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Ex vivo effect of gold nanoparticles on porcine synovial membrane

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Abbreviations: AuNP, gold nanoparticle; AuNPs, gold nanoparticles; LPS, lipopolysaccharide; IL-1β, interleukin-1β; PGE2, prostaglandin E2; HA, hyaluronic acid; MMP, matrix metalloproteinase; LDH, lactate dehydrogenase

Gold nanoparticles (AuNPs) have great potential as carriers for local drug delivery and as a primary therapeutic for treatment of inflammation. Here we report on the AuNP-synovium interaction in an ex vivo model of intra-articular application for treatment of joint inflammation. Sheets of porcine femoropatellar synovium were obtained post mortem and each side of the tissue samples was maintained in a separate fluid environment. Permeability to AuNPs of different sizes (5–52 nm) and biomarker levels of inflammation were determined to characterize the ex vivo particle interaction with the synovium. Lipopolysaccharide or recombinant human interleukin-1β were added to fluid environments to assess the ex vivo effect of pro-inflammatory factors on permeability and biomarker levels. The synovium showed size selective permeability with only 5 nm AuNPs effectively permeating the entire tissue’s width. This process was further governed by particle stability in the fluid environment. AuNPs reduced matrix metalloproteinase and lactate dehydrogenase activity and hyaluronic acid concentrations but had no effect on prostaglandin E2 levels. Exposure to pro-inflammatory factors did not significantly affect AuNP permeation or biomarker levels in this model. Results with ex vivo tissue modeling of porcine synovium support an anti-inflammatory effect of AuNPs warranting further investigation.

Introduction

The medicinal use of gold can be traced back through many centuries, although historical proof for its efficacy is lacking.1 Today’s evidence supports the systemic use of gold salts in man for the treatment of inflammatory arthritis2-5 but the practical utility is decreased by the potential cytotoxic side effects.6 There are reports of reduced pain and improved limb use in human and canine patients with arthropathies that receive gold beads implanted at acupuncture sites.7,8 The suggested beneficial effect is likely attributable to the local liberation of gold ions that can be traced to the surrounding connective tissues.9

As an alternative to the use of gold salts or locally applied gold particles, intra-articular administration of gold nanoparticles (AuNPs) has recently been investigated.10,11 These studies showed that AuNPs reduced synovial pro-inflammatory cytokines and cellular infiltrates and resulted in fewer clinical and radiographic abnormalities in a rodent model of collagen induced arthritis. In addition to a potential primary therapeutic use, AuNPs may also serve as carriers for local drug delivery and thereby increase the half-life of therapeutic levels of select drugs.12-14 Future intra-articular application of AuNPs will require specific knowledge of the synovial barrier-carrier interaction, including the permeability of the synovium to nanoparticles. Ex vivo tissue modeling techniques are well established in a variety of biomedical fields and form an important step in advancing bench top discoveries to in vivo applications and ultimately clinical practice.15,16 Advantages associated with their use include the control and standardization of the experimental environment while maintaining physiologic tissue responses.17,18 We are not aware of the use of similar techniques as described herein for modeling of synovial pathophysiology. On the basis of these techniques we report on the AuNP-synovium interaction and synovial membrane permeation for potential future intra-articular application in treating arthritis. We hypothesized that by using ex vivo tissue modeling methods we could evaluate the size-selective permeability of the synovium to AuNPs and determine whether this permeability pattern changes in the presence of lipopolysaccharide (LPS) or interleukin-1β (IL-1β), activators of synovial inflammatory cascades.19,20 Furthermore, we hypothesized that tissues exposed ex vivo to AuNPs would show a reduction in inflammatory biomarkers compared with control tissues.

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Results

Details on experimental groups and sampling protocol are shown in Figure 1. Tissues exposed to AuNPs received either pro-inflammatory factors (Group AuNP/P-Infl), LPS and IL-1β or their respective vehicle (Group AuNP/V). Groups that were not exposed to AuNPs (control group) received either IL-1β (C/IL-1β) or IL-1β vehicle (C/IL1V).

AuNP permeation through the synovium. AuNP permeation through the synovial membrane from the articular to the non-articular chamber compartment was evaluated on the basis of the following measures: The AuNP dose reduction (H1 minus H2 sample concentration), the sequential and cumulative amount of diffusing elemental gold (individual or sum of sample concentrations S1-S10) and the histomorphometric quantitation of gold specific tissue stains. The average AuNP dose (H1 sample) was 7.63 mg/L (range 3.17–18) which at the end of experiments (H2 sample) was reduced to 6.48 mg/L (H2 sample; range 1.11–14.08 mg/L). The average percent reduction in articular AuNP concentration was 23% (SEM 2.63%) for 5 nm, 22.6% (SEM 5.5%) for 10 nm, 3.2% (SEM 2.88%) for 20 nm and 7.2% (SEM 3.73%) for 52 nm AuNPs.

