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# Evaluation of colloidal platinum on cytotoxicity, oxidative stress and barrier permeability across the gut epithelium

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## Abstract

Colloidal platinum (Pt) is widely consumed due to its health promoting benefits. However, the exact biological effects of these nanoparticles have not been studied in detail, particularly in the gut. In the present study we observed that colloidal Pt was not cytotoxic towards three different epithelial colon cancer cell lines. Co-treatment of the colon cancer cell line Caco-2 with the oxidative stress inducing agent hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and colloidal Pt resulted in a significant decrease in H<sub>2</sub>O<sub>2</sub> induced oxidative stress. Colloidal Pt by itself did not induce any oxidative stress. Additionally, both overnight pretreatment of

Caco-2 cells with colloidal Pt followed by 1 h treatment with H<sub>2</sub>O<sub>2</sub>, or co-treatment of cells for 1 h with colloidal Pt and H<sub>2</sub>O<sub>2</sub> resulted in a significant recovery of cell death. Of note, the same protective effects of colloidal Pt were not observed when the oxidative stress was induced in the presence of 2, 2-azobis (2-amidinopropane) dihydrochloride, indicating that the source of free radicals may define the outcome of anti-oxidant activity of colloidal Pt. Colloidal Pt was also able to cross a model intestinal barrier formed *in vitro* with differentiated Caco-2 cells easily. Overall, our data indicate that colloidal Pt was not toxic towards intestinal epithelial cells, reduced H<sub>2</sub>O<sub>2</sub> induced oxidative stress, protected from oxidative stress related death of intestinal epithelial cells and could pass a model gut barrier easily. Colloidal Pt can therefore be consumed orally for its anti-oxidant and other health promoting benefits.

Keywords: Molecular biology, Cell biology

## 1. Introduction

Platinum (Pt) is an important but non-essential metal that has found biological value as glucose sensors [1] and in cancer therapy [2]. The mode of action of platinum in cancer therapy is attributed to its ability to crosslink DNA thereby causing DNA damage and eventual apoptosis [2]. According to the Woodrow Wilson Nanotechnology Consumer Products Inventory 2011, Pt nanoparticles are some of the most commonly used nanomedicines in consumer products (<http://www.nanotechproject.org/inventories/consumer/>). Nanoparticles are defined as solid colloidal particles that can range in size from 10 nm to 1.0 µm [3]. Colloidal systems for dispersion of metals provide advantage of small size (1–50 nm diameter) that allows for easy absorption [4]. Platinum nanoparticles have been reported to be toxic in various model systems such as algae [5] and mammalian cells [6]. On the other hand, the size of the nanoparticle was reported to be important for bactericidal activities, with particles as low as 3 nm having strong bactericidal properties; larger particles were largely compatible with the growth of bacteria [7]. A similar size dependent effect of Pt nanoparticles in the induction of DNA damage has also been indicated [6]. Colloidal Pt suspended in hydrogen dissolved (HD) water was reported to be very effective against human tongue cancer epithelial cells by reducing cell proliferation and colony formation but had no effect on normal tongue epithelial cells [8]. Additionally, colloidal Pt was demonstrated to enhance apoptotic cell death of esophageal squamous cell carcinoma cells by sensitizing the cells to gamma irradiation [9]. Mechanistically, colloidal Pt was shown to induce apoptosis by enhancing the protein levels of p53, p21 and reducing the levels of proliferating cell nuclear antigen [10]. Furthermore, colloidal Pt nanoparticles were found to be highly hemocompatible and could enhance the fluidity of red blood cells [11].

Many of the beneficial effects of colloidal Pt stem from its anti-oxidant activity [11]. Superoxide anion radical ( $\text{O}_2^{\cdot-}$ ), singlet oxygen ( $^1\text{O}_2$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radical ( $\cdot\text{OH}$ ) are typical reactive oxygen species (ROS) generated due to metabolic reactions. The reduction of oxygen to water during the production of ATP in the mitochondrial electron transport chain results in the leakage of a small number of electrons, which can directly react with oxygen to generate ROS [12]. Superoxide anion is reduced to  $\text{H}_2\text{O}_2$  by the antioxidant superoxide dismutase (SOD), which can be transported across membranes. Although ROS are required to maintain several cellular functions including proliferation, host defense, signal transduction and gene expression [13], when steady-state ROS concentration is transiently or chronically increased, it can induce several pathological conditions such as atherosclerosis, neurodegenerative diseases and aging as well as certain types of human cancers including lung, breast, and colon cancer [14, 15]. To control the steady-state levels of ROS, cells possess finely regulated antioxidant systems: anti-oxidant enzymes such as SOD, catalase and glutathione peroxidase as well as endogenous and exogenous non-enzymatic small molecules including glutathione,  $\beta$ -carotene, vitamin C and E [16]. Colloidal Pt, due to the presence of unpaired electrons, is highly reactive and is known to exhibit intrinsic SOD/catalase-like activity by converting the superoxide radical to  $\text{H}_2\text{O}_2$  and subsequently  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  and  $\text{O}_2$  under neutral and alkaline conditions [17, 18].

