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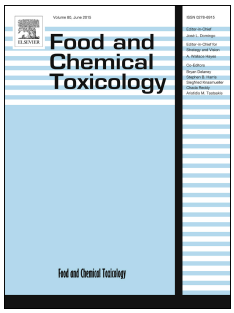

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Oral exposure to silver nanoparticles increases oxidative stress markers in the liver of male rats and deregulates the insulin signalling pathway and p53 and cleaved caspase 3 protein expression

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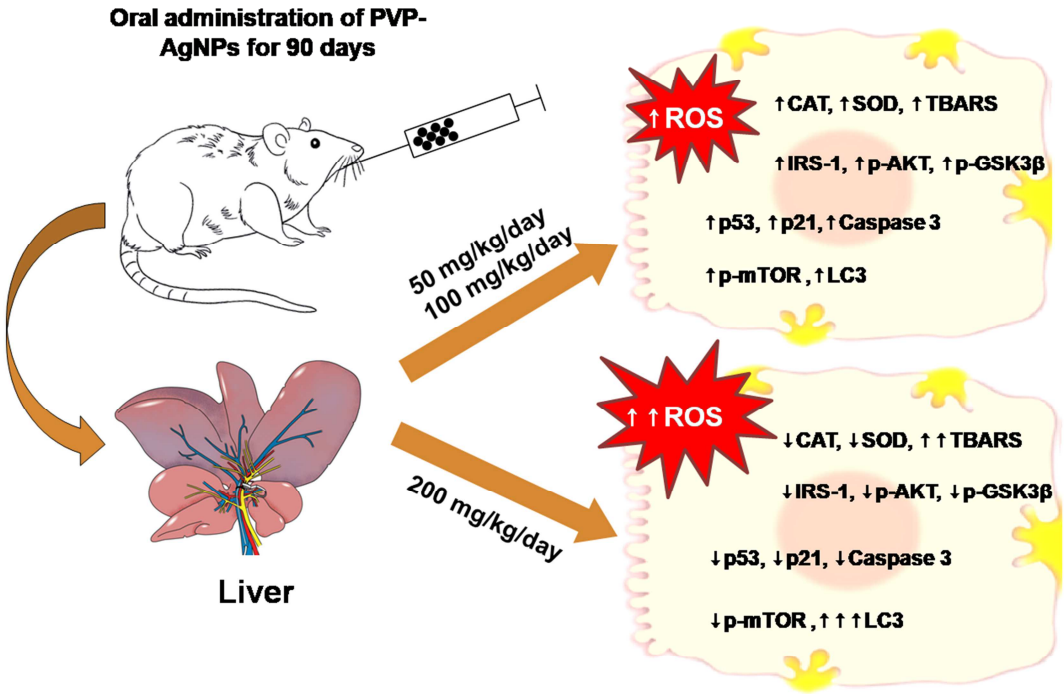

Oral exposure to silver nanoparticles increases oxidative stress markers in the liver of male rats and deregulates the insulin signalling pathway and p53 and cleaved caspase 3 protein expression

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ABSTRACT

The present study was aimed at assessing the impact of AgNPs on the liver of male rats orally exposed to 0, 50, 100 and 200 mg/kg/day of polyvinyl pyrrolidone coated AgNPs (PVP-AgNPs) for 90 days.The induction of apoptotic cell death -by measuring the protein levels of the active form of caspase 3- and the levels of the microtubule-associated protein 1A/1B-light chain (LC3) protein were measured as a marker of the induction of autophagy. PVP-AgNPs caused an increase of the activity of superoxide dismutase (SOD) and catalase (CAT) in the liver of male rats. However, the activity decreased after exposure to high amounts of PVP-AgNPs. Increased protein levels of IRS-1, AKT, GSK3β and mTOR proteins were observed in a dose-dependent manner. However, these proteins showed a decrease at 200 mg/kg/day. The same pattern was observed for the p53, p21 and cleaved caspase 3 protein levels. The current results suggest that the increase of ROS production by PVP-AgNPs stimulated SOD and CAT activity, as well as IRS-1, AKT, mTOR, p53, p21 and caspase 3 as protective mechanisms of cell survival and preserve DNA fidelity. However, cellular damage by excessive ROS production might induce the depletion of these survival mechanisms at 200 mg/kg/day.

Keywords

Silver nanoparticles, insulin pathway, authophagy, oxidative stress

1. Introduction

Nanotechnology is part of the so-called “fourth industrial revolution”. During the last two decades, this multidisciplinary field has been essential to the progress of industry, giving new proprieties to existing materials (Piperigkon et al., 2016; Seal and Karn, 2014). Among the different commercial nanomaterials, silver nanoparticles (AgNPs) are the most used in industrial applications (Austin et al., 2014; Pinzaru et al., 2018). Inclusion of AgNPs into materials confers antifungal and antimicrobial proprieties (Maillard and Hartemman, 2012). These advantages are useful for improving technologies used in agricultural (biopesticides, food preservation) and biomedical (wound dressings, catheters, dental materials, bone cement, drugs) areas, among others (Duhan et al., 2017; Jacob et al., 2017; Kuskov et al., 2016; Mei et al., 2017; Shishatskaya et al., 2016; Sussman et al., 2015; Zhang et al., 2016). As evidence of this, many different day-to-day consumer products that contain AgNPs contribute to enhancing our quality of life, such as textiles, food packaging, cosmetics, water purifiers, paints, and electronics (Jiravova et al., 2016; McGillicuddy et al., 2017). Despite this, there is a growing concern about AgNPs, because the consequences to human health remain still unknown.

The increased production and use of AgNPs are leading to the increased human exposure to these nanomaterials. For example, recent studies have demonstrated that AgNPs can migrate from packaging into food under several usage conditions (Choi et al., 2018; Mackevica et al., 2016). Moreover, AgNPs released from urban and industrial effluents enter into aquatic ecosystems, and these nanoparticles can accumulate along trophic chains (Gambardella et al., 2015; McGillicuddy et al., 2017). Thus, the ingestion of AgNPs may be the most relevant route of human exposure, with inhalation and dermal absorption likely also making a significant contribution.

In vitro and in vivo studies showed that AgNPs can form aggregates that stimulate cellular endocytosis. Once inside, AgNPs stimulate the autophagic process by accumulating in autophagosomes, being partially dissolved into silver ions (Ag+) by autophagosome-lysosome

fusion. Disruption of the lysosomal membrane integrity occurred, which triggers the permeabilization of lysosomal membranes and the cytosolic release of Ag+ and AgNPs (Mishra et al., 2018; Stern et al., 2012). Upon release, these can directly interact with macromolecules and cell organelles, thus disturbing proper cell function. AgNPs interact with mitochondria, thereby blocking the respiratory electron transport chain by inhibiting respiratory chain enzymes (Bressant et al., 2013; Costa et al., 2017). As result of this, there are increased levels of radical oxygen species (ROS) and oxidative cell damage, causing genotoxicity and depleting ATP levels (AshaRani et al., 2009; Huang et al., 2014). It stimulates the activation of apoptotic or non-apoptotic pathways, finally leading to cell death (Brkić Ahmed et al., 2017; Ergin et al., 2017; Kumar et al., 2015; Lee et al., 2014).

In view of these biological actions, evaluation of the health effects of AgNPs must be investigated. Recently, we assessed the potential toxic effects of an in vivo oral subchronic exposure to polyvinyl pyrrolidone coated AgNPs (PVP-AgNPs) in adult male rats (Garcia et al., 2016; Lafuente et al., 2016). After 90 days of treatment, animals showed an increase of sperm morphology abnormalities, an accumulation of PVP-AgNPs within hepatic and ileum cells, a higher number of binucleated hepatocytes, and an increased number of immunoreactive cells in the liver.

The present study was aimed at assessing the impact of AgNPs on the liver of male rats from a molecular perspective, after oral administration for 90 days. The activity of two important antioxidant enzymes, catalase (CAT) and superoxide dismutase (SOD), was determined. The level of thiobarbituric acid reactive substances (TBARS) was also measured to evaluate the lipid peroxidation. Disruption of the insulin signalling pathway was also assessed by measuring the protein levels of insulin receptor substrate 1 (IRS-1), protein kinase B (AKT), glycogen synthase kinase-3 beta (GSK3β) and the mammalian target of rapamycin (mTOR). In turn, the levels of p53 and p21 proteins were measured because of their roles in DNA damage accumulation and modulation of the cell cycle. We also assessed the induction of apoptotic cell death by measuring the protein levels of the active form of caspase 3. Finally, the levels of the

microtubule-associated protein 1A/1B-light chain (LC3) protein were also evaluated as a marker of the induction of autophagy.

2. Materials and methods

2.1. Nanoparticle preparation

PVP-AgNPs (0.2 wt % PVP; SkySpring Nanomaterials, Inc., Houston, USA), with an average size of 20-30 nm were obtained as a dry powder (Ag, 99.95%, PVP coated). PVP-AgNPs were resuspended in 0.9% saline and administered at concentrations of 0, 50, 100 or 200 mg AgNPs/kg/day. The main criteria for the selection of these doses was based on previous studies by Kim et al. (2010). PVP-AgNPs were dispersed by sonication on ice for 30 min at 35-40 W. The nanoparticle solutions were freshly prepared each day immediately before treatment.

2.2. Animals

Adult male Sprague Dawley rats (262 ± 17.70 g) were purchased from Charles River (SantGermain-L'Arbresle, France). Animals were housed in a room equipped with automatic light cycles (12-h light/dark) and maintained at 22 ± 2 °C and 40%-60% humidity. Food (Panlab rodent chow, Barcelona, Spain) and tap water were offered ad libitum throughout the study. The experiment was approved by the Ethics Committee of Animals Research, Universitat Rovira i Virgili (Tarragona, Spain).

2.3. Experimental design

After 10 acclimatization days, rats were weighed and randomly divided into four different groups (n = 6 per group). Each experimental group received 0 (control group), 50 (low treated group), 100 (medium treated group) or 200 (high treated group) mg/kg/day of PVP-AgNPs. To evaluate the toxicological effects of PVP-AgNPs, animals were treated daily by gavage for 90 days at a dose-volume of 4 mL/kg body weight with either vehicle (0.9% saline) or the specific dose of PVP-AgNP. At the end of the experimental period, animals were weighed and anaesthetized by an intraperitoneal injection of 75 mg/kg ketamine and 0.5 mg/kg medetomidine. Livers of the animals were aseptically excised, frozen in liquid nitrogen, and stored at −80°C for analysis.

2.4. Characterization of the nanoparticle suspension

The morphological characteristics of the PVP-AgNPs were analysedwith a JEOL JEM-1011 (JEOL, Tokyo, Japan) transmission electron microscope (TEM), operating at an acceleration range of voltages of 100-800 kV. The morphology of the PVP-AgNPs was analysed using carbon film-coated Cu grids in contact with a droplet of 4 mg/mL of PVP-AgNPs resuspended in 0.5% aqueous carboxymethylcellulose (Sigma Aldrich, San Louis, MO, USA). To reduce the risk of possible artefact formation, all samples for TEM evaluation were prepared and analysed on the same day in which the grids were prepared. The size of 200 particles was analysed with a particle analysis tool to establish size distributions using the ImageJ software (Version 1.48). Results of morphological characteristics of the PVP-AgNPs were previously reported (Garcia et al., 2016).

2.5. Oxidative stress markers

A fraction of the male rat liver was used to assess the activity of the antioxidant enzymes SOD and CAT, the level of TBARS, and the protein content. Tissue samples were thawed and

homogenized in 0.2M sodium phosphate buffer (pH 6.25, 1:20 wt/vol) in a Potter-Elvehjem homogenizer fitted with a Teflon pestle (Braun, Melsungen, Germany). The supernatant was collected after centrifugation at 105000 × g for 1 h and used for biochemical analyses. The protein content was measured using the Bradford spectrophotometric method (Sigma Chemical Co, St Louis, MO, USA), with bovine serum albumin as a standard (Merck, Darmstadt, Germany). The activities of SOD and CAT were determined according to Mulero et al. (2006), and the total TBARS level was determined according to Zupan et al. (2008).

2.6. Western blot analysis

Aliquots containing 30 µg of protein per sample of liver lysate were analysed by Western blot analysis. Briefly, samples were placed in sample buffer (0.5M Tris-HCl pH 6.8, 10% glycerol, 2% [wt/vol] SDS, 5% [vol/vol] 2-β-mercaptoethanol, 0.05% bromophenol blue) and denatured by boiling at 95°C–100°C for 5min. Samples were then separated by electrophoresis on 10% acrylamide gels. Proteins were subsequently transferred to Immobilon-P PVDF sheets (Millipore Corp, Bedford, Massachusetts) using a transblot apparatus (Bio-Rad, Madrid, Spain). The membranes were blocked for 1 h with 5% non-fat milk dissolved in TBS-T buffer (50mM Tris, 1.5% NaCl, 0.05% Tween 20, pH 7.5). They were then incubated overnight with primary monoclonal antibodies (Abcam, Cambridge, UK) against total IRS-1 (molecular weight [MW]: 180 kDa), IRS-1 phosphorylated on Tyr608 (MW: 180 kDa), total AKT (MW: 60 kDa), AKT phosphorylated on Ser473 (MW: 60 kDa), total GSK3β (MW: 46 kDa), GSK3β phosphorylated on Ser9 (MW: 46 kDa), total mTOR (MW: 289 kDa), mTOR phosphorylated on Ser2448 (MW: 289 kDa), total p53 (MW: 53 kDa), p53phosphorylated on Ser15 (MW: 53 kDa), p21 (MW: 21 kDa), caspase 3 (MW: 19 and 17 kDa), LC3 (MW: 16 and 14 kDa), β-actin (MW: 45 kDa), or GAPDH (MW: 37 kDa). The blots were washed thoroughly in TBS-T buffer and incubated for 1h with a peroxidase-conjugated immunoglobulin G antibody. Immunoreactive proteins were visualized using an Immun-Star Chemiluminescence Kit (Bio-Rad, Madrid, Spain) according to

the manufacturer's instructions. Digital images were taken with a Versadoc (Bio-Rad, Madrid, Spain), which permits semiquantification of the band intensity. The protein load was periodically monitored via the immunodetection of β-actin, with the exception of GSK3β, which was monitored via immunodetection of GAPDH.

2.7. Statistics

Statistical analysis was performed using the software Statistical Package for the Social Sciences (SPSS v.22). The homogeneity of variances was analysed with Levene's test. If variances were homogenous, ANOVA was then used followed by Bonferroni's test in order to analyse all dose groups simultaneously. The Kruskal-Wallis test was used when variances were not homogeneous. Significance was set at $p < 0.05$.

3. Results

3.1. Oral exposure to PVP-AgNPs increased oxidative stress markers

ROS are inevitably produced in mammalian cells as a by-product of aerobic metabolism. Moderate levels of ROS act as second messengers in signalling cascades involved in cell proliferation and differentiation. However, excessive levels of ROS formation can unbalance redox homeostasis, thereby generating oxidative stress, cell damage, and cell death. To oppose the increased ROS production, cells can up-regulated intrinsic antioxidant defences, such as the SOD and CAT enzymes. The SOD enzyme catalyses the conversion of superoxide radicals to molecular oxygen (O_2) and hydrogen peroxide, while the CAT enzyme neutralizes hydrogen peroxide by dismuting them to H_2O and O_2 (Birben et al., 2012). In the current study, the production of ROS in the male rat liver was indirectly evaluated by measuring the activity of the antioxidant enzymes SOD and CAT. Total levels of TBARS were also measured as an indicator

of lipid peroxidation generated by the interaction of ROS with polyunsaturated fatty acids in lipid membranes.

The increase of SOD and CAT activity occurred in a dose-dependent manner, being significant ($p < 0.05$) at 100 and 200 mg/kg/day, compared with the control group (Figures 1A and B, respectively). The same effect was also observed for the TBARS levels, which was significantly increased in the 100 and 200 mg/kg/day groups ($p < 0.001$), compared with the control group (Fig. 1C). These results suggest that increased oral exposure to AgNPs generates increased ROS formation -dose-dependently- in male rat livers.

3.2. Oral exposure to PVP-AgNPs deregulates the insulin signalling pathway

Insulin signalling pathways are involved in the control of nutrient metabolism, cell growth and survival in mammalian organisms. The binding of insulin to the insulin receptor tyrosine kinase recruits and activates different substrate adaptors, such as IRS-1, by phosphorylation. Phosphorylation of IRS-1 on Tyr608 promotes activation of the AKT/mTOR signalling pathway via previous activation of PI3K (Boucher et al., 2014). AKT is involved in the regulation of various processes, such as the inhibition of apoptosis and cell proliferation. In liver, AKT plays an important role in glucose metabolism, inactivating GSK3β via phosphorylation on Ser9, allowing the activation of glycogen synthesis.

The ratio of phosphorylated and total protein levels of IRS-1 did not show significant changes (Fig. 2A). However, the analysis performed for both forms independently showed that the phosphorylated (52 ± 12 %, $p = 0.001$) and total (54 ± 18 %, $p = 0.002$) IRS-1 levels increased significantly in the 100 mg/kg/day group, compared to the control group. Surprisingly, decreased levels of both forms were produced in the 200 mg/kg/day group compared to the 100 mg/kg/day group. The same pattern was observed for the ratio between phosphorylated and total AKT, the ratios of which were significantly increased in the 50 (194 ± 52 %, $p = 0.030$) and 100 mg/kg/day (225 ± 22 %, $p = 0.010$) groups compared to the control group. However, their ratio

also showed a decreased level at 200 mg/kg/day (Fig. 2B). Similarly, significant increases of the GSK3β (134 ± 16 %, p = 0.001) and mTOR (48 ± 9 %, p = 0.001) ratios were also observed at 100 mg/kg/day. However, the ratio was decreased in the 200 mg/kg/day group compared to the 100 mg/kg/day (Figures 2C and D, respectively). These results suggest that oral exposure to PVP-AgNPs, first stimulates induction of the insulin signalling pathway by increasing the total and active form of IRS-1 protein. However, a high concentration of PVP-AgNPs has an opposite effect, creating a state similar to that observed in hepatic insulin resistance.

3.3. Oral exposure to PVP-AgNPs dysregulates the protein levels of p53, p21 and cleaved caspase 3

Among various cellular actions, p53 stimulates the expression of p21. The p21 protein induces cell cycle arrest through the inhibition of cyclin-dependent kinases, allowing cellular mechanisms to detect and repair damaged DNA (Engeland, 2017). Notwithstanding, when the amount of damaged DNA exceeds the capacity to repair it, p53 can stimulate apoptotic signalling pathways, which finally induces the activation of caspase 3 proteins.

Although the ratio of the phosphorylated and total p53 did not show significant differences (Fig. 3A), the independent analysis showed that both forms (31 ± 8 %, p = 0.009; 34 ± 5 %, p = 0.001; respectively) significantly increased in the 100 mg/kg/day group compared to the control group. Moreover, the levels of total p21 (53 ± 14 %, p = 0.008) and cleaved caspase 3 (62 ± 8 %, p = 0.001) proteins were also significantly increased compared with the control group (Figures 3B and 4A, respectively). Similar to the results of the previous section, decreased levels of the evaluated proteins were observed in the 200 mg/kg/day group compared to the 100 mg/kg/day group. These results suggest that increased DNA damage and apoptotic cell death induction was caused by oral exposure to PVP-AgNPs in a dose-dependent manner. However, excess damaged DNA could affect the expression of the evaluated proteins, triggering a decrease of their expression due to the action of high amounts of PVP-AgNPs in the cell.

3.4. Oral exposure to PVP-AgNPs induced the increase of the autophagy marker LC3

Autophagy is a self-degradative process that targets damaged organelles to the lysosome for degradation and recycling of cellular components. Although autophagy can prolong survival during nutrient deprivation, overstimulated autophagy can also lead to an alternate death pathway, which facilitates type II programmed cell death. During the autophagy process, the level of the LC3-II protein correlates with the number of autophagosomes. Therefore, the level of conversion of LC3-I to LC3-II can be used as an indicator of autophagic activity (Tomás-Hernández et al., 2018).

In spite of the increased levels of the conversion of LC3-I to LC3-II that occurred in a dose-dependent manner (Fig. 4B), the LC3-II/LC3-I ratio only showed a significant increase at 200 mg/kg/day (128 ± 46 %, $p = 0.001$) compared to the control group. The excessive cell damage caused by exposure to high concentrations of PVP-AgNPs could lead to the depletion of cellular survival and metabolic mechanisms, inducing an increase of autophagy to remove damaged organelles and as a compensatory mechanism to obtain energy.

4. Discussion

The results of present study show that oral subchronic exposure to PVP-AgNPs stimulated the increase of markers of hepatotoxicity in a dose-dependent manner. The effect of PVP-AgNPs on the insulin signalling pathway, and the increase of ROS production may play an essential role, contributing to the modulation of apoptotic and autophagic molecular processes.

The increased levels of TBARS here found, along with higher induction of CAT and SOD activity, suggest that PVP-AgNPs promoted the production of ROS in a dose-dependent

manner. In vitro and in vivo studies have reported that a primary consequence of AgNPs exposure was the increased production of ROS (Blanco et al., 2017; Juling et al., 2018; Piao et al., 2011a; Zhu et al., 2017). Nevertheless, at 200 mg/kg/day, the activity of both antioxidant enzymes was decreased compared to the 100 mg/kg/day group. It is possible that impaired nuclear translocation of the nuclear factor erythroid 2 related factor 2 (Nrf2) could be partially responsible for the decreased antioxidant activity. The transcription factor Nrf2 regulates the transcription of genes encoding for cyto-protective proteins, such as CAT and SOD (Zhao et al., 2018). Downregulation of the expression of Nrf2 and the inhibition of transcriptional activity was described by various authors in different types of cells after AgNPs exposure (Kang et al., 2012; Lee et al., 2016; Piao et al., 2011b). By contrast, SOD and CAT activity could be also affected by the inhibition of hydrogen peroxide formation. Specifically, hydrogen peroxide causes a peroxidase reaction, which cleaves the Pro112–His113 peptide bond of SOD, triggering the dissociation of copper from the active site, thus losing the enzymatic activity (Gottfredsen et al., 2013).

On the other hand, another interesting finding of the present study was that the total and phosphorylated form of the IRS1 protein was increased in a dose-dependent manner in the treated groups. Specifically, the 100 and 200 mg/kg/day groups showed a higher level of the active IRS1 form, which triggers activation of the AKT/mTOR pathway and the inactivation of GSK3β by AKT. Unexpectedly, decreased levels of the total and active form of the IRS protein were observed at 200 mg/kg/day compared to the 100 mg/kg/day group, decreasing the phosphorylated form of AKT, and consequently, the phosphorylation of their substrate proteins, GSK3β and mTOR.

Thus, the massive amount of ROS produced in the liver at 200 mg/kg/day, as indicated by the increased levels of TBARS, may be related to the reduced levels of the IRS-1 protein. Potashnik et al. (2003) demonstrated that oxidative stress induced the increase of IRS-1 degradation, similar to prolonged insulin and other inducers of cellular insulin resistance. Based on this, increased ROS production may inhibit IRS-1 signalling via AMPK-dependent phosphorylation

of IRS-1 at Ser794, leading to the dissociation of IRS-1 from its upstream transmembrane insulin receptor (Tzatsos and Tsichlis, 2007). Furthermore, an epigenetic action trigger by AgNPs could be also key in the downregulation of the total IRS-1 concentration. Recently, Huang et al. (2014) reported that human dermal fibroblasts exposed to high concentrations of AgNPs increased the levels of the microRNAs miR-30a/30d/30e/200b. These microRNAs could bind to IRS-1 mRNA, promoting their degradation and the consequent decrease in the levels of IRS-1 protein translation.

Moreover, the inactivation or downregulation of theIRS-1 protein by PVP-AgNPs could have a direct impact on the inactivation of AKT. Because of the importance of AKT in hepatic glycogen storage via the inhibition of GSK3β, high amounts of PVP-AgNPs could interfere in hepatic glucose metabolism to create a similar situation described in the liver in insulin-resistant states. In relation to this, Ostaszewska et al. (2018) reported a glycogen depletion of rainbow trout (Oncorhynchus mykiss) hepatocytes after 28 days of exposure to 1.5 mg/L of AgNPs. Similarly, Lee et al. (2016) showed that a treatment of 5 nm AgNPs decreased glucose consumption in a hepatoma cell line by producing high levels of ROS.

In contrast, the decreased levels of phosphorylated AKT in the 200 mg/kg/day group may also cause increased levels of the autophagic marker LC3-II. Activated AKT prevents the inhibition of phosphorylation of mTOR, which acts as a negative regulator of autophagy. Therefore, the inhibition of AKT activity could stimulate the decrease of phosphorylated mTOR, thus increasing autophagic processes (Heras-Sandoval et al., 2014). Moreover, high ROS levels can also induce autophagy via the activation of AMPK, which in addition to its negative regulation of the IRS-1/PI3K/AKT signalling pathway, also enhances the stimulation of negative regulators of mTOR, such as the tuberous sclerosis complex proteins (Zhang et al., 2013). Zhu et al. (2017) showed that a decrease of phosphorylated and total mTOR protein was produced in murine pro-B cells in relation to an increase of LC3-II and ROS levels after 24h of exposure to various doses of AgNPs. These authors reported that the effect of AgNPs co-administered with antioxidant substances restored mTOR levels and decreased cell autophagy.

In accordance with this, increased levels of the total and active form of p53 and total p21 were also observed at 100 and 200 mg/kg/day, which is related to the increase of markers of ROS production. Among its multiple functions in the cell, p53 preserves genomic stability by blocking cell cycle progression, by stimulating the expression of p21. The increase of p21 maintains the cells in the G1 phase, which protects the cells from replicating damaged DNA, thus facilitating their repair (Engeland, 2017). Recently, Garcia et al. (2016) showed that higher doses of PVP-AgNPs caused an increase of the number of binucleated hepatocytes in a histopathological examination of male rats liver under the same experimental conditions of the present study. It has been recently reported that hepatocytes maintained in the G1 phase over time are associated with increased levels of p53 and p21, higher rates of binucleated cells and the accumulation of damaged DNA (Tormos et al., 2015). It suggests that increased levels of intracellular PVP-AgNPs and ROS in hepatocytes could generate DNA damage in the 50 and 100 mg/kg/day groups, promoting the expression of p53 and cell cycle arrest by p21. Thus, PVP-AgNPs could also promote the increased levels of cleaved caspase 3 because of the difficulty of repairing damaged DNA. Moreover, excess damaged DNA may have occurred in the 200 mg/kg/day group, affecting the expression of caspase 3, p53 and p21,which were all decreased with respect to the 100 mg/kg/day group. Supporting this hypothesis, several studies have reported the downregulation of mRNA and/or protein expression of caspase 3, p53 and p21 after exposure to high doses of AgNPs over time (Arora et al., 2008; Blanco et al., 2017; Mytych et al., 2015).

In summary, the results of present investigation showed that oral administration of PVP-AgNPs caused an increase of the activity of SOD and CAT in the liver of male rats. However, the activity was decreased after exposure to high amounts of PVP-AgNPs. In addition, TBARS levels increased in a dose-dependent manner, which suggest that increased levels of ROS were produced by the increased presence of PVP-AgNPs in liver. Furthermore, increased protein levels of IRS-1, AKT, GSK3β and mTOR proteins were also observed in a dose-dependent manner. However, these proteins showed a decrease at 200 mg/kg/day group with respect to the

100 mg/kg/day group. The same pattern was observed for the p53, p21 and cleaved caspase 3 protein levels, whose concentrations increased in a dose-dependent manner, but also decreased in the 200 mg/kg/day group. The decrease of mTOR may be related to the autophagy marker ratio LC3-II/LC3-I, which presented higher levels in the high treated group. These results suggest that the increase of ROS production by PVP-AgNPs stimulated SOD and CAT activity, as well as IRS-1, AKT, mTOR, p53, p21 and caspase 3, as protective mechanisms of cell survival and preserve DNA fidelity. However, cellular damage by excessive ROS production may induce the depletion of these survival mechanisms in liver of rats receiving 200 mg/kg/day, thereby enhancing autophagy and inducing a similar situation described in the liver in insulin-resistant states.

References

Arora, S., Jain, J., Rajwade, J.M., Paknikar, K.M., 2008. Cellular responses induced by silver nanoparticles: In vitro studies. Toxicol. Lett. 179, 93–100.

AshaRani, P. V., Mun, G.L.K., Hande, M.P., Valiyaveettil, S., 2009. Cytotoxicity and genotoxicity of silver nanoparticles in human cells. ACS Nano 3, 279–290.

Austin, L.A., MacKey, M.A., Dreaden, E.C., El-Sayed, M.A., 2014. The optical, photothermal, and facile surface chemical properties of gold and silver nanoparticles in biodiagnostics, therapy, and drug delivery. Arch. Toxicol. 88, 1391-417.

Birben, E., Murat, U., Md, S., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative Stress and Antioxidant Defense. WAO J. 5, 9–19.

Blanco, J., Lafuente, D., Gómez, M., García, T., Domingo, J.L., Sánchez, D.J., 2017. Polyvinyl pyrrolidone-coated silver nanoparticles in a human lung cancer cells: time- and dose-dependent influence over p53 and caspase-3 protein expression and epigenetic effects. Arch. Toxicol. 91, 651–666.

Bressan, E., Ferroni, L., Gardin, C., Rigo, C., Stocchero, M., Vindigni, V., Cairns, W., Zavan, B., 2013. Silver nanoparticles and mitochondrial interaction. Int. J. Dent. 2013, 312747.

Brkić Ahmed, L., Milić, M., Pongrac, I.M., Marjanović, A.M., Mlinarić, H., Pavičić, I., Gajović, S., VinkovićVrček, I., 2017. Impact of surface functionalization on the uptake mechanism and toxicity effects of silver nanoparticles in HepG2 cells. Food Chem. Toxicol. 107, 349–361.

Boucher, J., Kleinridders, A., Ronald Kahn, C., 2014. Insulin receptor signaling in normal and insulin-resistant states. Cold Spring Harb. Perspect. Biol. 6(1).

Choi, J.I., Chae, S.J., Kim, J.M., Choi, J.C., Park, S.J., Choi, H.J., Bae, H., Park, H.J., 2018. Potential silver nanoparticles migration from commercially available polymeric baby products into food simulants. Food Addit. Contam. Part A 0, 1–10.

Duhan, J.S., Kumar, R., Kumar, N., Kaur, P., Nehra, K., Duhan, S., 2017. Nanotechnology: The new perspective in precision agriculture. Biotechnol. Reports. 15,11-23.

Engeland, K., 2017. Cell cycle arrest through indirect transcriptional repression by p53: I have a DREAM. Cell Death Differ. 25, 114–132.

Engin, A.B., Nikitovic, D., Neagu, M., Henrich-Noack, P., Docea, A.O., Shtilman, M.I., Golokhvast, K., Tsatsakis, A.M., 2017. Mechanistic understanding of nanoparticles' interactions with extracellular matrix: The cell and immune system. Part. Fibre Toxicol. 14(1), 22.

Garcia, T., Lafuente, D., Blanco, J., Sánchez, D.J., Sirvent, J.J., Domingo, J.L., Gómez, M., 2016. Oral subchronic exposure to silver nanoparticles in rats. Food Chem. Toxicol. 92, 177–187.

Gambardella, C., Costa, E., Piazza, V., Fabbrocini, A., Magi, E., Faimali, M., Garaventa, F., 2015. Effect of silver nanoparticles on marine organisms belonging to different trophic levels. Mar. Environ. Res. 111, 41–49.

Gottfredsen, R.H., Larsen, U.G., Enghild, J.J., Petersen, S. V., 2013. Hydrogen peroxide induce modifications of human extracellular superoxide dismutase that results in enzyme inhibition. Redox Biol. 1, 24–31.

Heras-Sandoval, D., Pérez-Rojas, J.M., Hernández-Damián, J., Pedraza-Chaverri, J., 2014. The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. Cell. Signal. 26, 2694–2701.

Huang, Y., Lü, X., Ma, J., 2014. Toxicity of silver nanoparticles to human dermal fibroblasts on MicroRNA level. J. Biomed. Nanotechnol. 10, 3304–3317.

Jacob, S.J.P., Prasad, V.L.S., Sivasankar, S., Muralidharan, P., 2017. Biosynthesis of silver nanoparticles using dried fruit extract of Ficus carica - Screening for its anticancer activity and toxicity in animal models. Food Chem. Toxicol. 109 (Pt2), 951-956.

Jiravova, J., Tomankova, K.B., Harvanova, M., Malina, L., Malohlava, J., Luhova, L., Panacek, A., Manisova, B., Kolarova, H., 2016. The effect of silver nanoparticles and silver ions on mammalian and plant cells in vitro. Food Chem. Toxicol. 96, 50–61.

Juling, S., Böhmert, L., Lichtenstein, D., Oberemm, A., Creutzenberg, O., Thünemann, A.F., Braeuning, A., Lampen, A., 2018. Comparative proteomic analysis of hepatic effects induced by nanosilver, silver ions and nanoparticle coating in rats. Food Chem. Toxicol. 113, 255–266.

Kang, S.J., Ryoo, I. geun, Lee, Y.J., Kwak, M.K., 2012. Role of the Nrf2-heme oxygenase-1 pathway in silver nanoparticle-mediated cytotoxicity. Toxicol. Appl. Pharmacol. 258, 89–98.

Kim, Y.S., Song, M.Y., Park, J.D., Song, K.S., Ryu, H.R., Chung, Y.H., Chang, H.K., Lee, J.H., Oh, K.H., Kelman, B.J., Hwang, I.K., Yu, I.J., 2010. Subchronic oral toxicity of silver nanoparticles. Part. FibreToxicol. 7, 20.

Kumar, G., Degheidy, H., Casey, B.J., Goering, P.L., 2015. Flow cytometry evaluation of in vitro cellular necrosis and apoptosis induced by silver nanoparticles. FoodChem. Toxicol. 85, 45–51.

Kuskov, A.N., Kulikov, P.P., Shtilman, M.I., Rakitskii, V.N., Tsatsakis, A.M., 2016. Amphiphilic poly-N-vynilpyrrolidone nanoparticles: Cytotoxicity and acute toxicity study. Food Chem. Toxicol. 96, 273–279.

Lafuente, D., Garcia, T., Blanco, J., Sánchez, D.J., Sirvent, J.J., Domingo, J.L., Gómez, M., 2016. Effects of oral exposure to silver nanoparticles on the sperm of rats. Reprod. Toxicol. 60, 133–139.

Lee, M.J., Lee, S.J., Yun, S.J., Jang, J.-Y., Kang, H., Kim, K., Choi, I.-H., Park, S., 2016. Silver nanoparticles affect glucose metabolism in hepatoma cells through production of reactive oxygen species. Int. J. Nanomedicine 11, 55–68.

Lee, Y.H., Cheng, F.Y., Chiu, H.W., Tsai, J.C., Fang, C.Y., Chen, C.W., Wang, Y.J., 2014. Cytotoxicity, oxidative stress, apoptosis and the autophagic effects of silver nanoparticles in mouse embryonic fibroblasts. Biomaterials 35, 4706–4715.

Mackevica, A., Olsson, M.E., Hansen, S.F., 2016. Silver nanoparticle release from commercially available plastic food containers into food simulants. J. Nanoparticle Res. 18, 1–11.

Maillard, J.-Y., Hartemann, P., 2012. Silver as an antimicrobial: Facts and gaps in knowledge. Crit. Rev. Microbiol. 39, 373-83.

McGillicuddy, E., Murray, I., Kavanagh, S., Morrison, L., Fogarty, A., Cormican, M., Dockery, P., Prendergast, M., Rowan, N., Morris, D., 2017. Silver nanoparticles in the environment: Sources, detection and ecotoxicology. Sci. Total Environ. 575, 231–246.

Mei, L., Teng, Z., Zhu, G., Liu, Y., Zhang, F., Zhang, J., Li, Y., Guan, Y., Luo, Y., Chen, X., Wang, Q., 2017. Silver Nanocluster-Embedded Zein Films as Antimicrobial Coating Materials for Food Packaging. ACS Appl. Mater. Interfaces 9, 35297–35304.

Mishra, A.R., Zheng, J., Tang, X., Goering, P.L., 2016. Silver nanoparticle-induced autophagic-Lysosomal disruption and NLRP3-inflammasome activation in HepG2 cells is size-dependent. Toxicol. Sci. 150, 473–487.

Mytych, J., Pacyk, K., Pepek, M., Zebrowski, J., Lewinska, A., Wnuk, M., 2015. Nanoparticle-mediated decrease of lamin B1 pools promotes a TRF protein-based adaptive response in cultured cells. Biomaterials 53, 107–116.

Ostaszewska, T., Sliwinski, J., Kamaszewski, M., Sysa, P., Chojnacki, M., 2017. Cytotoxicity of silver and copper nanoparticles on rainbow trout (Oncorhynchus mykiss) hepatocytes. Environ. Sci. Pollut. Res. 25(1), 908-915

Piao, M.J., Kang, K.A., Lee, I.K., Kim, H.S., Kim, S., Choi, J.Y., Choi, J., Hyun, J.W., 2011. Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis. Toxicol. Lett. 201, 92–100.

Piao, M.J., Kim, K.C., Choi, J.Y., Choi, J., Hyun, J.W., 2011. Silver nanoparticles down-regulate Nrf2-mediated 8-oxoguanine DNA glycosylase 1 through inactivation of extracellular regulated kinase and protein kinase B in human Chang liver cells. Toxicol. Lett. 207, 143–148.

Pinzaru, I., Coricovac, D., Dehelean, C., Moacă, E.A., Mioc, M., Baderca, F., Sizemore, I., Brittle, S., Marti, D., Calina, C.D., Tsatsakis, A.M., Şoica, C., 2018. Stable PEG-coated silver nanoparticles – A comprehensive toxicological profile. Food Chem. Toxicol. 111, 546–556.

Piperigkou, Z., Karamanou, K., Engin, A.B., Gialeli, C., Docea, A.O., Vynios, D.H., Pavão, M.S.G., Golokhvast, K.S., Shtilman, M.I., Argiris, A., Shishatskaya, E., Tsatsakis, A.M., 2016. Emerging aspects of nanotoxicology in health and disease: From agriculture and food sector to cancer therapeutics. Food Chem. Toxicol. 91, 42-57.

Potashnik, R., Bloch-Damti, A., Bashan, N., Rudich, A., 2003. IRS1 degradation and increased serine phosphorylation cannot predict the degree of metabolic insulin resistance induced by oxidative stress. Diabetologia 46, 639–648.

Seal, S., Karn, B., 2014. Safety aspects of nanotechnology based activity. Saf. Sci. 63, 217-225.

Shishatskaya, E.I., Nikitovic, D., Vasilievich, A.S., Tzanakakis, G.N., Tsatsakis, A.M., Menzianova, N.G., 2016. Short-term culture of monocytes as an in vitro evaluation system for bionanomaterials designated for medical use. Food Chem. Toxicol. 96, 302–308.

Sussman, E.M., Jayanti, P., Dair, B.J., Casey, B.J., 2015. Assessment of total silver and silver nanoparticle extraction from medical devices. Food Chem. Toxicol. 85, 10–19.

Stern, S.T., Adiseshaiah, P.P., Crist, R.M., 2012. Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity. Part. FibreToxicol. 9, 20.

Tomas-Hernández, S., Blanco, J., Rojas, C., Roca-Martínez, J., Ojeda-Montes, M.J., Beltrán-Debón, R., Garcia-Vallve, S., Pujadas, G., Arola, L., Mulero, M., 2018. Resveratrol Potently Counteracts Quercetin Starvation-Induced Autophagy and Sensitizes HepG2 Cancer Cells to Apoptosis. Mol. Nutr. Food Res. 1700610, 1700610.

Tormos, A.M., Taléns-Visconti, R., Sastre, J., 2015. Regulation of cytokinesis and its clinical significance. Crit. Rev. Clin. Lab. Sci. 52, 159–167.

Tzatsos, A., Tsichlis, P.N., 2007. Energy depletion inhibits phosphatidylinositol 3-kinase/Akt signaling and induces apoptosis via AMP-activated protein kinase-dependent phosphorylation of IRS-1 at Ser-794. J. Biol. Chem. 282, 18069–18082.

Zhang, J., Kim, J., Alexander, A., Cai, S., Tripathi, D.N., Dere, R., Tee, A.R., Tait-Mulder, J., Di Nardo, A., Han, J.M., Kwiatkowski, E., Dunlop, E.A., Dodd, K.M., Folkerth, R.D., Faust, P.L., Kastan, M.B., Sahin, M., Walker, C.L., 2013. A tuberous sclerosis complex signalling node at the peroxisome regulates mTORC1 and autophagy in response to ROS. Nat. Cell Biol. 15, 1186–1196.

Zhang, X.-F., Liu, Z.-G., Shen, W., Gurunathan, S., 2016. Silver Nanoparticles: Synthesis, Characterization, Properties, Applications, and Therapeutic Approaches. Int. J. Mol. Sci. 17, 1534.

Zhao, Y., Song, W., Wang, Z., Wang, Z., Jin, X., Xu, J., Bai, L., Li, Y., Cui, J., Cai, L., 2018. Resveratrol attenuates testicular apoptosis in type 1 diabetic mice: Role of Akt-mediated Nrf2 activation and p62-dependent Keap1 degradation. Redox Biol. 14, 609–617.

Zhu, L., Guo, D., Sun, L., Huang, Z., Zhang, X., Ma, W., Wu, J., Xiao, L., Zhao, Y., Gu, N., 2017. Activation of autophagy by elevated reactive oxygen species rather than released silver ions promotes cytotoxicity of polyvinylpyrrolidone-coated silver nanoparticles in hematopoietic cells. Nanoscale 9, 5489–5498.

Zupan, G., Pilipović, K., Hrelja, A., Peternel, S., 2008. Oxidative stress parameters in different rat brain structures after electroconvulsive shock-induced seizures. Prog. Neuropsychopharmacol. Biol. Psychiatry 32, 771–7.

Figure legends

Fig. 1. Oral subchronicexposure to PVP-AgNPs increased oxidative stress markers. Activity of (A) SOD and (B) CAT enzymes, and (C) TBARS levels was measured in the liver tissue from male rats orally exposed to 0,50, 100 and 200 mg/kg/day of PVP-AgNPs for 90 days. Data are expressed as means ± SD. Significant differences relative to the control (0 mg/kg/day) group were analysed by one-way ANOVA followed by Bonferroni's post hoc test: $*p < 0.05$, $**p < 0.01$.

Fig. 2. Oral subchronicexposure to PVP-AgNPs deregulates the insulin signalling pathway. Total protein were extracted from livers of male rats orally exposed to 0, 50, 100 and 200 mg/kg/day of PVP-AgNPs for 90 days. The ratio of the relative protein expression of phosphorylated and total (A) IRS-1, (B) AKT, (C) GSK3β and (D) mTOR was measured by Western blot analysis. The intensity of the bands was determined by densitometric analysis. Data were normalized using β-actin or GADPH as an internal control. A representative Western blot is shown in the upper panel. Data from the experiments are presented in the lower panel. All data are expressed as means ± SD. Significant differences relative to the control group were analyzed by one-way ANOVA followed by a Bonferroni's post hoc test: $*p < 0.05$ and $**p < 0.01$.

Fig. 3. Oral subchronicexposure to PVP-AgNPs dysregulates the protein levels of p53, p21. Total protein were extracted from livers of male rats orally exposed to 0, 50, 100 and 200 mg/kg/day of PVP-AgNPs for 90 days. The ratio of the relative protein expression of phosphorylated and total (A) p53 and total (B) p21 was measured by Western blot analysis. The intensity of the bands was determined by densitometric analysis. Data were normalized using β-actin as an internal control. A representative Western blot is shown in the upper panel. Data from the experiments are presented in the lower panel. All data are expressed as means ± SD. Significant differences relative to the control group were analyzed by one-way ANOVA followed by a Bonferroni's post hoc test: $*p < 0.05$.

Fig. 4. Oral subchronicexposure to PVP-AgNPs dysregulates the protein levels of cleaved caspase 3 and induced the increase of the autophagy marker LC3. Total protein were extracted from livers of male rats orally exposed to 0, 50, 100 and 200 mg/kg/day of PVP-AgNPs for 90 days. The relative protein expression of total (A) cleaved caspase 3 and the ratio of total (B)

LC3-II and LC3-I were measured by Western blot analysis. The intensity of the bands was determined by densitometric analysis. Data were normalized using β-actin as an internal control. A representative Western blot is shown in the upper panel. Data from the experiments are presented in the lower panel. All data are expressed as means ± SD. Significant differences relative to the control group were analyzed by one-way ANOVA followed by a Bonferroni's post hoc test: $*p < 0.01$.

Figures

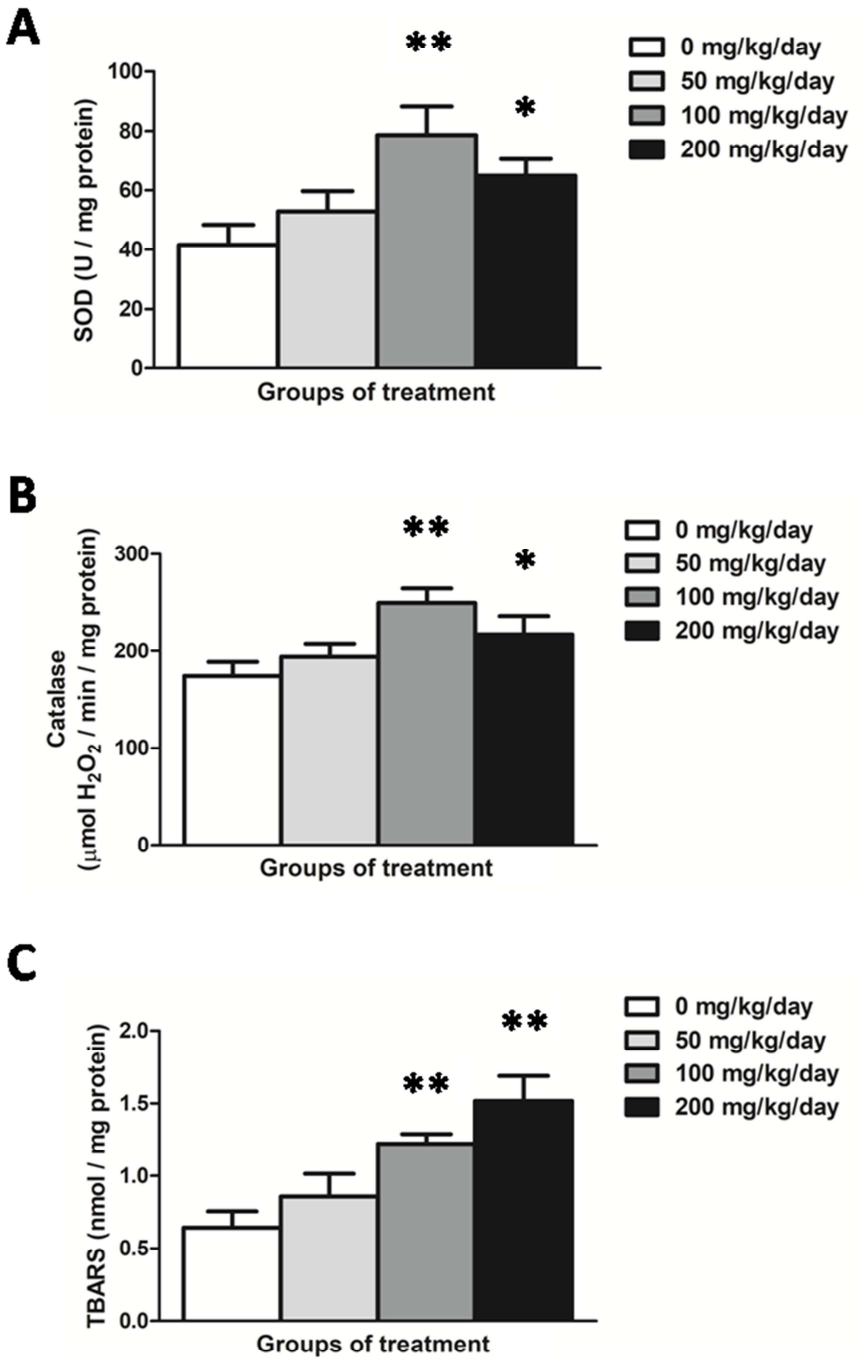

Fig. 1.

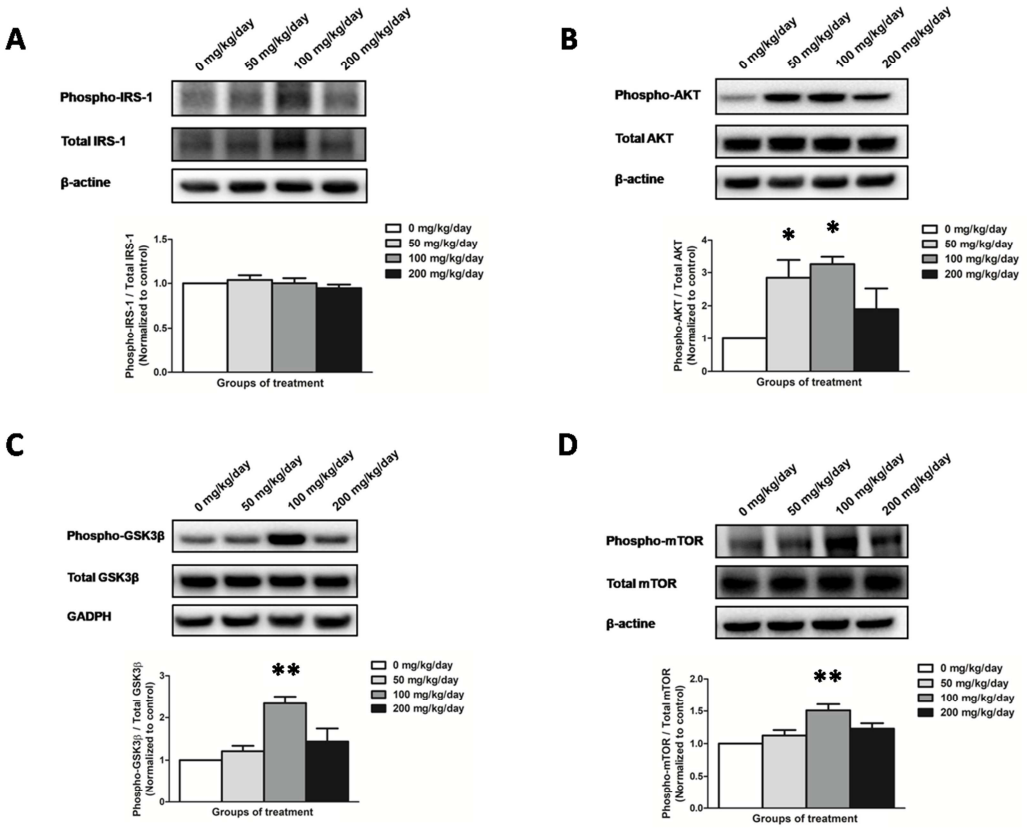

Fig. 2.

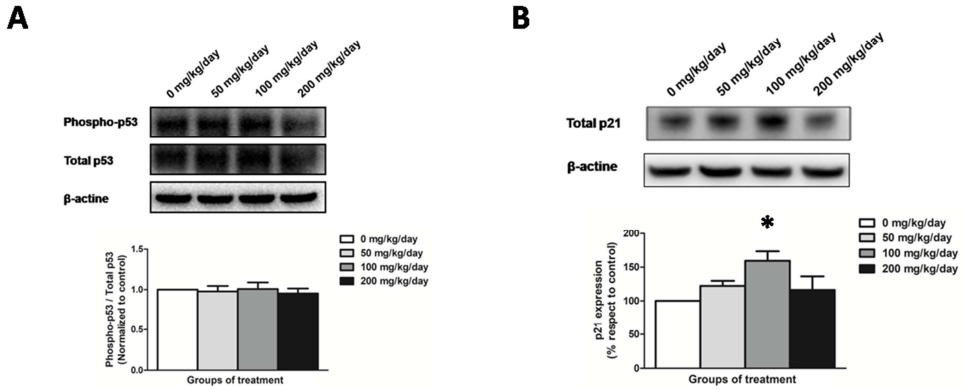
A
0 mg/kg/day
50 mg/kg/day
100 mg/kg/day
200 mg/kg/day
Phospho-p53
Total p53
β-actine
Phospho-p53 / Total p53 (Normalized to control)
Groups of treatment
B
Total p21
β-actine
p21 expression (% respect to control)
Groups of treatment
*

Fig. 3.

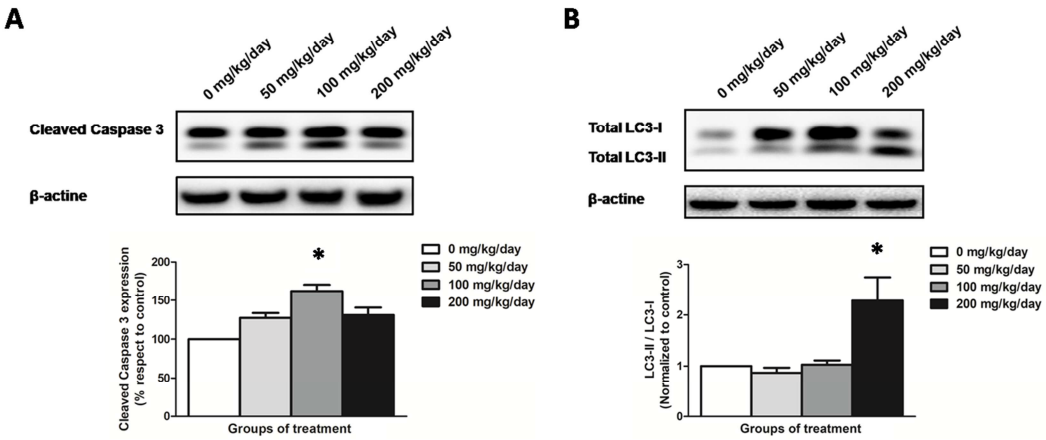
A
0 mg/kg/day
50 mg/kg/day
100 mg/kg/day
200 mg/kg/day
Cleaved Caspase 3
β-actine
Cleaved Caspase 3 expression (% respect to control)
Groups of treatment
*
B
Total LC3-I
Total LC3-II
β-actine
LC3-II / LC3-I (Normalized to control)
Groups of treatment
*

Fig. 4.

Highlights

- AgNPs are the most used nanoparticles in industrial applications.
- The impact on the liver of male rats orally exposed to PVP-AgNPs was investigated.
- 50 and 100 mg/kg/day of PVP-AgNPs promoted protective mechanisms of cell survival.
- 200 mg/kg/day of PVP-AgNPs may induce cellular damage by excessive ROS production.
- 200 mg/kg/day of PVP-AgNPs enhanced autophagy and depleted insulin signalling pathway.