Dry state particle size significantly determined the amount of AuNP diffusion assessed by both histomorphometry and cumulative and sequential elemental gold concentrations (p = 0.001). While lesser diffusion was appreciated with greater AuNP size, only comparisons with 5 nm particles reached significance. This illustrated the fact that effective tissue permeation only occurred with the smallest particles (Fig. 2). Next to particle size, the time of sampling also exerted a significant effect in all kinetic data analyses (p < 0.001). As stated previously, this is explained by 5 nm particles permeating through the entire tissues’ width, which at the most measured 379.5 µm (SEM 20.9 µm) and at the least 216.3 µm (SEM 17.4 µm).
Examples of the size-dependent permeation of the synovial membrane are demonstrated in Figure 4. Transmission electron microscopy was performed as an alternate method to gold staining to validate the presence of AuNPs in tissues, confirming the presence of AuNPs in extra- and intracellular locations. Relatively few particles were observed in the interstitium, presumably due to particle loss during sample preparation. Figure 5 shows synoviocytes and surrounding interstitium after exposure to 5 nm AuNPs and demonstrates that particles were diffusely distributed throughout the cytoplasm. This observation suggests uptake of the AuNPs through the cytoplasmic membrane independent of endocytosis (Fig. 5C). Evidence suggestive of possible endosomal uptake in the form of a membrane bound electron lucent vesicle containing AuNPs (white arrow) and mitochondrial penetration (asterisk) was also seen (Fig. 5D). Extracellular locations of larger agglomerates are indicated by black arrows (Fig. 5A and B). Nuclear penetration was not observed.

Effect of pro inflammatory factors on AuNP permeation. When examining the individual effect of the two pro-inflammatory factors (LPS or IL-1β) and their vehicle controls (LPSV or IL1V) on synovial permeability, chambers containing IL-1β vehicle (1% bovine serum albumin solution) displayed significantly greater sequential and cumulative AuNP permeation (p = 0.001). Figure 3 illustrates this effect for 5 nm AuNP permeation. When compared with each of their vehicle controls, neither of the two factors exerted a measurable effect on ex vivo synovial AuNP permeation. With respect to the effect of “time,” a significant difference in permeating AuNPs concentrations over baseline values was noted for vehicle and IL-1β use, 105 min and 90 min respectively after dosing (p = 0.0119; p = 0.0232).

AuNP effect on biomarkers of inflammation. To assess the effect of permeating AuNPs on synovial tissues, biomarkers of inflammation (prostaglandin E₂, PGE₂; matrix metalloproteinases, MMP), cell integrity (lactate dehydrogenase, LDH) and synoviocyte function (hyaluronic acid, HA) were assessed in non-articular fluid samples. There was

![Figure 2. Size dependent permeation of the synovial membrane assessed by histomorphometry. Bars represent the average surface area of the gold specific tissue stain (pixel²) for each size of gold nanoparticle (AuNP) used (5, 10, 20 and 52 nm). Significance was reached for all comparisons with 5 nm AuNPs (p < 0.001). Error bars represent the standard error for the mean.](image1)

![Figure 3. Time-dependent permeation of 5 nm gold nanoparticles through the synovial membrane. A significant difference in synovial AuNP permeation was observed between chambers receiving IL-1β vehicle and LPS or LPS vehicle. Significantly different permeation curves (p < 0.05) are indicated by different letters. Stars above time points indicate when and for what group sample concentrations were significantly different from baseline values. Error bars represent the standard error for the mean (LPS, lipopolysaccharide; IL-1β, interleukin-1β; V, vehicle).](image2)
no significant association of the experimental group with PGE$_2$ concentrations. Tissues that were exposed to AuNPs showed significantly less MMP (p < 0.001) and LDH (p < 0.001) activity and lower HA concentrations (p < 0.001). For LDH and HA this significance was limited to individual comparisons with Group C/IL1V (Fig. 6). Positive correlations between biomarkers were

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**Figure 4.** Representative images of the size dependent permeation of the synovial membrane. Tissues had been exposed to (A) 5 nm AuNPs, (B) 10 nm AuNPs, (C) 20 nm AuNPs and (D) 52 nm AuNPs prior to gold enhancement. Five nanometer AuNPs can be seen penetrating all tissue layers. Elemental gold appears black (AuNPs, gold nanoparticles; scale bar = 50 μm).

**Figure 5.** Representative transmission electron microscopic images of AuNP localization. By enlarge AuNPs were scattered throughout the cytoplasm and organelles of synoviocytes including the smooth endoplasmatic reticulum and mitochondria (C and D). Extracellular location of large particle agglomerates were also appreciated (A and B). Evidence for larger AuNPs agglomerates in endosomes (white arrow) is illustrated in (D).
with AuNPs added. PGE2 and HA concentration standards with AuNPs added resulted in an average increase equal to 56 and 4.5% of the value when AuNPs were not present (corrected for dilution). MMP and LDH activity decreased by 22.7 and 0.77%.

**Effect of pro-inflammatory factors on biomarkers of inflammation.** No significant difference in biomarker levels was observed between tissues exposed to pro-inflammatory factors and their controls (Fig. 6).

**Histopathology of the synovial membrane.** Histologic changes were assessed on the basis of a semi-quantitative grading scale for which examples are shown in Figure 7. Notable ex vivo changes were limited to synovial ulcerations. Only one of the synovial membrane samples was categorized as edematous (Grade 2). When Group C/IL1β was considered the reference standard, the proportion of histologic grades differed significantly among the three remaining groups with 63% in Group AuNP/P-Infl, 59% in Group AuNP/V and 0% in Group C/IL-1β showing ulceration greater than 5% of the sections’ surface.
contrast to our expectations, ex vivo exposure to pro-inflammatory factors did not significantly affect biomarker levels. This is likely the result of the inherent activation associated with tissue collection that cannot be surpassed by further ex vivo stimulation. A similar phenomenon has been observed when using porcine gastrointestinal tissues, which required pre-treatment with a cyclooxygenase inhibitor to study subsequent prostaglandin-dependent outcome measures. The porcine and murine IL-1β cDNA sequence show 74% homologies with the human form and use of human recombinant IL-1β has been effective in tissues from both species. Nevertheless, heterology between human and porcine cytokines should also be considered in the absence of a significant IL-1β effect. Concerning the selection of IL-1β as the pro-inflammatory factor, the role of this cytokine in joint disease has been well documented. The dose applied was chosen taking previous in vitro and in vivo heterologous use of recombinant human IL-1β into consideration and adjusting for the typical fluid volume in injected joints vs. the Ussing chamber system. Despite these considerations the dose selection may have been inappropriate. The authors are aware of these specific limitations and future investigations will include the use of recombinant porcine IL-1β and dose-response experiments to further elucidate the pro-inflammatory potential of this cytokine in an ex vivo model of synovial tissue.