Platinum has also been used for a long time as colloidal dispersions; the latter was approved as a food additive of natural origin by the Ministry of Health, Labor and Welfare of Japan in 1995 [9]. Due to the size of the colloidal nanoparticles, their diffusion across the plasma membrane is unlikely [19]; although it has been shown that these particles are internalized [20], most likely through the process of pinocytosis. Several studies have shown that colloidal Pt can scavenge ROS *in vitro* [11, 18, 20], although it is not clear whether the same effect can be seen inside cells. Additionally, since colloidal Pt is approved for oral consumption, here we aimed to better establish the role of colloidal Pt on the gut. For this, we examined the effect of different concentrations of colloidal Pt on gut epithelial cells, examining its cytotoxicity, cytoprotection from oxidative stress, free radical quenching ability and passage through the gut barrier. Our data indicate that colloidal Pt is not cytotoxic, can reduce oxidative stress, and can cross the gut barrier effectively.

## 2. Materials and methods

### 2.1. Colloid Pt and its characterization

Colloidal Pt at 400 ppm concentration was provided by platinsai® (Turkey, [www.londoh.co.jp](http://www.londoh.co.jp)). To determine whether filtration through 0.22  $\mu\text{m}$  or 0.45  $\mu\text{m}$  polyethersulfone (PES) filters or incubation in solution at different pH values

affected the size, charge or dispersion of colloidal Pt, the size distribution and zeta potential of the colloidal Pt samples were determined using a Zetasizer (NanoZS, Malvern, UK). To determine the effect of filtration, the undiluted samples were filtered through the different filters or left unfiltered and immediately measured for size and zeta potential. To determine the effect of pH on colloidal Pt, the samples were diluted 1:10 (v/v) in deionized water containing 2 g/L of NaCl (to mimic the ionic strength of gastric fluids [21]) and the pH was adjusted to 1.2, 2.0, 3.0, 4.0 and 5.0 with HCl. As a control, the colloidal Pt samples were diluted in deionized water containing 2 g/L NaCl at pH 7.5. The samples were incubated in solutions at the respective pH values for 2 h and then the size distribution and zeta potential were measured with no further modifications. The hydrodynamic diameter of colloidal Pt was reported as  $Z_{avg}$  and the zeta potential as mV. Polydispersity index (PdI) values indicated the homogeneity of the sizes of the particles. The average of 10 measurements was used for each data point.

## 2.2. Cell culture

Caco-2 and HT-29 cells were obtained from ŞAP Enstitüsü (Ankara, Turkey). HCT-116 cells were received from DSMZ (Braunschweig, Germany). HT-29 and HCT-116 cells were grown in RPMI-1640 without phenol red (Biological Industries, Beit Haemek, Israel) supplemented with 10% Fetal Bovine Serum (FBS) (Biological Industries, Beit Haemek, Israel, Cat. No: 04-127-1A), 2 mM L-glutamine (Biological Industries, Cat. No: 03-020-1B) and 1% penicillin/streptomycin (Biological Industries, Cat. No: 03-031-1B). Caco-2 cells were grown in EMEM-Minimum Essential Medium without phenol red with Earl's salt (Thermo Fisher Scientific, Boston, MA, USA, and Cat. No: 51200038) containing 20% FBS (Biological Industries), 1% penicillin/streptomycin (Biological Industries), 2 mM L-glutamine (Biological Industries), 1X-NEA-Non-Essential Amino Acids (Biochrom GmbH, Berlin, Germany, Cat. No: K 0293) and 1 mM Na pyruvate (Biowest, Florida, USA, cat. No: L0642-100). All cell lines were grown in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C.

When the cultures reached 60–70% confluency, media were removed and the cells were washed with PBS (Dulbecco's Phosphate Buffered Saline, without Ca<sup>2+</sup> and Mg<sup>2+</sup>, Biological Industries, Cat. No: 02-023-1A). The cells were then detached by adding Trypsin/EDTA solution (0.25%/0.02% w/v, Biochrom GmbH, Cat. No: L2163) and incubated at 37 °C for 5 min. Finally, trypsin was inhibited with at least 3-fold of the growth media and appropriate aliquots of the cell suspension were added to new culture vessels.

## 2.3. Spontaneous differentiation of Caco-2 cells

Caco-2 cells were plated at high cellular density. Proliferating cells were collected the day after seeding when the cultures were 60–70% confluent (sub-confluent).

After about 48 hours of plating, the cells generally reached 100% confluency. To differentiate the cells, 100% confluent cells (day 0) were grown for a further 10 days [22], during which the medium was changed every other day. For every set of differentiation, the expression of sucrose isomaltase (SI) and carcinoembryonic antigen (CEA) were assayed as differentiation markers. To generate the intestinal barrier using differentiated Caco-2 cells,  $6 \times 10^5$  cells were plated in Transwell inserts (Costar 0.4  $\mu\text{m}$ , Corning, USA Cat. No: 3470) and grown for a further 10 days. Differentiation was confirmed by collecting the cells from the Transwells and determining the mRNA and protein expression of SI and CEA, respectively.

## 2.4. FITC-dextran permeability assay

To assess barrier formation in differentiated Caco-2 cells, the permeability of FITC Dextran was determined [23]. For this, Caco-2 cells were differentiated on Transwells for 10 days. FITC-dextran (FD4, Sigma Aldrich, St. Louis, Missouri, USA, Cat No: 60642-46-8) was dissolved in complete EMEM medium containing 10% FBS at 500  $\mu\text{g/mL}$  concentration and then applied to the apical side of the cell monolayers on the Transwells. FD4 permeability was determined by sampling the basolateral solution for every 30 min for 3 h, while replacing the sampled amount with fresh media without FD4 at each time point. The amount of FD4 that passed the Caco-2 barrier across the Transwell membrane was quantified by fluorescence spectroscopy at 535 nm using a calibration curve using a range of concentrations of FD4 between 10  $\mu\text{g/mL}^{-1}$  mg/mL. As a control, proliferating Caco-2 cells that did not form the barrier were used.

## 2.5. RNA isolation, cDNA synthesis and RT-qPCR assays

Cells were collected in cell culture-grade PBS by scraping. Total RNA was isolated using an RNA Extraction Kit (NucleoSpin RNA, Macherey Nagel, Germany, Cat. No: 740955.250) according to the manufacturer's guidelines. RNA concentrations were measured with BioDrop  $\mu\text{LITE}$  spectrophotometer (BioDrop, Cambridge, UK). To reversely transcribe 1  $\mu\text{g}$  of total RNA with random hexamers, cDNA synthesis was carried out using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, cat no: K1622). The cDNAs were stored at  $-20^\circ\text{C}$ .

For RT-qPCR mixtures, 0.25  $\mu\text{M}$  forward and reverse primers (shown in Table 1) and 5  $\mu\text{l}$  of 2X GO Taq QPCR Master Mix containing SYBR Green (Promega, Madison, Wisconsin, USA, Cat. No: A6001) were mixed in 8  $\mu\text{l}$  volume, then 2  $\mu\text{l}$  of 1:20 diluted cDNA was added.  $\beta$ -Actin was used as an internal control. Reactions were carried out in Rotor GeneQ 6000 (Qiagen, Germany). Standard curves were prepared for each set of primers which were then used to calculate  $C_t$  values of the unknown samples. Fold changes with respect to the internal controls were

calculated using Pfaffl method [24]. MIQE guidelines were followed in the RT-qPCR reactions [25].

## 2.6. Western blotting

M-PER Mammalian Protein Extraction Buffer (Thermo Fisher Scientific, Cat. No: 78501) was used to extract proteins. Protease inhibitors (Roche, Basel, Sweden, Cat. No: 11836153001) and phosphatase inhibitors (Roche PhosStop Cat No: 4906845001) were added to the lysis buffer. After incubation on ice for 30 min, cells were centrifuged at  $14000 \times g$  for 10 min; then supernatants containing the total protein lysate were collected in fresh eppendorf tubes. Protein concentration was determined using Coomassie Protein Assay Reagent (Thermo Fisher Scientific, Cat. No: 23200) by reference to a standard curve generated with known concentrations of BSA (Bovine Serum Albumin).

For the separation of proteins by SDS-PAGE, the proteins were boiled at 95 °C for 6 min in a 6X loading dye (12% SDS, 30%  $\beta$ -mercaptaethanol, 30% glycerol, 0.02% bromophenol blue, 375 mM Tris–HCl pH 6.8). Electrophoresis was carried out at 100 V using 10% SDS-polyacrylamide gels loaded with equal amounts of protein (20  $\mu$ g). For the transfer of proteins onto polyvinylidene fluoride membranes (Roche, Cat no: 3010040001), wet transfer was performed with constant current at 380 mA, 115 V for 75 min. The membranes were blocked by using 5% skim milk (Sigma Aldrich) in 1X TBS containing 0.1% Tween-20 (Appllichem, Germany) for 1 h at room temperature on a shaker. Membranes were incubated with primary antibodies at 4 °C overnight on a shaking platform. Next day, membranes were washed with TBS-T, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, on a shaker. List of primary and secondary antibodies are given in Table 2. Visualization of the blots was performed using WesternBright ECL HRP substrate (Advansta, Menlo Park, CA, USA) and imaged on a Chemi-Doc MP (Bio-Rad, Hercules, CA, USA).

**Table 1.** Primer sequences used in RT-qPCR and their properties.

| Name                    | Primer sequences (5'-3')                                              | Annealing temperature (°C) | Product size (bp) | Target mRNA ID |
|-------------------------|-----------------------------------------------------------------------|----------------------------|-------------------|----------------|
| Sucrase isomaltase (SI) | Forward: CAAATGGCCAAACACCAATG<br>Reverse: CCACCACTCTGCTGTGGAAG        | 59                         | 160               | NM_001041.3    |
| $\beta$ -Actin          | Forward: CAGCCATGTACGTTGCTATCCAGG<br>Reverse: AGGTCCAGACGCAGGATGGCATG | 60                         | 151               | NM_003900.4    |

## 2.7. Determination of cell viability

The colloidal Pt sample was filtered through a 0.22  $\mu\text{M}$  PES filter for sterilization. Vybrant® MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) Cell Proliferation Assay (Life Technologies, Carlsbad, CA, USA, Cat. No: M6494) was used for determination of cell viability of Caco-2, HCT-116 and HT-29 cell lines in the presence of different concentrations of colloidal Pt. First,  $5 \times 10^3$  cells per well were plated in 96-well plates in their corresponding growth medium. Cell proliferation was determined under the following conditions:

1. Treatment with colloidal Pt only (1%–20% v/v diluted in the corresponding cell culture medium) for 48 h to determine the effect of colloidal Pt on cell viability.
2. Treatment first with 20% colloidal Pt for 24 h then 1 h with 250  $\mu\text{M}$ –5 mM  $\text{H}_2\text{O}_2$  (Sigma-Aldrich, Cat No: 216763) after removal of Pt containing growth medium.
3. Co-treatment with Pt (20%) and  $\text{H}_2\text{O}_2$  or co-treatment with Pt (20%) and 5 mM–80 mM 2, 2-azobis (2-amidinopropane) dihydrochloride (AAPH, dissolved in  $\text{dH}_2\text{O}$ , Sigma-Aldrich, Cat. No: 440914) for 1 h.

Following the treatments, the medium was removed and the MTT cell viability assay was carried out according to the manufacturer's instructions. Briefly, 12 mM MTT stock solution was diluted with growth medium in 1:10 ratio and distributed to each well as 100  $\mu\text{l}$ . Empty wells containing only 100  $\mu\text{l}$  of diluted MTT were used as blank. The plates were incubated at 37 °C for 4 h. In order to dissolve the formazan crystals, 100  $\mu\text{l}$  of 1/10 (g/mL) SDS/ $\text{dH}_2\text{O}$  (containing 0.01 N HCl) solution was added for each well followed by incubation at 37 °C for 16 h. Finally, measurements were taken at 570 nm in MultiSkan GO Microplate Spectrophotometer (Thermo Scientific) microplate reader. Sterile  $\text{dH}_2\text{O}$  was used as vehicle in colloidal Pt and AAPH treatments. Experiments were carried out with at least two biological replicates each with eight technical replicates.

**Table 2.** List of the antibodies and conditions for western blot.

| Antibodies                  | Source                                      | Dilutions                       |
|-----------------------------|---------------------------------------------|---------------------------------|
| $\beta$ -Actin              | Santa Cruz Biotechnology, Cat. No: sc-47778 | 1:4000 in 5% skim milk in TBS-T |
| Mouse anti-CEA (Clone Col1) | Invitrogen Cat. No: 180057                  | 1:500 in 5% skim milk in TBS-T  |
| Goat anti-rabbit IgG HRP    | Advansta Cat. No: R-05072-500               | 1:4000 in 5% skim milk TBS-T    |
| Goat anti-mouse IgG HRP     | Advansta Cat. No: R-05071-500               | 1:4000 in 5% skim milk TBS-T    |



## 2.8. Determination of oxidative stress: dihydroethidium (DHE) staining assay

In the presence of free radicals, oxidization of DHE that normally exhibits blue fluorescence in the cytosol results in the intercalation of DNA and formation of red fluorescence in the nucleus, which can then be measured by flow cytometry [26]. Caco-2 cells were treated with either 20% (v/v) colloidal Pt or 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , or co-treated with 20% (v/v) colloidal Pt and 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 1 h. Cells ( $5 \times 10^5$ ) were trypsinized, washed with PBS and centrifuged at  $400 \times g$  for 7 min and the pellets were resuspended in 0.5 mL DHE (Thermo Fischer Scientific, Cat. No: D1168) staining solution (3.2  $\mu\text{M}$  in 1X cell culture grade PBS). The samples were incubated for 20 min at 37 °C in dark and then analyzed in a BD Accuri C6 Flow cytometer (Becton-Dickinson, Franklin Lakes, New Jersey, USA) in FL-2 channel immediately.

## 2.9. Inductively coupled plasma mass spectrometry (ICP-MS)

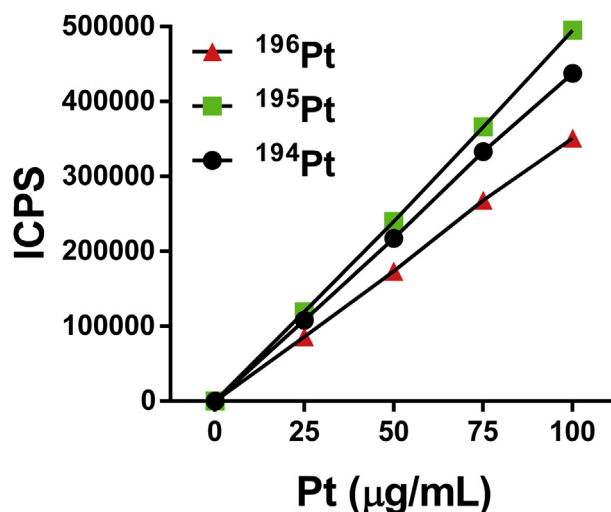
To determine the amount of colloidal Pt that can cross the Caco-2 barrier (mimicking the intestinal barrier function [27]) ICP-MS (Thermo Scientific X series) with a concentric nebulizer and Peltier effect cooled spray chamber was used. Caco-2 cells ( $6 \times 10^5$ ) were seeded on Transwells and differentiated for 10 days. After differentiation, growth medium containing 20% (v/v) colloidal Pt was added to Transwells inserts. After 3 h, samples were collected from both the apical and basolateral side of the differentiated cells and subjected to ICP-MS. The permeability of the differentiated Caco-2 cells to colloidal Pt was compared to the permeability of sub-confluent Caco-2 cells.

Instrumental working parameters were optimized using “Tune A” mixed standard solution with a concentration of 10  $\mu\text{g/L}$  for each element as suggested by the manufacturer. Tune A solution contains Li(I), Co(II), In(III), Pb(II), Bi(V) and U(IV) ions in 0.5 mol/L nitric acid solution. Continuous flow mode with 100 ms dwell time was used for Pt determination. Performance of the instrument was regularly monitored using this solution; if there was a change in the instrument sensitivity with time, autotune sequence was run again. Furthermore, a fine tuning of the target isotopes was performed using 10  $\mu\text{g/L}$  Pt solution. The calibration graph for Pt is shown in Fig. 1. Quantification was carried out with  $^{194}\text{Pt}$ ,  $^{195}\text{Pt}$  and  $^{196}\text{Pt}$  isotopes. Plasmalab and Microsoft Excel software were used for evaluation of the data.

## 2.10. Statistical analyses

Results are expressed as the mean  $\pm$  standard error of mean (SEM). Data analyses and graphing were carried out with Graphpad Prism 6.0. t-test was used for statistical analysis, and differences at  $p \leq 0.05$  were considered significant (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ; \*\*\*\* $p \leq 0.0001$ ).





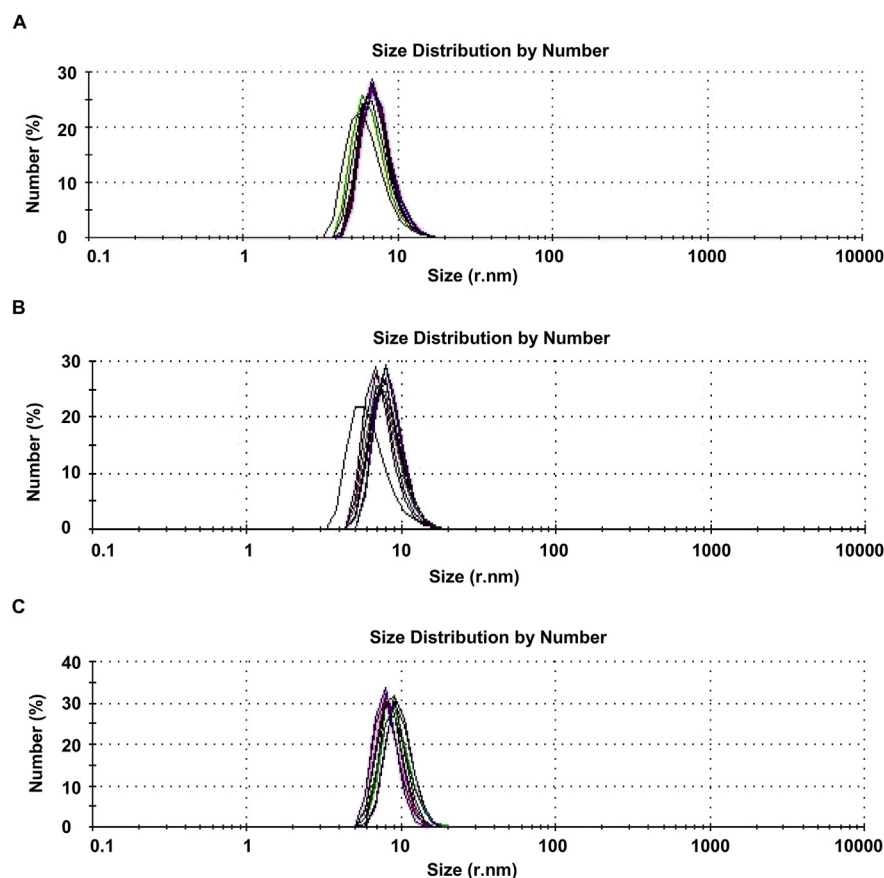
**Fig. 1.** Calibration graph and the best line equation for  $^{194}\text{Pt}$ ,  $^{195}\text{Pt}$  and  $^{196}\text{Pt}$  isotopes by ICP-MS.

### 3. Results and discussion

#### 3.1. Characterization of colloidal platinum nanoparticles

We first determined whether sterilization of colloidal Pt by filtering with 0.22  $\mu\text{m}$  or 0.45  $\mu\text{m}$  filters affected the size of the colloidal Pt nanoparticles by using dynamic light scattering (DLS) (Fig. 2). This is a technique for understanding the diffusion behavior of macromolecules in solution, thus, this behavior is affected by the size and shape of macromolecules [28]. The size of the colloidal Pt was calculated as  $7.4 \text{ nm} \pm 0.08$ ,  $8.1 \text{ nm} \pm 0.14$  and  $8.9 \pm 0.22$  for unfiltered, 0.45  $\mu\text{m}$  and 0.22  $\mu\text{m}$  filtered samples, respectively at 25  $^{\circ}\text{C}$ . The results show a slight variation in the hydrodynamic size of the particles before and after filtration (Fig. 2). These variations may have resulted from the dynamic nature and rapid movement of the very small particles medium resulting in a fluctuating intensity signal [29]. It should also be kept in mind that this is the hydrodynamic size that includes layer of water around the nanoparticles. It is likely that the actual size of the particles is lower. For all future experiments, the colloidal Pt was filtered through a 0.22  $\mu\text{m}$  filter for sterilization. