).

In the present study, NMs suspended into culture medium with great size heterogeneity particles were found, as 5.92–316.90 nm, 7.40–217.90 nm, 9.25–4,675.00 nm to TiO_2NP, AgNP and AgNS (PVP coated), respectively. In other words, particles smaller than 80 nm and bigger than 120 nm into the culture medium smaller were confirmed. Once the oxidative DNA damage happened, it might be expected that the NMs were internalized for RTG-2 cells, because is necessary the generation of reactive oxygen species into cytosol for targeting the DNA and thus causing lesions. In relation to the crosslinking DNA damage (*i.e.* DNA-DNA or DNA-protein), it might be expected that they target the nucleus of the RTG-2. Additionally, it is known that biomolecules (such as proteins) can be adsorbed at nanoparticle surface in a biological environment, resulting in a nanoparticle-corona complex. The biomolecular nanoparticle-corona can interact with the cell machinery promoting uptake facilitated of them and disrupting cell homeostasis (MONOPOLI et al., 2012).

The comprehension about NMs' toxicokinetic has been a great challenge, due to their many variables in physicochemical properties. Furthermore, the methodologies that have been suggested to confirm the uptake of NMs demand a

long study time and their costs are very high (ZHU et al., 2012). Thus, herein these the genotoxic biomarker alterations and the characterization analysis – showing very small NM sizes – may be evidenced the internalization of TiO_2NP, AgNP and AgNS (PVP coated) into RTG-2 cells and their nuclei.

Many studies report the oxidative DNA damage by NMs and most of them are in human cells with FPG modified Comet assay (PETKOVIĆ et al., 2011; KERMANIZADEH et al., 2012; KRUSZEWSKI et al., 2013; HUK et al., 2014; VALES et al., 2015; ARMAND et al., 2016; IGLESIAS et al., 2017). The method using FGP endonuclease does not elucidate the MoA specifically, because it identify many damages, as 8-oxo-7,8-dihydroguanine (8-oxoGua), 2,6- diamino-4-hydroxy-5-formamidopyrimidine (FaPyGua) and 4,6-diamino-5-formamidopyrimidine (FaPyAde), AP sites and ring-opened N7 guanine adducts (KARAKAYA et al., 1997, SPEIT et al., 2004, DUSINSKA & COLLINS 2008). In contrast, the method with hOGG1 identifies only 8-oxoGua and FaPyGua (SMITH et al., 2006). Hence, we can confirm that the TiO_2NP, AgNP and AgNS (PVP coated) (PVP coated) of our study promoted the alterations in the DNA through nitrogenous bases oxidation, modifying guanine in 8-oxoGua or FaPyGua. Few studies about nanomaterial genotoxicity have investigated the oxidative DNA damage through hOGG1 endonuclease method. The hOGG1-modified alkaline Comet assay to identify oxidative DNA damage by NMs was only reported to human cells. Both Hudecová et al. (2012) and Rinna et al. (2015) studies demonstrated oxidative DNA damage in 1, 25 and 100µg/ml after 30 minutes of exposition of Silver nanoparticles in human embryonic kidney (HEK) 293 and human epithelial embryonic cell (EUE) lines, respectively. With fish cells, our study is the first to use the hOGG1-modified alkaline Comet assay and to demonstrate the oxidative DNA damage, 8-oxoGua and FaPyGua specifically, in lower concentrations than studies with human cells aforementioned.

The present study is so far the first to report other genotoxic MoA of NMs, the crosslink DNA-DNA or DNA-protein. In standard alkaline Comet assay, the crosslink is detected for the migration decrease of the DNA fragments in relation to negative control (TICE et al., 2000). The migration decreased happens with the DNA-DNA interstrand crosslink, producing fragments with double-strand DNA (KLAUDE et al., 1996); with the DNA-DNA intrastrand crosslink and with DNA-proteins crosslink (as the histones or larger proteins) that both increase the size of the break fragments (MERK & SPEIT 1999).

In the work of the Pfuhler and Wolf (1996) was tested different crosslinkers as mitomycin C, cisplatinum, formaldehyde, dimethylol urea and diazolidinyl urea. Firstly they performed the standard Comet assay with the isolated substances, and all did not present a high damage in relation to clastogenic agent known (MMS) alone. However, in the next step, when they tested the co-exposition of the substances with the clastogenic agent, all they decreased the tail moment. In other words, the chemicals made crosslink with the DNA fragments produced by the clastogenic agent and the size of them increased and the migration was reduced in the gel. Some of these substances are crosslinkers known, as the formaldehyde (DNA-protein crosslink), the cisplatin (DNA-DNA intrastrand crosslink) and mitomycin C (DNA-DNA interstrand crosslinks) (MERK & SPEIT, 1999).

