Laboratorio de Toxicología Celular[1], Departamento de Ciencias Biológicas, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México; Laboratorio de enfermedades Neuromusculares[2], División de Neurociencias, Instituto Nacional de Rehabilitación "Luis Guillermo Ibarra", Secretaría de Salud, Tlalpan; Synovio-analysis Molecular Laboratory[3], Instituto Nacional de Rehabilitación "Luis Guillermo Ibarra", Secretaría de Salud, Tlalpan, México

Oxidative stress modulation induced by chitosan-glutathione nanoparticles in chondrocytes

L. D. López-Barrera, R. Díaz-Torres[1,*], A. López Macay[2], A. G. López-Reyes[3], S. Piña Olmos[1], P. Ramírez-Noguera[1,*]

Received September 12, 2018, accepted March 4, 2019

**Corresponding authors: Roberto Díaz-Torres and Patricia Ramírez-Noguera, Laboratorio de Toxicología Celular, Departamento de Ciencias Biológicas, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México, 54714 Cuautitlan Izcalli, Mexico*
diaztorres_r@hotmail.com; ramireznoguera@unam.mx

Pharmazie 74: 406-411 (2019) *doi: 10.1691/ph.2019.8166*

The use of nanometric systems to deliver biologically active substances is a successful tool in different fields. In this study, we investigated nanometric systems with antioxidant capacity to modulate events associated with the redox state in human chondrocytes. We used nanoparticles (NPs) prepared with chitosan and glutathione (GSH) and an in vitro model: primary cultures of human chondrocytes were extracted from hyaline cartilage. The cells were exposed to $CdCl_2$ in the presence or absence of NPs. $CdCl_2$ is a widely known oxidizing agent. Fluorescence and confocal microscopy showed the location of the NPs within the cells. The results obtained showed that the NPs did not significantly affect cell viability. We studied the antioxidant capacity of the NPs by estimating the GSH, TBARs, and Cell Rox content and the enzymatic activity of glutathione peroxidase (GPx). In vitro assays showed that GSH levels, GPx activity and reactive oxygen species (Cell Rox) levels were modified with both concentrations of NPs, while lipoperoxidation (TBARs) decreased when cells exposed to $CdCl_2$ were in contact with the NPs. All these results suggest the ability of NPs to modulate the cell redox state in a dose-dependent manner.

1. Introduction

Oxidative stress has been suggested to be involved in several pathological processes in which an imbalance exists between oxidants and antioxidants at the cellular level, and these effects are related to the production of reactive oxygen species (ROS) and free radicals, which damage proteins, lipids, and nucleic acids.

Osteoarthritis and osteoarthrosis are degenerative, progressive and disabling diseases of the articular cartilage associated with oxidative stress. This disease is accompanied by changes in underlying bone and soft tissue and is primarily characterized by progressive erosion of articular cartilage accompanied by a series of cellular events that contribute to the inflammatory process. The oxidation-reduction imbalance and the release of proinflammatory cytokines promote degradation of the extracellular matrix and the death of chondrocytes, among other associated biological effects (Maneesh et al. 2005; Dycus 2013).

The synovial fluid is responsible for providing nutrition to chondrocytes and is highly susceptible to osmotic changes and oxygen and glucose concentration because it is highly permeable. An alteration in the composition of the cartilage and synovial fluid induced by the initial degradation of cartilage leads to inadequate functioning of chondrocytes due to an increase in the production of free radicals (synthase cNOS), which among other factors, decreases the release of proteoglycans, further aggravating cartilage degeneration (Naranjo et al. 2011).

Under normal conditions, at a low concentration, ROS play an important role in the physiological processes of proliferation, differentiation, cell regeneration and matrix synthesis. However, an increase in ROS levels is involved in the ageing process, which contributes to degenerative and inflammatory processes through the release of cytokines and metalloproteinases (Rebrin et al. 2008) and increases the concentration of malondialdehyde by increasing lipid lipoperoxidation. Malondialdehyde binds to the histidine of extracellular matrix proteins and forms stable adducts, altering the composition and stability of the matrix and thus the activity of chondrocytes (Naranjo et al. 2011; Sandell et al. 2001).

Recent studies have suggested that the use of antioxidant substances in synovial fluid might have important effects in osteoarthrosis treatment. Specifically, the substances that regulate the oxidative process contribute to decreasing the amount and effect of oxygen and nitrogen free radicals, favouring suppression of the inflammatory process through signalling mediated by cytokines and antioxidant molecules (Rebrin et al. 2008). One of these antioxidants is glutathione, a tripeptide that plays a vital role in several cellular events related to maintenance of the oxidative stress status of the cell by interacting directly with free radicals or helping in the catalytic reaction of glutathione peroxidase (Lushchak 2012). Maintaining the state of oxide reduction in human chondrocytes is important, and the main antioxidant of cellular origin is glutathione. In addition, this antioxidant can only be synthesized in the intracellular environment to exert its activity. In this work, we utilized chitosan nanoparticles to deliver glutathione to act as an antioxidant and free radical scavenger and evaluated the redox modulatory effect of the NPs in human chondrocyte cultures.

2. Investigations and results

Nanoparticles (NPs) were prepared with chitosan-GSH using the method of ionic gelation, in which the positive charge of chitosan interacts with the polyanion sodium tripolyphosphate (STPP) to form the NPs (De Pinho Neves et al. 2014). To characterize the average particle size and zeta potential, we used a Zeta Sizer Nanoseries instrument from Malvern (Table). To determine the final concentration of nanoparticles, we use a Nanosight NS300 instrument from Malvern Panalytical. We observed NP morphology via transmission electron microscopy (TEM) (Fig. 1).

The zeta potential indicates the magnitude of charge repulsion or attraction between particles and therefore is a fundamental parameter to determine the stability of a suspension of obtained NPs (Wu et al. 2011). The results shown in the Table indicate that there was repulsion between the obtained NPs, preventing the system from agglomerating and precipitating and thus allowing a stable suspension. We ultracentrifuged the NPs in glycerol to determine the amount of glutathione in the nanoparticles. The concentration of soluble (not encapsulated) GSH in the supernatant was estimated using the DTNB technique with a GSH concentration curve (Hu 1994). The obtained results indicate that the non-encapsulated percentage of GSH in the NPs was 0.86 %, and therefore, the remaining 99.14 % of GSH was encapsulated in the NPs.

Table: Values obtained for average particle size, Z potential from CHI-GSH NPs using the Zetasizer- Nanoseries from Malvern, Nanosight NS300 from Malvern Panalytical, and percentage of GSH inside NP.

NANOPARTICLES	Average particle Size (nm)	Average Z potential (Vm)	% GSH	Nanoparticles/ mL
CHI-GSH	170.1	17.5	99.14	$3.718x10^{10}$
CHI-GSH/ Rhodamine	156.8	18.6	99.14	$5.343x10^{10}$

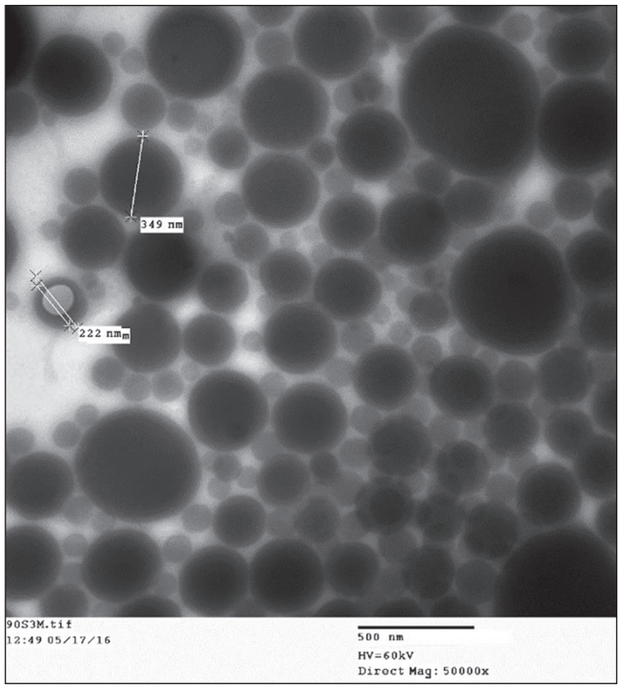

Fig. 1: Transmission Microscopy (TEM) of CHI-GSH Nanoparticles. TEM analysis of nanoparticles suspension having an average size of 170.1 nm.

2.1. Preparation and characterization of primary cultures of human chondrocytes

Primary cultures of human chondrocytes were cultured to the third passage and then characterized. The presence of type II collagen, which is the main structural component of hyaline cartilage, and Sox 9, which is an essential transcription factor during chondrocyte differentiation (Poole 2003), was detected by immunofluorescence (Fig. 2A and 2B) and western blot analysis (Fig. 2C).

As seen in Fig. 2A, type II collagen was present in the cytoplasm of the chondrocytes, whereas as shown in Fig. 2B, the transcription factor Sox 9 was observed in the nucleus of chondrocytes.

Some reports have noted that as the number of passages of chondrocyte cultures increases, the cells maintain their phenotype; however, the expression of certain genes, such as type II collagen and aggrecan, can decrease. However, this may be reversible when

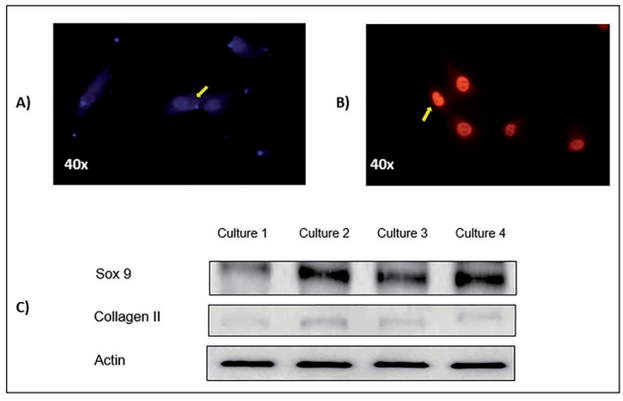

Fig. 2: Results of human chondrocyte immunostaining A) Ab-Anti collagen II with Ab-secondary Alexa 350. B) Ab-anti Sox 9 with Ab-secondary Alexa 647. C) Western blot identification of total typical proteins of the chondral phenotype. Each column represents a different culture of human chondrocytes up to the third cell pass.

chondrocytes are cultured in a three-dimensional environment with the addition of transcription factors and other components that mimic the function of the cell matrix (Lin et al. 2008; Stokes et al. 2001). Therefore, the presence of type II collagen is reasonable because the cells were cultured in a monolayer (as was the case with the samples analysed) and not with a scaffold (Fig. 2C).

Sox 9 is an essential protein that induces the expression of other proteins, such as L-Sox 5 and Sox 6, which participate as transcription factors and induce the expression of genes characteristic of articular chondrocytes: COL2A1, COL9A1, COL11A2 and ACAN (the latter encodes for aggrecan, the major proteoglycan component of the extracellular matrix and articular cartilage). Additionally, Sox 9 inhibits the differentiation of proliferative chondrocytes to hypertrophic chondrocytes; therefore, Sox 9 is mostly expected to be present in the nucleus, as shown in Fig. 2B, indicating that these are not dedifferentiated chondrocytes (Demoor et al. 2014).