A previous in vivo study on rabbit synovium illustrated a loss in barrier function and increase in diffusion of hyaluronic acid molecules with enzymatic degradation of extracellular matrix proteins. In our study, use of IL-1β did not initiate a similar effect on AuNP permeation. This may have been caused in part by factors described in the preceding section. However, inability

\[ p = 0.0087 \]. However in statistical analyses the histologic grade had no effect on biomarker levels.

**Particle stability in the fluid environment.** The hydrodynamic size of AuNPs in components of articular fluid media was assessed to interpret the particles’ ability to permeate tissues in chamber experiments in the context of physical and functional size. When AuNPs were added to articular media, an increase in hydrodynamic size was noted which was attributed to particle agglomeration. This effect was most pronounced for 5 nm particles and when LPS was added to the media (Table 1). Hydrodynamic sizes of AuNPs in IL-1β vehicle containing fluids remained relatively unchanged confirming a stabilizing effect of the 1% bovine serum albumin solution and supporting findings shown in Figure 3.

**Discussion**

Confirming the authors’ hypothesis, samples of synovial membrane were successfully mounted in the Ussing chamber and demonstrated size selective permeability to AuNPs both on inductively coupled plasma mass spectrometry and histomorphometry. However, effective permeation of tissue samples measured with mass spectrometry was only observed using AuNPs of 5 nm dry state size. Previous in vivo work in rabbit stifles suggests that effective matrix pore size ranges between 66 and 118 nm allowing smaller molecules to leave the joint environment freely. Our observation that 52 nm particles were unable to breach the subintima may be in line with these findings but the propensity of bare AuNPs to agglomerate and result in greater hydrodynamic size may make this conclusion less relevant (Table 1).

Figure 7. Representation of synovial ulceration. The degree of synovial ulceration was graded based on the estimated percentage of synovium missing over the length of the sample evaluated. (A) shows a representation of the synovial lining at 60× magnification that was considered normal; a continuous lining of synovial cell is present. (B) Represents tissue at the same magnification with ulceration of the synovium; a discontinuous lining of synovial cell is observed over the subsynovial connective tissue (bar = 30 μm).
to recruit vascular effector cells promoting tissue inflammation and degradation in an ex vivo model may also be involved. In this study LPS exposure appeared to reduce synovial permeability to AuNPs compared with IL-1β use. However this effect was predominantly driven by the absence of IL-1β vehicle, leading to particle agglomeration and reduced permeation.

Nanoparticles show complex behavior in biological systems based on their composition, size, shape, surface charge and chemistry. Derived from the intensity of scattered light the hydrodynamic size describes the dimension of a spherical particle in fluid media surrounded by the solvation layer. It provides information on the particle’s stability or agglomeration state which is dependent on surface charge and ultimately the particle’s interactions with different fluid components (ions and proteins). From the hydrodynamic sizing of AuNPs in different chamber fluid components it can be concluded that particles agglomerated in saline solution and that this was increased with the presence of LPS and decreased with the use of 1% bovine serum albumin solution. These effects have in part been reported previously and are supported by our conclusion that presence of IL-1β vehicle increased AuNP permeation.

From the results it may be further concluded that AuNPs promoted cell viability (reduced LDH activity), reduced MMP activity and HA concentrations while exerting no significant effect on PGE₂ concentrations. These findings need to be interpreted with caution as evidenced by the limited investigation of the AuNP effect on performed assays. Nanoparticles have the ability to interfere with biological assays based on adsorbance of reagents or reporter dyes, optical absorbance and interaction with the primary analyte. Although LDH assay performance was not affected by AuNPs in our study, this potential bias has been demonstrated for other nanoparticles. In contrast, PGE₂ assays overestimated true concentrations by an average of 56%. Prostaglandin immunoassays are based on the competitive antibody binding of sample PGE₂ and acetylcholinesterase bound PGE₂ molecules. Enzyme driven conversion of Ellman’s reagent results in a yellow color reaction that is inversely proportional to sample PGE₂ concentrations. Considering the great affinity of nanoparticles to acetylcholinesterase, overestimation of the actual PGE₂ content was likely caused by AuNP-dependent enzyme inhibition. Assuming that PGE₂ concentrations are in fact lower than reported, they may be in line with the observed trend that AuNPs reduce biomarkers of inflammation.

Based on the absence of a marked nanoparticle effect on HA assay performance, the conclusion that AuNPs reduced HA concentrations is likely valid. IL-1β stimulation has the potential to increase HA production via activation of synthases located on the inner surface of cell membranes. In this process synthases continuously assemble disaccharide molecules to form longer chains, extruding from the cell surface into the extracellular space. Possible explanations for the observed reduction in HA levels may include AuNP dependent inhibition of synthases or IL-1β adsorption. Alternatively, low concurrent LDH activity levels may be indicative of fewer HA chains being dissociated from the cell surface due to greater membrane stability when AuNPs were present. However, this potential explanation is contradicted by the histological finding that exposure to AuNPs resulted in greater synovial ulceration.

As alluded to previously, nanoparticles have the ability to interfere with cellular function based on their reactivity and binding of key proteins. This reportedly includes vascular endothelial growth factor and IL-1β. Exposure of LPS-activated macrophages, a cell line similar to synoviocytes (Type A), to polyethylene glycol coated AuNPs suggests that direct effects also include inhibition of inducible nitric oxide synthase expression. Besides these effects, administration of gold particles may lead to gold ion elution which in turn could result in therapeutic effects comparable to those of other gold-based pharmaceuticals. Most importantly these effects could include inhibition of NF-kappa B and downregulation of the p38 MAPK pathway. In the present experiments, synovial tissue samples were maintained in Ussing chambers for 195 min. From other applications it is known that treatment-induced changes in PGE₂ concentrations occur within this experimental time frame. Whether this time is sufficient for the observed changes in HA or MMP levels to develop remains to be determined but causes that involve downstream interference at the level of enzyme function rather than the signaling pathway may be more plausible. In fact, significant in vitro IL-1β activation of intracellular signaling pathways to increase MMP synthesis was detected one hour post stimulation. Recent evidence obtained in vivo demonstrated a significant change in MMP and PGE₂ concentrations 4 h post IL-1β exposure. Taken together this may suggest that our experimental time frame may have been too short to illustrate significant upstream effects on biomarker concentrations.

Given that in our experiments, ability to permeate tissues was not associated with a greater effect on biomarkers, in vivo use of larger AuNPs may be preferred in an attempt to minimize systemic absorption and potential toxic side effects. Support for this conclusion may be found in the only other study available for comparison, which demonstrated that a positive in vivo treatment

### Table 1. Hydrodynamic size of gold nanoparticles (AuNPs) in different fluid environments

| Dry state size (nm) | In distilled water (nm) | In simulated chamber fluid (nm) | In simulated chamber fluid + IL1β (nm) | In simulated chamber fluid + IL1β-C (nm) | In simulated chamber fluid + LPS (nm) |
|---------------------|------------------------|-------------------------------|--------------------------------------|-----------------------------------------|-------------------------------------|
| 5                   | 30                     | 700                           | 157                                  | 72                                      | 790                                 |
| 10                  | 24                     | 432                           | 98                                   | 78                                      | 495                                 |
| 20                  | 25                     | 345                           | 69                                   | 62                                      | 489                                 |
| 52                  | 64                     | 284                           | 88                                   | 84                                      | 293                                 |

IL1β-C, interleukin-1β vehicle; LPS, Lipopolysaccharide.
effect of AuNPs was also not dependent on particle size. Interestingly, next to other dramatic positive effects of AuNPs in this study, a reduction in cell necrosis was also reported, further validating our observation of reduced LDH activity.

Regarding systemic side effects, murine studies suggest that after intravenous administration biodistribution of AuNPs is in part size dependent and that particles predominantly accumulate in the liver, lung and spleen but can also be found in the brain if smaller than 50 nm. Gene expression profiling of liver part size dependent and that particles predominantly accumulate after intravenous administration biodistribution of AuNPs is in vivo validated our observation of reduced LDH activity. In this study, a reduction in cell necrosis was also reported, further validating our observation of reduced LDH activity.

Interestingly, next to other dramatic positive effects of AuNPs in vivo, it is further confirmed that AuNPs are low in the context of total body exposure and potential systemic biodistribution (0.00148 mg/kg; by approximation). Based on this and previous work it is further confirmed that AuNPs are low in the context of total body exposure and potential systemic biodistribution (0.00148 mg/kg; by approximation).

In summary, the results herein suggest that intra-articular application of 5 nm AuNPs (dry state size) leads to particle permeation through the joint capsule, facilitating systemic bioavailability. Based on this and previous work it is further confirmed that nanoparticles leave the joint cavity in a size dependent manner either unassisted or within cells and accumulate in the reticuloendothelial system. Beyond a yet to be determined size but likely in the range of 50 nm, we anticipate AuNPs to be largely retained within the synovial environment. We consider this to be a relevant characteristic in light of the potential use of AuNPs as drug carriers or primary therapeutics and the potential for systemic side effects. In this investigation, we associate AuNP administration with a significant reduction in inflammatory biomarkers except PGE 2, but limitations of this study have to be considered. Future studies will seek to validate our ex vivo experiments by including more analytical time points, in vivo exposure to homologous cytokines and AuNP while still collecting ex vivo outcome data. This approach will minimize potential bias of AuNPs on assays, increase the stimulatory effect of pro-inflammatory cytokines and allow time dependent assessment of biomarkers.

**Materials and Methods**

**Tissue acquisition.** Synovial membrane samples were obtained from pigs sacrificed at the authors’ institution for unrelated research or teaching purposes approved by the Institutional Animal Care and Use Committee. Animals weighed between 22 and 31 kg and were euthanized with an intravenous barbiturate overdose. In each animal synovial membrane was harvested from one femoropatellar joint. To do so the patella was released from its proximal and distal attachments and bisected to open the joint in a “barn-door” fashion resulting in two areas of preserved synovial membrane located between each femoral trochlear ridge and hemi-patella. Continuous tissue samples were obtained by blunt dissection underlining the synovial membrane in that area. Within one hour, samples were transported to the laboratory in oxygenated (95% O 2 and 5% CO 2) Ringer’s solution (323.9 mOsml/l; Na+ 154.1, K+ 6.3, Cl− 137.3, H 2PO 4− 0.3, Ca 2+ 1.2, Mg 2+ 0.7, HCO 3− 24 mmol/l) at room temperature and mounted in Ussing chambers with a 1.14 cm 2 aperture.

**Ussing chambers.** Synovial tissue samples were bathed on the intima (articular) and subintima (non-articular) side with 5 ml of oxygenated Ringer’s solution maintained at 37°C via water-jacketed reservoirs. Glucose was added to the non-articular side and mannitol to the articular side (10 mmol/L) of the Ussing chamber for oncotic balance. At time “0,” the articular side of the chambers were either treated with 100 ng/ml of LPS in Ringer’s solution (from *E. coli* 0111:B4, Sigma-Aldrich), 20 ng/ml of human recombinant IL-1β (SRP3083, Sigma-Aldrich) suspended in a 1% solution of bovine serum albumin or vehicle control solution (Ringer’s solution or bovine serum albumin solution). After one hour the articular fluid compartments were either dosed with an unconjugated spherical gold nanoparticle (AuNP) solution (Nanopartz, Accurate Spherical Gold Nanoparticles, mean particle sizes 5, 10, 20 and 52 nm) that was previously sonicated for one minute or with a saline control. Subsequently the bathing fluid of the non-articular side was sampled (1 ml) and every 15 min thereafter until completion of the experiment (S1–S10, 60–195 min post tissue mounting). Fluid of the articular compartment was sampled twice (0.5 ml at time “60” (H1) and “195” (H2)). Non-articular reservoir fluids were replenished using the Ringer’s and glucose solution. All fluid samples were immediately frozen in liquid nitrogen and stored at -80°C until they were thawed on ice, vortexed and further analyzed.

**AuNP hydrodynamic size.** Samples of the nanoparticle dosing solution were mixed with the simulated articular fluid media or distilled water (1:4), sonicated for 5 min and the hydrodynamic size was assessed in a 100 μl sample using dynamic light scattering methods (ZetaSizer, Malvern Instruments). Size measurements were averaged across 60 repeated measurements obtained in triplicate runs of the same sample.

**AuNP quantitation.** Elemental gold concentration in articular and non-articular fluid samples was determined using inductively coupled plasma mass spectrometry (Varian 820) with an estimated detection limit of 0.1 μg/L. Gold concentrations of samples (S1–S10) were summed to give a cumulative amount of permeating AuNPs for each chamber.

**Prostaglandin E 2 (PGE 2) quantitation.** A competitive ELISA kit (Cayman Chemical, Item No 514010) was used with samples analyzed in triplicate. Outliers that resulted in a coefficient of variance greater than 30% were excluded from the analysis.

**Hyaluronic acid (HA) quantitation.** An enzyme linked binding protein assay was used to determine HA concentrations (ng/ml) of articular fluid samples in duplicate analyses (Corgenix Inc., 029-001).

The effect of gold particles on the PGE 2 and HA assays was determined by running the standards (S4 and medium molecular weight HA) with and without AuNPs added. This was performed in duplicate for each nanoparticle size.
Matrix metalloproteinase (MMP) activity. Using a previously described technique\textsuperscript{3} MMP activity (MMP 2, 3, 7, 9, 12 and 13) was determined using an activatable near infrared (NIR) fluorescent probe added to articular fluid samples in a 96-well plate (MMPSense 750 FAST, PerkinElmer) and a NIR fluorescence reader (Ivis Lumina II, PerkinElmer).

The effect of AuNPs on MMP measurements was investigated by repeated analysis of three fluid samples with and without 5 nm AuNPs added.

Lactate dehydrogenase (LDH) activity. A toxicology assay kit (TOX7, Sigma-Aldrich) was used to determine the enzyme’s activity in articular fluid samples based on a stoichiometric colorimetric reaction measured at a wavelength of 490 nm. Samples of Group C/IL1V were rerun in duplicate with AuNPs (all sizes) added.

Tissue processing. Tissue samples were fixed using MacDowell’s and Trump’s 4F:1G solution, embedded in paraffin and three cross sections per synovial membrane were acquired. One section was stained with hematoxylin and eosin (H&E) only, two underwent autometallurgical gold gold enhancement for 20 min (Goldenhance-LM/Blot, Nanoprobes) and one of these was later also stained with H&E. Tissue samples were also embedded in Spurr resin followed by ultrathin sectioning for transmission electron microscopy.

Histomorphometry. Slides were anonymized, randomized and for each synovium sample, the entire length of the sample was photographed at 10× and 40× using an Olympus DP 25 digital microscope camera with the CellSens digital imaging software mounted on an Olympus BH 41 microscope (Olympus Corporation, Tokyo, Japan). Using Adobe Photoshop CS4 (PhotoshopCS4; Adobe Systems) and via batch processing, 40× images were converted to 6,000 × 4,500 pixels (100 pixels/cm) and black and white in order to highlight the gold particle. The batch processing procedure allowed the same setting to be applied to all images in a given data set. The best setting was determined empirically by testing images with the lowest and highest amount of gold particles. Finally, images were thresholded in the “intermodes” setting and the area fraction covered with the gold particles, expressed as a percentage of the picture surface area, was measured using an open source image analysis system (ImageJ, NIH). 10× images were used to measure the tissue’s maximum and minimum width in each image and measurements were subsequently averaged to give an average maximum and minimum width for each sample.

Histology. A board certified pathologist, blinded to the identity of each slide graded the degree of synovial ulceration (0 = no ulcer or less than 5%; 1 = up to 25%; 2 = up to 50%; 3 = up to 75% and 4 = more than 75% of the synovial surface) and sub-synovial connective tissue edema (0 = no edema; 1 = mild; 2 = moderate; 3 = severe). Tissue samples that were neither stimulated nor had gold exposure (Group C/IL1V) were used as a reference standard.

Transmission electron microscopy. Synovial samples were fixed in 4F:1G, dehydrated in a graded series of ethanol and embedded in Spurr resin.\textsuperscript{74} Semithin sections, 90 nm thick, stained with 1% toluidine blue in 1% sodium borate, were examined under a light microscope. Ultrathin sections, 90 nm thick, of appropriate blocks were examined with a FEI/Philips EM 2085 transmission electron microscope. Only tissue samples exposed to 5 nm AuNPs were examined.

Statistical analyses. A Fisher’s exact test was used to compare proportions of histologic grades by experimental group (AuNP/P-Infl, AuNP/V, C/IL1-B). Using the mixed procedure (SAS 9.2, SAS Institute Inc.) the significance of individual independent variables, AuNP presence (yes or no), AuNP size (5, 10, 20, 52 nm), use of pro-inflammatory factor (yes or no), type of pro-inflammatory factor (IL-1B or LPS), experimental group (AuNP/P-Infl, AuNP/V, C/IL1V, C/IL1-B), was determined. Variables showing co-linearity were analyzed separately. Models were fitted separately for each biomarker (PGE\textsubscript{2}, MMP, LDH, gold stain area, cumulative elemental gold concentration). Relevant comparisons were performed using an LSMEANS statement and the Tukey adjustment for multiple testing. Relationships between dependent variables were investigated using Pearson correlations.

Kinetic data obtained with mass spectroscopic gold determinations were analyzed using repeated measures ANOVA with “time” as the repeated variable. For all statistical analyses the critical p value was set at 0.05.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.

References
1. Higby GJ. Gold in medicine: a review of its use in the West before 1900. Gold Bull 1982; 15:130-40; PMID:1614517; http://dx.doi.org/10.1007/BF0321618.
2. Rau R. Have traditional DMARDs had their day?\textsuperscript{5}\textsuperscript{5}\textsuperscript{5}\textsuperscript{5}\textsuperscript{5}
3. Higby GJ. Gold in medicine: a review of its use in the West before 1900. Gold Bull 1982; 15:130-40; PMID:1614517; http://dx.doi.org/10.1007/BF0321618.
4. Lehman AJ, Edaile JM, Klinkhoff AV, Grant E, Fitzgerald A, Canvin J; METGO Study Group. A 48-week, randomized, double-blind, double-blinded, placebo-controlled multicenter trial of combination methotrexate and intramuscular gold therapy in rheumatoid arthritis: results of the METGO study. Arthritis Rheum 2005; 52:1-160;70; PMID:15880810; http://dx.doi.org/10.1002/art.21018.
5. Smolen JS, Landewé R, Breedveld FC, Dougados M, Emery P, Gaujoux-Viala C, et al. EULAR recommen- dations for the management of rheumatoid arthritis with synthetic and biological disease-modifying anti-rheumatic drugs. Ann Rheum Dis 2010; 69:964-75; PMID:20444750; http://dx.doi.org/10.1136/ard.2009.126532.
6. Sandor O, Herborn G, Bock E, Rau R. Prospective six year follow up of patients withdrawn from a randomised study comparing parenteral gold salt and methotrexate. Ann Rheum Dis 1999; 58:281-7; PMID:10225812; http://dx.doi.org/10.1136/ard.58.5.281.
7. Nejrup K, Olivarius NdB; Jacobsen JL, Siersma V. Randomised controlled trial of extraarticular gold bead implantation for treatment of knee osteoarthritis: a pilot study. Clin Rheumatol 2008; 27:1363-9; PMID:18500437; http://dx.doi.org/10.1007/s10067-008-0918-9.
8. Jaeger GT, Larsen S, Sali N, Moe L. Double-blind, placebo-controlled trial of the pain-relieving effects of the implantation of gold beads into dogs with hip dysplasia. Vet Rec 2006; 158:722-6; PMID:16731702; http://dx.doi.org/10.1136/vr.158.21.722.
9. Danscher G. In vivo liberation of gold ions from gold implants. Autometallographic tracing of gold in cells adjacent to metallic gold. Histochem Cell Biol 2006; 132:475-512; PMID:16774760; http://dx.doi.org/10.1007/s00418-006-0450-x.
10. Tsai CY, Shiu AL, Chen SY, Chen YH, Cheng PC, Chang MY, et al. Amelioration of collagen-induced arthritis in rats by nanogold. Arthritis Rheum 2007; 56:544-54; PMID:17265489; http://dx.doi.org/10.1002/art.22401.
21. Sabaratnam S, Arunan V, Coleman PJ, Mason RM, Morton AJ, Campbell NB, Gayle JM, Redding J, Davies J. Potential Advantages of Using Biomimetic Hydrogels for Optimizing Physicochemical Parameters for Intra-articular Drug Delivery Systems for the Treatment of Rheumatic Diseases: A Review of the Factors Influencing Their Performance. Eur J Pharm Biopharm 2019; 73:205-18; PMID:35495624; http://dx.doi.org/10.1016/j.ejpb.2019.06.009.

Squier CA, Mantz MJ, Schievert PM, Davis CC. Interleukin-1 beta induces coronary intimal lesions and vasoplastic responses in pigs in vivo. The role of platelet-derived growth factor. J Clin Invest 1996; 97:769-76; PMID:8692934; http://dx.doi.org/10.1172/JCI18476.

Bai LJ, Tuch BE, Hering B, Simpson AM. Fetal pig beta cells are resistant to the toxic effects of human cytokines. Transplantation 2002; 73:74-82; PMID:11907416; http://dx.doi.org/10.1097/00007890-200203150-00010.

Jana B, Kozlowska A, Andronowska A, Jędrzewska-Krakowska M. The effect of tumor necrosis factor-alpha (TNF-alpha), interleukin (IL)-1 beta and IL-6 on the monomeric and dimeric prostaglandins (PGI2, alpha 2 and E2 in pigs. Reprod Biol 2008; 8:57-68; PMID:18432307; http://dx.doi.org/10.1016/S1642-4131(06)00004-7.

Kojima F, Kato S, Kawai S. Prostaglandin E synthase in the pathophysiology of arthritic. Fundam Clin Pharm 2005; 19:255-61; PMID:15910650; http://dx.doi.org/10.1111/j.1472-8206.2005.00316.x.

Kapoor M, Martel-Pelletier J, Laujaresu D, Pelletier JP, Fahimi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol 2011; 7:33-42; PMID:21136968; http://dx.doi.org/10.1038/nrrheum.2010.196.

Bertone AL, Palmer JL, Jones J. Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. Vet Surg 2001; 30:528-38; PMID:11704984; http://dx.doi.org/10.1111/j.1532-4933.2001.284.x.

Richich C. Fibroblast biotage. Effectors signals released by the synovial fibroblast in arthritus. Arthritis Res 2000; 2:356-60; PMID:11094448; http://dx.doi.org/10.1186/ar12.

Artur M, Samuels J, Krasnokosty S, Abramson SB. Targeting the synovium for treating rheasititits (OA): where is the evidence? Best Pract Res Clin Rheumatol 2010; 24:71-9; PMID:20129201; http://dx.doi.org/10.1016/j.berh.2009.08.011.

Byron CR, Stewart MC, Stewart AA, Pondeen HC. Effects of glycosaminoglycans on glucose-amine on equine chondrocytes and synoviocytes in vito. Am J Vet Res 2008; 69:1229-34; PMID:18764682; http://dx.doi.org/10.2460/ajvr.69.9.1129.

May SA, Hooke RE, Lees P. Interleukin-1 stimulation of equine articular cells. Res Vet Sci 1993; 52:342-8; PMID:16029688; http://dx.doi.org/10.1016/0034-528X(92)90035-Z.

Hardy J, Bertone AL, Weisbrode SE, Muir WW, O’Dorio TM, Matty J. Cell trafficking, mediator release, and acute inflammation in artificial membrane of invertebrate and dervisated isolated equine joints. Am J Vet Res 1998; 59:88-100; PMID:9442251.

Pearson W, Orth MW, Lindinger MI. Evaluation of inflammatory responses induced via intra-articular injection of interleukin-1 in horses receiving a dietary nutraceutical and assessment of the effects of long-term nutraceutical administration. Am J Vet Res 2009; 70:848-61; PMID:19566670; http://dx.doi.org/10.2460/ajvr.70.7.848.

Sabaratnam S, Coleman PJ, Mason RM, Leivick JR. Interleukin-1 beta reduces the transcriptional and post-translational regulation of hyalurain synthesis. FEBS Lett 2005; 571:181-6; PMID:15909399; http://dx.doi.org/10.1016/j.febslet.2005.02.039.

Brewer SH, Glomm WR, Johnson MC, Krag MK, Franzen S. Probing BSA binding to citrate-coated gold nanoparticles and surfaces. Langmuir 2005; 21:9303-7; PMID:16171565; http://dx.doi.org/10.1021/la042939-5.

Mahl D, Greulich C, Meyer-Zaik A, Koller M, Epple M. Gold nanoparticles: dispersibility in biological media and cell-biological effect. J Mater Chem 2010; 20:6176-81; http://dx.doi.org/10.1039/c0jm01071e.

Kroll A, Pilkull MH, Hahn D, Schneikerb J. Current in vivo methods in nanoparticle risk assessment: limitations and challenges. Eur J Pharm Biopharm 2009; 72:370-7; PMID:18775492; http://dx.doi.org/10.1016/j.ejpb.2008.08.009.

Monteiro-Riviere NA, Inman AO, Zhang I. Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. Toxicol Appl Pharmacol 2009; 234:222-35; PMID:18938864; http://dx.doi.org/10.1016/j.taap.2008.09.050.

Kroll A, Pilkull MH, Hahn D, Schneikerb J. In vitro and in vivo toxicity of engineered nanoparticles with in vivo toxicity assays. Angew Chem Int Ed 2012; 86:1123-36; PMID:22407301; http://dx.doi.org/10.1002/anie.201102872.

Wang G, Zhang J, Dewide AH, Pal AK, Bello D, Therien JM, et al. Uptake binding and correcting for carbon nanotube interference with a commercial LHD cytotoxicity assay. Toxicology 2012; 299-99; PMID:22634321; http://dx.doi.org/10.1016/j.tox.2012.05.012.

Wang Z, Zhao J, Li J, Fu G, Dang X. Adsorption and inhibition of acetyicholinesesterase by different nanoparticles. Chemosphere 2009; 77:67-73; PMID:19540350; http://dx.doi.org/10.1016/j.chemosphere.2009.05.015.

Hye A, Oseieka-Iwawa A, Nideka-Biheloto J, Jaskowska-Stefan E, Moskalke S. Pro- and anti-inflammatory cytokines increase hyaluronan produc- tion by rat synovial membrane in vivo. Int J Mol Med 2009; 24:579-85; PMID:19724900.

Tammi RH, Pasi AG, Rilla K, Koutouev E, Viggirti D, Mikkonen K, et al. Transcriptional and post-translational regulation of hyaluronan synthesis. FEBs J 2011; 278:1419-28; PMID:21362137; http://dx.doi.org/10.1111/j.1472-6602.2011.08070.x.

Sumbayev VV, Yasinska IM, Garcia CP, Gilliland D, Lall GS, Gibbs BF, et al. Gold nanoparticles down- regulate interleukin-1b induced pro-inflammatory responses. Small 2013; 9:472-7; PMID:23121317; http://dx.doi.org/10.1002/smll.201201528.

Bhattacharya R, Mukherjee P, Xiong Z, Atala A, Sokol S, Mukhopadhyay D. Gold nanoparticles inhibit VEGF-165 induced proliferation of HUV E2C cells. Nano Lett 2004; 4:874-81; http://dx.doi.org/10.1021/nl0348379.

Ma JS, Kim WI, Kim JH, Kim TJ, Ye SK, Song MD, et al. Gold nanoparticles attenuate LPS-induced NO production through the inhibition of NF-kB and iNOS beta/TAT1 pathways in RAW264.7 cells. Nicot Oxide 2010; 23:214-9; PMID:20547236; http://dx.doi.org/10.1016/j.innox.2010.06.005.

Larsen A, Stolberg M, Danchev G. In vitro libration of charged gold atoms and topological chiralging of gold ions released by macrophages grown on metal- lic gold surfaces. Histochem Cell Biol 2007; 128:1-6; PMID:17549510; http://dx.doi.org/10.1007/s00418-007-0295-5.
56. Elder RC, Zhao Z, Zhang YF, Dorsey JG, Hess EV, Tepperman K. Dicyanogold(I) is a common human metabolite of different gold drugs. J Rheumatol 1993; 20:268-72; PMID:8474063.
57. Graham GG, Dale MM. The activation of gold complexes by cyanide produced by polymorphonuclear leukocytes—II. Evidence for the formation and biological activity of aurocyanide. Biochem Pharmacol 1990; 39:1697-702; PMID:2160818; http://dx.doi.org/10.1016/0006-2952(90)90113-3.
58. Graham GG, Kettle AJ. The activation of gold complexes by cyanide produced by polymorphonuclear leukocytes. III. The formation of aurocyanide by myeloperoxidase. Biochem Pharmacol 1998; 56:307-12; PMID:9744567; http://dx.doi.org/10.1016/10.016/S0006-29529800031-8.
59. Yanni G, Nabil M, Farahat MR, Poston RN, Panayi GS. Intramuscular gold decreases cytokine expression and macrophage numbers in the rheumatoid synovial membrane. Ann Rheum Dis 1994; 53:315-22; PMID:8017985; http://dx.doi.org/10.1136/and.53.3.315.
60. Yang JP, Merin JP, Nakano T, Kato T, Kitade Y, Okamoto T. Inhibition of the DNA-binding activity of NF-kappa B by gold compounds in vitro. FEBS Lett 1995; 361:89-96; PMID:7890047; http://dx.doi.org/10.1016/0014-5793(95)00157-5.
61. Yoshida S, Kato T, Sakurada S, Kuroko C, Yang JP, Matsui N, et al. Inhibition of IL-6 and IL-8 induction from cultured rheumatoid synovial fibroblasts by treatment with aurothioglucose. Int Immunol 1999; 11:151-8; PMID:10069413; http://dx.doi.org/10.1093/immunol/11.2.151.
62. Nieminen R, Korhonen R, Moilanen T, Clark AR, Moilanen E. Aurothiomalate inhibits cyclooxygenase 2, matrix metalloproteinase 3, and interleukin-6 expression in chondrocytes by increasing MAPK phosphatase 1 expression and decreasing p38 phosphorylation: MAPK phosphatase 1 as a novel target for anti-rheumatic drugs. Arthritis Rheum 2010; 62:1650-9; PMID:2078133; http://dx.doi.org/10.1002/art.27409.
63. Tomlinson JE, Bilskager AT. Effects of cyclooxygenase inhibitors flunixin and dexamethasone on permeability of ischemia-injured equine jejunum. Equine Vet J 2005; 37:75-80; PMID:15651739; http://dx.doi.org/10.2746/025104954406865.
64. Hill TL. A Canine Gastric Macosa Injury Model. Raleigh, North Carolina: North Carolina State University, 2012.
65. Chang CC, Hsieh MS, Liao ST, Chen YH, Cheng CW, Huang PT, et al. Hyaluronan regulates PPARγ and inflammatory responses in IL-1β-stimulated human chondrosarcoma cells, a model for osteoarthritis. Carbohydr Polym 2012; 90:1168-75; http://dx.doi.org/10.1016/j.carbpol.2012.06.071.
66. Ross TN, Kisiday JD, Hess T, McIlwraith CW. Evaluation of the inflammatory response in experimentally induced synovitis in the horse: a comparison of recombinant equine interleukin 1 beta and lipopolysaccharide. Osteoarthritis Cartilage 2012; 20:1583-90; PMID:22917743; http://dx.doi.org/10.1016/j.joca.2012.08.008.
67. Sonavane G, Tomoda K, Sakurada S, Ohshima H, Makino K. In vitro permeation of gold nanoparticles through rat skin and rat intestine: effect of particle size. Colloids Surf B Biointerfaces 2008; 65:1-10; PMID:18499408; http://dx.doi.org/10.1016/j.colsurfb.2008.07.004.
68. Sonavane G, Tomoda K, Sano A, Ohshima H, Terada H, Makino K. In vivo permeation of gold nanoparticles through rat skin and rat intestine: effect of particle size. Colloids Surf B Biointerfaces 2008; 65:1-10; PMID:18499408; http://dx.doi.org/10.1016/j.colsurfb.2008.02.013.
69. Klehrbnoy N, Dykman L. Biodistribution and toxicity of engineered gold nanoparticles: a review of in vitro and in vivo studies. Chem Soc Rev 2011; 40:1647-71; PMID:21082078; http://dx.doi.org/10.1039/c000081c.