In the present work, the NMs were assessed using the same methodology of co-exposition with a clastogenic agent (MMS). All the NMs promoted the crosslink and decreased the Tail Intensity in relation to the clastogenic agent treatment alone. However, concentration-dependent decrease was not observed for any of tested NMs. All the concentrations had the same response, as well as the comparison among TiO_2NP, AgNP and AgNS (PVP coated). It may means that NMs seem to have behaved similarly and caused damage in any concentration probably due to a wide particle size range distribution found. Chemicals can cause different DNA lesions at the same time, as demonstrated by Hartmann co-worker (1995) in the genotoxicity assessment of cyclophosphamide using the alkaline Comet assay and sister chromatid exchange test. According to the authors, in order to obtain a positive result in the Comet assay, the cells had to be exposed to a concentration 100 times higher than the one used in the sister chromatid exchange test, since the cyclophosphamide is a crosslinker that could prevented the migration of DNA fragments in the alkaline Comet assay.

4.3.5 Conclusion

The present study showed that NMs can be able to cause oxidation and crosslinking in the DNA, the latter being the first report of other mode of action. Both nano-TiO_2 and -Ag had the same behavior in relation to DNA damage, also no difference among concentrations, showing a difficulty in the finding of responses dependent of the concentration. Unpredictability in NMs behavior may be due to the

fact of particle agglomeration in suspension. However, if the agglomerates have a size range up to 120 nm, the particle internalization in both cytoplasm and nucleus is possible, lead to genotoxic effects. In addition, our study demonstrated that the use only of standard Comet assay is not sufficient to detect the accurate genotoxicity of NMs. This show that they are not safe, and, probably, there are many studies with false-negative results indicating that NMs are not hazardous substances. Therefore, the variations in the Comet assay methods — as hOGG1- and crosslinking-modified versions shown herein — are very efficient to detect the mode of actions and the hazard of NMs.

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4 CONSIDERAÇÕES FINAIS

A combinação dos ensaios *in vivo* com a espécie de peixe *H. intermedius* e dos ensaios *in vitro* com a linhagem de células de peixe RTG-2 confirmou o perigo dos nanomateriais no ambiente aquático, uma vez que em baixas concentrações causaram efeitos tóxicos.

Foi possível observar que as nanoesferas de prata podem causar danos sistêmicos em peixes, sendo principalmente neurotóxicas. Com exposição via trófica, observou-se que podem atingir o sistema nervoso, causando alterações no sistema visual e danos ao DNA de células do cérebro. Além disso, causaram efeitos tecido específico, tanto após 1 administração, sendo genotóxicas em células do fígado, do rim e do cérebro, quanto após 10 administrações, com predominância de genotoxicidade em células do rim e do cérebro. E o estresse oxidativo, efeito que comumente é referido na literatura, foi detectado em células do fígado.

Com maior importância, o resultado do ensaio Cometa alcalino detectou o dano do tipo *crosslink* DNA-DNA ou DNA proteína nas células dos tecidos analisados de *H. intermedius*, com a observação da diminuição do escore em relação ao controle negativo. Este tipo de dano foi confirmado nos ensaios *in vitro* com a linhagem de células de peixe RTG-2. Estas foram expostas a três nanomateriais diferentes — nanopartículas de dióxido de titânio, nanopartículas de prata e a nanoesfera de prata — que causaram tanto o *crosslink* DNA-DNA ou DNA proteína como também a oxidação do DNA, mecanismos estes, identificados por meio de modificações na metodologia do ensaio Cometa.

Por fim, este trabalho tem importância para estudos futuros na área de nanotoxicologia, uma vez que demonstrou tanto os efeitos sistêmicos de um nanomaterial em um organismo representante do ambiente aquático, quanto os mecanismos de ação de nanomateriais no DNA. A partir disso, outros métodos podem ser aplicados objetivando elucidar com maior precisão esses efeitos e mecanismos de ação encontrados, além da possibilidade de aplicar a mesma metodologia para o estudo de outros nanomateriais.

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