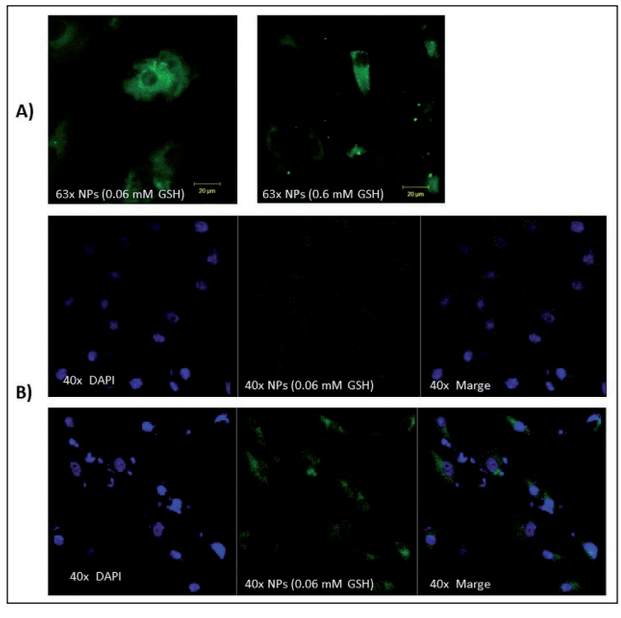

Fig. 3: Live cells exposed for 2 hrs at NPs (0.06 mM GSH) and NPs (0.6 mM GSH) by confocal microscopy. A) Live cells B) Fixed cells

2.2. Localization of NPs in human chondrocytes

Once we characterized the chondrocytes, the presence of the NPs within them was analysed to estimate their uptake into these cells. Rhodamine-coupled GSH chitosan NPs were constructed and characterized previously (Table), and then, the cells were exposed to these NPs for 2 h.

We determined the tested NP concentration according to the total amount of GSH that gets into the well: a low concentration of NPs (0.06 mM GSH) and high concentration of NPs (0.6 mM GSH). Fig. 3A shows the chitosan-GSH/Rhodamine NPs in living cells. The NPs were primarily distributed in the cytoplasm and close to the periphery of the nucleus. To visualize whether some of the NPs were present in the nucleus, the cells were fixed with paraformaldehyde and stained with DAPI. In the first image presented in Fig. 3B, the cells can be observed, followed by the chondrocytes exposed to NPs, and finally, both images are merged, showing where the NPs are located in both the cytoplasm and the nucleus.

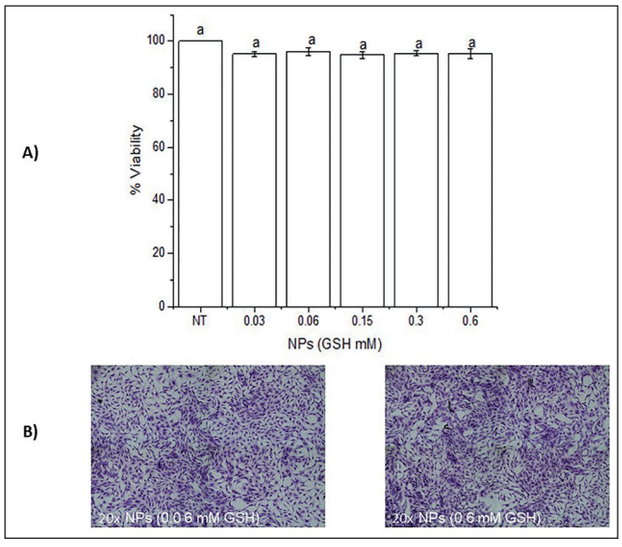

Fig. 4: The viability of chondrocytes exposed to NPs 0.06 mM GSH and 0.6 mM GSH. A) Bars with different letters indicate the significant differences between the means (Tukey test, $p<0.05$). B) Chondrocytes exposed to NPs with GSH.

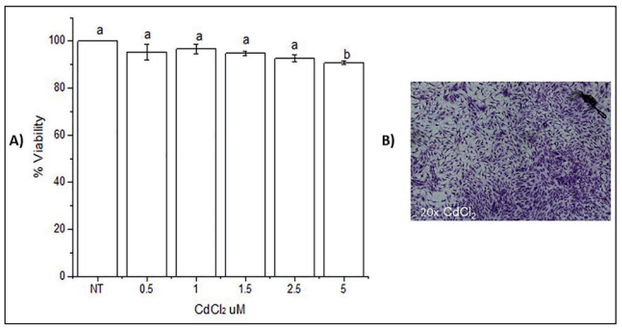

Fig. 5: The viability of chondrocytes exposed to different concentrations of CdCl2. A) Bars with different letters indicate that there are significant differences between the means (Tukey test, $p<0.05$). B) Chondrocytes exposed to 1uM CdCl2.

2.3. Evaluation of the cytotoxicity of NPs in human chondrocytes

Once we verified that the chitosan-GSH NPs were inside the chondrocytes, their effect on cell viability was evaluated using the crystal violet technique (Nakagawa et al. 1996). We tested dose-response effects. As shown in Fig. 4A, the different concentrations of NPs tested did not induce significant cytotoxic effects or morphologic changes in cultured cells (Fig. 4B).

Cadmium chloride ($CdCl_2$) can induce the formation of reactive species that cause lipoperoxidation, and the produced lipoperoxides can react with DNA and proteins and alter cellular functions, including inhibiting the activity of some antioxidant enzymes (Nuran Ercal et al. 2001). Because in this work the chitosan-GSH NPs are proposed as a biocompatible system capable of carrying GSH into the cell, thus triggering modulatory effects associated with the intracellular oxidative-reduction status, we estimated the viability of human chondrocytes exposed to the xenobiotic agent cadmium chloride ($CdCl_2$), which induces oxidative stress.

Chondrocytes were exposed to $CdCl_2$ in dose-range experiments for 2 h. Significant cytotoxic effects were observed using 5 mM $CdCl_2$ compared with untreated cells and the other $CdCl_2$ doses tested (Fig. 5A). Therefore, a concentration of 1 µM was used for the experiments, which was a concentration that did not compromise cell viability or chondrocyte morphology (Fig. 5B).

2.4. Estimation of NP ability to deliver GSH into chondrocytes

As mentioned before, GSH is a molecule synthesized in the cytoplasm of cells, and it cannot re-enter the cells once released into the extracellular space. Therefore, it is crucial to determine the amount of GSH carried from the NPs into the cells. We exposed cells to NPs for 2 h and then added $CdCl_2$ and incubated the cells for another 2 h. We lysed the cells and estimated intracellular GSH levels according to a previously described assay (Hu 1994).

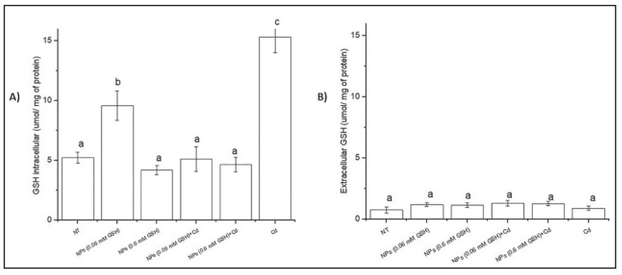

Fig. 6: Effects of NPs GSH on GSH levels. A) Intracellular GSH. B) Extracellular GSH. Untreated cells, (NT). Bars with different letters indicate that there are significant differences between the means (Tukey test, $p<0.05$).

As seen in Fig. 6A, intracellular GSH levels were increased in cells exposed to the low concentration of NPs, whereas at high concentrations, the NPs did not induce significant differences in GSH levels compared with untreated cells. This could be related to the findings of previous reports suggesting that certain physicochemical characteristics of NPs under study, especially their size, concentration and GSH loading, could be a limiting factor for their inclusion into cells because they tend to agglomerate due to the composition of the medium in which they are dispersed, among other effects inherent to the nanoparticles (Díaz-Torres et al. 2015; Monopoli et al. 2013). When chondrocytes were exposed to $CdCl_2$ and subsequently to NPs, we observed that GSH levels were not significantly modified relative to the untreated control cultures. $CdCl_2$ has been reported to increase ROS and free radical levels by activating cellular mechanisms to counterbalance the oxidation-reduction imbalance, which can be observed by increasing levels of intracellular GSH (Ogasawara et al. 2014; Delalande et al. 2010). The results of this work showed that the amount of intracellular GSH was increased in chondrocytes exposed to cadmium. These effects in response to Cd toxicity and during the time exposure may have promoted cadmium-induced gene modulation towards genes involved in GSH biosynthesis (Nzenge et al. 2008). The amount of GSH remaining in the culture medium was estimated to determine if the nanoparticles could deliver glutathione to the chondrocytes in culture. Fig. 6B shows that there were no significant differences in the amount of GSH in cells between the cultures with different exposure doses. According to previous and current confocal analyses, our results suggest that NPs could be taken up by chondrocytes and the intracellular and extracellular GSH levels may be associated with the GSH introduced by the NPs.

2.5. Lipoperoxidation

As an oxidative status-related effect, we estimated the lipoperoxidation level through quantification of reactive species to thiobarbituric acid (TBARS) (Ohkawa et al. 1979).

Several pieces of evidence suggest that cadmium-induced ROS (Hussain et al. 1987) contribute to the increased lipid peroxidation promoted by Cd exposure. Additionally, ROS can be generated via cytokines because of the pro-inflammatory effects of Cd(II) in liver tissue.

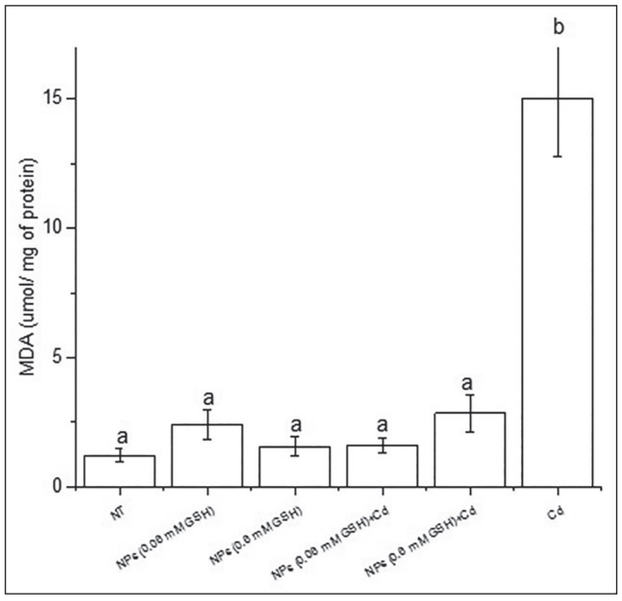

Fig. 7: Effect of NPs at 0.06 mM GSH and NPs at 0.6 mM GSH on TBARS levels. Untreated cells, (NT). Bars with different letters indicate that there are significant differences between the means (Tukey test, p<0.05).

As shown in Fig. 7, cells exposed to NPs with or without cadmium did not show a significant increase in the TBARS amount; however, exposure of the cells to $CdCl_2$ did increase the TBARS level. It is known that the oxidizing potential of cadmium can be directly related to inactivation of antioxidant enzymes and other antioxidant molecules through interaction with the thiol groups present in these molecules, contributing to disruption of cellular homeostasis by compromising the antioxidant mechanisms of the cell. This inactivation increases the ROS and free radicals levels, which results in lipid oxidation (Nzengue et al. 2008).

The GSH concentration was also significantly increased by the need to respond to the increase in the oxidative status reflected by the amount of TBARS. These effects decreased when chondrocytes were exposed to nanoparticles with antioxidant capacity prior to cadmium exposure. Nanoparticles did not significantly modify ($p < 0.05$ %) the GSH levels in chondrocytes. GSH biosynthesis is a complex and very sensitive cellular event that depends on a vast, sensitive and precise enzymatic machinery that maintains the best cellular redox homeostasis conditions (Lushchak 2012). Human chondrocytes reacted to the xenobiotic-induced aggression and the modulatory conditions induced by the effect of the nanoparticles in the study.

Our results suggest that the studied NPs significantly modified the redox state induced by cadmium exposure, decreasing the amount of ROS and free radicals and thus reducing lipid peroxidation in the exposed human chondrocytes. Cd(II) has been shown to deplete GSH in rat liver and kidney cells, possibly by activating g-glutamyl transpeptidase (g-GT) or by depleting NADPH. The system of intracellular disposition of biologically active substances, such as GSH, whose biochemical nature does not allow their access to the cell once synthesized in the intracellular environment and disposed to the extracellular space, is beneficial when maintenance of the oxide reduction state depends on its activity.

2.6. Evaluation of the antioxidant capacity of NPs in chondrocytes exposed to oxidizing agents

A Life Technologies Cell ROX fluorescence test was used to qualitatively and quantitatively evaluate the oxidative reactive species. This test is designed to measure ROS levels, which are proportional to the fluorescence emitted by each cell. For quantification, we used a Talli image-based cytometer. According to the results presented in Fig. 8, when the chondrocytes were exposed to the NPs for 2 h prior to cadmium exposure, we observed that the fluorescence significantly decreased, suggesting an antioxidant effect of the NPs, which reduced the amount of ROS at the intracellular level.

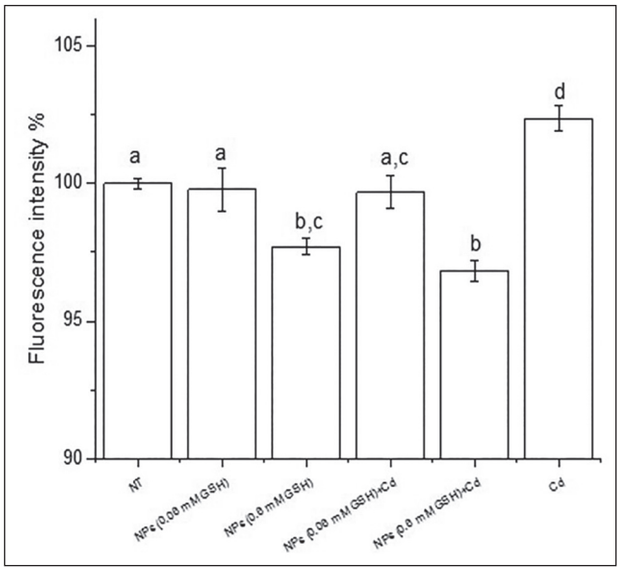

Fig. 8: Effect of NPs at 0.06 mM GSH and NPs at 0.6 mM GSH on the production of ROS in chondrocytes exposed to 1 uM CdCl2. A) Data obtained from the Tali image-based cytometer. The signal generated in untreated cells (NT) is considered as 100%. Bars with different letters indicate that there are significant differences between the means (Tukey test, p<0.05).

In Fig. 8, it can be seen that in cells exposed to NPs, the induced fluorescence was less than that in the cells exposed to cadmium, suggesting that the ROS level was decreased by the NPs. There is sufficient evidence showing that oxidative stress induced by cadmium is related to inhibition of the antioxidant capacity of the cell. Cadmium decreases the activity of antioxidant enzymes, such as catalase, glutathione reductase, glutathione peroxidase, ascor-

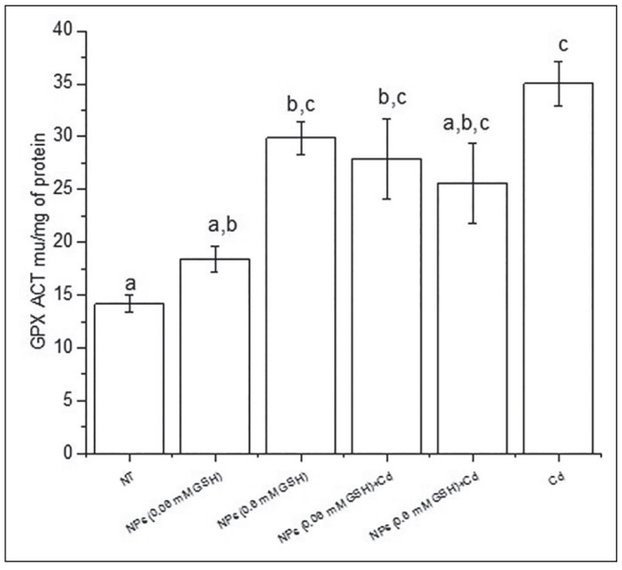

Fig. 9: Effect of NPs at 0.06 mM GSH and NPs at 0.6 mM GSH on GPx activity in chondrocytes exposed to 1 uM CdCl2. Untreated cells, (NT). Bars with different letters indicate that there are significant differences between the means (Tukey test, p<0.05).

bate peroxidase and superoxide dismutase, as well as the interaction of the sulfhydryl groups of glutathione and other antioxidant molecules, contributing to accumulation of reactive oxygen species (Delalande et al. 2010). The number of free radicals according to the results obtained seemed to decrease in chondrocytes exposed to NPs carrying GSH, which could suggest an intracellular redox balance modulating effect of the NPs.

The enzyme glutathione peroxidase (GPx) is one of several molecules involved in the central antioxidant mechanisms at the cellular level. This enzyme is dependent on selenium to support detoxification of endogenously formed hydrogen peroxide and hydroperoxides. GPx reduces peroxides by using GSH as a reducing agent, which is oxidized and converted into glutathione disulfide (GSSG) and then regenerated by the enzyme glutathione reductase (GRx) (Mohamed et al. 2005). To determine the GPx activity in chondrocytes, we exposed the cells to NPs and cadmium (Esworthy et al. 1999).

As shown in Fig. 9, GPx activity was increased in chondrocytes exposed to both NPs and $CdCl_2$. There is also evidence that cadmium can bind to cysteine in reduced glutathione (GSH), resulting in inactivation of GSH-Px and failure to convert H_2O_2 to water (Acharya et al. 2008; Maiorino et al. 1995). When we exposed cells previously treated with GSH NPs to cadmium, GPx activity was modified. Moreover, there is increasing evidence that Cd interacts with Se and disrupts GSH-Px activity, and thus, according to the ROS analysis, TBARS and GSH levels are consequently related to the intracellular accumulation of ROS observed in this work in the absence of nanoparticles.

When the amount of GSH is increased by exposure of cells to the nanoparticles of interest, the cells strive to increase GPx activity. There are reports demonstrating that short-term exposure to cadmium temporarily increases the activity of GPx in response to the presence of hydroperoxides and free radicals produced by this metal in an effort by the cells to reestablish the oxide-reduction equilibrium (Ogasawara et al. 2014).

3. Discussion

Currently, there is no effective treatment to stop the progression of osteoarthritis. Joint injuries are conventionally treated with non-steroidal anti-inflammatories, as well as with glycosaminoglycans, to stop the inflammatory process, reduce pain and improve joint function. However, they only represent a symptomatic treatment whose effectiveness is still debated.

Recent studies have suggested that the use of antioxidant substances, among others, can promote important effects in the treatment of osteoarthrosis. In this regard, the use of substances that directly regulate the oxidative process, the elimination of free radicals or suppression of the inflammatory process have been of interest (Frisbie et al. 2009; Ngo et al. 2015; Rebrin et al. 2008; Adarmes et al. 2009).

Specifically, in this study, we examined the *in vitro* susceptibility of human chondrocytes to exposure to nanoparticles with glutathione and chitosan. The results obtained show that human chondrocytes respond to nanoparticle exposure, showing activation of significant cellular events that regulate oxide-reducing cell responses, evidenced by the intracellular decrease in ROS levels, the increase in glutathione peroxidase activity, the increase in the amount of intracellular GSH and the decrease in TBARS.

The properties of the nanoparticle components are important. Chitosan is a biopolymer obtained through alkaline deacetylation of chitin, which due to its positive charge can adhere to negatively charged surfaces. In addition, chitosan is non-toxic, biodegradable and biocompatible (Liu et al. 2007) and can prevent oxidative damage by interrupting the chain reaction of oxidation and increasing the activity of antioxidant enzymes, such as the enzyme glutathione peroxidase. Moreover, other properties have been attributed to it, such as anti-inflammatory capacity through its ability to inhibit metalloproteinases (Janes 2001).

Another component of the nanoparticles was GSH. This tripeptide is the major cellular antioxidant and participates in several cellular events, such as cell proliferation, apoptosis and maintenance of the cells in a reduced state to promote the maintenance of thiol groups in the proteins, which in turn allows the generation of various intracellular signalling cascades (Kalinina et al. 2014). The most important GSH functions are its ability to act directly on free radicals (ROS) and its activity as a glutathione peroxidase (GSH-Px) cofactor, which donates an electron in the reaction to reduce H_2O_2 or acid peroxides. Once we showed that the nanoparticles were in the cell cytoplasm, we supposed that the observed effects associated with the oxide reduction state studied in this work may be due to the modulatory effect exerted by the nanoparticles under study.

In vivo, there is evidence supporting the susceptibility of synovial fluid to osmotic changes and oxygen and glucose concentration, which puts the maintenance and nutrition of chondrocytes at risk. Alterations in synovial fluid have been associated with the initial degradation of cartilage, leading to inadequate functioning of the chondrocytes and increased redox imbalance, inflammation and cartilage degeneration through an increase in the production of free radicals (Naranjo et al. 2011).

Under optimal physiological conditions, the antioxidant cellular status is controlled through enzymatic and non-enzymatic reactions that contribute to the maintenance of a low ROS concentration. Cell signalling consequently promotes the execution of diverse physiological and biochemical events oriented to support appropriate cell proliferation, differentiation and matrix synthesis. Finally, the fact that in an *in vivo* experimental model, such as primary culture of human chondrocytes, the prepared chitosan and glutathione nanoparticles exhibited significant antioxidant properties is a very important finding with broad perspectives, which allows us to consider future *in vivo* studies to learn more about the effects of these nanometric systems in osteoarthrosis.

4. Experimental

4.1. Nanoparticles

NPs were prepared using an acetic acid solution (1 %) at pH 4.1-4.2, in which we dissolved chitosan at a concentration of 0.3 % and then added Pluronic F-68 (1 %) and GSH (1 %). In addition, a 0.1 % solution of tripolyphosphate (TPP) was prepared. The two solutions were mixed at a 1:1 ratio and stirred for 1 h. The NPs formed were vacuum filtered through a 0.65-µm and then a 0.45-µm cellulose membrane. To obtain fluorescent nanoparticles, we included rhodamine 123 at a concentration of 0.5 mg/ml. We mixed rhodamine 123 with the nanoparticles at a 1:4 ratio overnight. After that, the NPs were centrifuged and washed with an acetic acid solution (1 %).

For nanoparticle characterization, particle size and zeta potential were determined using a Zetasizer and a Nanosight NS300 instrument from Malvern Panalytical. Quantification of GSH inside nanoparticles was conducted indirectly via a colorimetric method using 2,2'-dinitro-5-5'dithiodibenzoic acid (DTNB), and the results were determined at a wavelength of 425 nm.

4.2. Isolation and culture of chondrocytes

Human chondrocytes obtained from human hyaline cartilage were used as an experimental model. The cartilage was obtained from patients who underwent cross-anterior knee ligament surgery. All donors signed a letter of informed consent approved by the Ethics Committee and Research of the National Institute of Rehabilitation (NIR) (Ref INR -. 08/11) to meet all the criteria contained in the Declaration of Helsinki.

The obtained cartilage was placed in PBS with 10 % Pen/Strep antibiotic and centrifuged at 3000 rpm/4 °C. We discarded the supernatant, and collagenase was added and resuspended in DMEM F12 culture medium with 1 % antibiotic and left stirring for 6 h at 37 °C. Chondrocytes were maintained until the third passage under controlled conditions (5 % CO_2, 37 °C and 98 % relative humidity). In the third passage, a cell count was performed using trypan blue, and cells were placed in 6-, 12-, 24- and 48-well plates depending on the determination to be carried out.

To characterize the chondrocytes, they were seeded on coverslips and fixed with 3 % paraformaldehyde (PFA); we then added 0.3 % Tris x100 (Sigma-Aldrich) and PBS with albumin at 0.75 %. Anti-Sox 9 (Abcam) and anti-collagen II (Abcam) antibodies were added at a 1:200 dilution, and Alexa 350 and Alexa 647 secondary antibodies were used at a 1:1000 dilution.

4.3. Cytotoxicity

Cytotoxicity was evaluated according to the assay described by Nakagawa et al. (1996) with some modifications. We fixed cells seeded in 48-well plates with 2.5 % glutaraldehyde, added 0.01 % crystal violet, washed the cells with PBS and added 10% acetic acid. Then, 100 ìL of each sample was placed in 96-well plates, and the absorbance was read on a spectrophotometer at a wavelength of ƛ = 594 nm. We express the results as percent viability relative to the absorbance obtained in cells without treatment.

4.4. GSH quantification

The technique is based on the reaction of GSH present in the sample with DTNB, which produces GS-TNB and TNB - a yellow product (Hu 1994). Confluent cells in

6-well plates were scraped and centrifuged at 12 000 rpm/4 °C. A protease inhibitor cocktail (PMSF 1 mM, 0.1 % Triton and 5 mM EDTA) was added to the obtained pellet, and the sample was again centrifuged at 1200 rpm/4 °C; a portion of the sample was reserved to determine the total protein concentration according to the Bradford method (Bradford 1976). Next, 5 % sulfosalicylic acid was added and incubated with the supernatant, and the sample was centrifuged at 12000 rpm/4 °C. DTNB was added and incubated with the supernatant at 37 °C. We performed the readings at a wavelength of ƛ = 425 nm.

4.5. Lipoperoxidation assay

The lipoperoxidation assay was performed according to those described by Ohkawa et al. (1979) with some modifications. The technique consists of the detection of malondialdehyde (MDA), which is a product of lipid oxidation. MDA reacts with thiobarbituric acid to produce a pink adduct. A protease inhibitor cocktail (PMSF, 0.1 % Triton and 5 mM EDTA) was added to the pellet collected from 6-well plates, and we reserved a portion of the sample to determine the total protein concentration according to the method of Bradford (1976). To the obtained supernatant, we added 2.5 % perchloric acid at room temperature. We centrifuged the samples at 12 000 rpm/4 °C. The supernatant was recovered and frozen until determination. To the treated sample, thiobarbituric acid was added at a concentration of 0.067 %, and the sample was incubated at 90 °C. We performed the readings at a wavelength of ƛ = 540 nm, and we determined the TBARS concentration with a standard malondialdehyde curve.

4.6. Measurement of reactive oxygen species

To estimate the amount of ROS within cells, a Cell ROX kit was used according to the protocol in the brochure provided by Life Technologies. We observed the fluorescence emitted by each cell in an Evos FL auto microscope (Life Technologies) and to determine the ROS amount, the fluorescence intensity emitted by each cell was measured quantitatively using a Tali-image-based Cytometer (Life Technologies) at a wavelength (ƛ) of 640/665 nm.

4.7. Glutathione peroxidase activity

We assessed glutathione peroxidase activity as described by (Esworthy et al. 1999). The technique is based on measuring the decreased NADPH absorption resulting from the coupled reaction of GPX, which utilizes GSH to convert H_2O_2 to H_2O, and the consequent reversal of GSSG production catalysed by glutathione reductase, leading to NADPH conversion to $NADP^+$. Confluent cells in 6-well plates were scraped and centrifuged at 12 000 rpm and 4 °C. The obtained pellet was resuspended in homogenization buffer (50 mM Tris HCl, 5 mM EDTA, 1 mM DTT), mechanically homogenized and stored at -20 °C for one day. Then, the samples were centrifuged at 12 000 rpm and 4 °C, and we collected the supernatant. The samples were placed in Reaction Buffer (GSH 10 mM, sodium azide 1.125 M, NADPH 2 mM, GRx 100 U/ml) in a 96-well plate, and the absorption was read at a wavelength of ƛ = 340 nm.

4.8. Western blotting

We lysed cell samples in buffer (MPER, PhosStop, Complete, DTT, PMSF 1 M) and measured protein concentration with a MICRO BCA TM Protein kit. Next, 30 µg of protein was placed on an 11 % acrylamide gel and allowed to run at 120 mV. Then, the gel was placed on a 0.2-µm nitrocellulose membrane and placed in the trans-blot instrument at 25 mv/1 Am. We probed the membranes with anti-collagen II antibody (Ab3092), anti-SOX 9 antibody (Ab76997), goat anti-rabbit IgG Fc (Ab97200), goat anti-mouse IgG (Ab7040) and monoclonal anti-β-Actin-Peroxidase, an antibody produced in mice (A3854).

4.9. Statistical analysis

We analysed the data using one-way analysis of variance (ANOVA) followed by multiple comparisons of means with a Tukey statistical test. $p<0.05$ was considered to indicate a significant difference.

Acknowledgements: The authors acknowledge the financial support of the following projects: PAPIME PE102118 and PIAPI 1823 from The National University of Mexico.
The authors acknowledge the unvaluable support of M en C Elizabeth Soria Castro in TEM analysis.

Conflicts of interest: None declared.

References

Acharya UR, Mishra M, Patro J, Panda MK (2008) Effect of vitamins C and E on spermatogenesis in mice exposed to cadmium. Reproduc Toxicol 25: 84-88.
Adarmes, H, Solís JP, Müller A, Galleguillos M (2009) Determinación de Nitrito Como Metabolito Estable Del Óxido Nítrico En El Líquido Sinovial de Articulación Metacarpofalángica Equina. Arch Med Vet 259: 255–259.
Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.
De Pinho Neves, Andréia Lange, Milioli CC, Müller L, Riella HG, Kuhnen NC, Stulzer HK (2014) Factorial design as tool in chitosan nanoparticles development by ionic gelation technique. Colloids Surf Physicochem Eng Aspects 445: 34-39.
Delalande O, Desvaux H, Godat E, Valleix A, Junot C, Labarre J, Boulard Y (2010) Cadmium–glutathione solution structures provide new insights into heavy metal detoxification. FEBS J 277: 5086-5096.
Demoor M, Ollitrault D, Gomez-Leduc T, Bouyoucef M, Hervieu M, Fabre H, Lafont J, Denoix J, Audigié F, Mallein-Gerin F (2014) Cartilage tissue engineering: Molecular control of chondrocyte differentiation for proper cartilage matrix reconstruction. Biochim Biophys Acta 1840: 2414-2440.
Díaz-Torres R, López-Arellano R, Escobar-Chávez JJ, García-García E, Domínguez-Delgado CL, Ramírez-Noguera P (2016) Effect of size and functionalization of pharmaceutical nanoparticles and their interaction with biological systems. In: Handbook of Nanoparticles, Springer, pp. 1041-1060.
Dycus DL, Au AY, Grzanna MW, Wardlaw JL, Frondoza CG (2013) Modulation of inflammation and oxidative stress in canine chondrocytes. Am J Vet Res 74: 983-989.
Ercal N, Gurer-Orhan H, Aykin-Burns N (2001) Toxic metals and oxidative stress part I: Mechanisms involved in metal-induced oxidative damage. Curr Top Med Chem 1:529-539.
Esworthy R, Chu F, Doroshow J (1999) Analysis of glutathione-related enzymes. Curr Prot Toxicol 7.1. 1-7.1. 32.
Frisbie DD, Kawcak CE, Wayne Mcilwraith C, Werpy NM (2009) Evaluation of polysulfated glycosaminoglycan or sodium hyaluronan administered with experimentally induced osteoarthritis. Am J Vet Res 70: 203–209.
Hu M (1994) Measurement of protein thiol groups and glutathione in plasma. In: Methods in Enzymology, Elsevier, pp 380-385.
Hussain T, Shukla GS, Chandra S (1987) Effects of cadmium on superoxide dismutase and lipid peroxidation in liver and kidney of growing rats: In vivo and in vitro studies. Basic Clin Pharmacol Toxicol 60: 355-358.
Janes KA, Fresneau MP, Marazuela A, Fabra A, Alonso MJ (2001) Chitosan nanoparticles as delivery systems for doxorubicin. J Control Release 73: 255-267.
Kalinina EV, Chernov NN, Novichkova MD (2014) Role of glutathione, glutathione transferase, and glutaredoxin in regulation of redox dependent processes. Biochemistry (Moscow) 79: 1562–1583.
Lin Z, Fitzgerald JB, Xu J, Willers C, Wood D, Grodzinsky AJ, Zheng MH (2008) Gene expression profiles of human chondrocytes during passaged monolayer cultivation. J Orthopaed Res 26: 1230-1237.
Liu C, Tan Y, Liu C, Chen X, Yu L (2007) Preparations, characterizations and applications of chitosan-based nanoparticles. J Ocean Univ China 6: 237-243.
Lushchak VI (2012) Glutathione homeostasis and functions: Potential targets for medical interventions. J Amino Acids 2012: 736837.
Maiorino FM, Brigelius-Flohé R, Aumann K, Roveri A, Schomburg D, Flohé L (1995) Diversity of glutathione peroxidases. In: Methods in Enzymology, Elsevier, pp. 38-53.
Maneesh M, Jayalekshmi H, Suma T, Chatterjee S, Chakrabarti A, Singh T (2005) Evidence for oxidative stress in osteoarthritis. Indian J Clin Biochem 20:129-130.
Mohamed A, Bakr MA, Farahat SE, El-Fattah EAA (2005) Glutathione peroxidase activity in patients with renal disorders. Clin Exp Nephrol 9:127-131.
Monopoli MP, Pitek AS, Lynch I, Dawson KA (2013) Formation and characterization of the nanoparticle–protein corona. In: Nanomaterial Interfaces in Biology, Springer, pp 137-155.
Nakagawa T, Sawada M, Gonzalez FJ, Yokoi T, Kamataki T (1996) Stable expression of human CYP2E1 in chinese hamster cells: High sensitivity to N, N-dimethylnitrosamine in cytotoxicity testing. Mutat Res 360:181-186.
Naranjo J, Sánchez C, López D (2011) Fisiopatología Celular de La Osteoartritis: El Condrocito Articular Como Protagonista. Iatreia 24: 167–178.
Ngo DH, Vo TS, Ngo DN, Kang KH, Je JY, Pham HND, Byun HG, Kim SK (2015) Biological effects of chitosan and its derivatives. Food Hydrocoll 51: 200–216.
Nzengue Y, Steiman R, Garrel C, Lefebvre E, Guiraud P (2008) Oxidative stress and DNA damage induced by cadmium in the human keratinocyte HaCaT cell line: Role of glutathione in the resistance to cadmium. Toxicology 243:193-206.
Ogasawara Y, Takeda Y, Takayama H, Nishimoto S, Ichikawa K, Ueki M, Suzuki T, Ishii K (2014) Significance of the rapid increase in GSH levels in the protective response to cadmium exposure through phosphorylated Nrf2 signaling in jurkat T-cells. Free Rad Biol Med 69: 58-66.
Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95: 351-358.
Poole AR (2003) Biochemical/immunochemical biomarkers of osteoarthritis: Utility for prediction of incident or progressive osteoarthritis. Rheum Dis Clinics North Am 29: 803-818.
Rebrin I, Sohal RS (2008) Pro-oxidant shift in glutathione redox state during aging. Adv Drug Deliv Rev 60:1545-1552.
Sandell LJ, Aigner T (2001) Articular cartilage and changes in arthritis. An introduction: Cell biology of osteoarthritis. Arthritis Res 63: 456–469.
Stokes DG, Liu G, Dharmavaram R, Hawkins D, Piera-Velazquez S, Jimenez SA (2001) Regulation of type-II collagen gene expression during human chondrocyte de-differentiation and recovery of chondrocyte-specific phenotype in culture involves sry-type high-mobility-group box (SOX) transcription factors. Biochem J 360: 461-470.
Wu L, Zhang J, Watanabe W (2011) Physical and chemical stability of drug nanoparticles. Adv Drug Deliv Rev 63: 456-469.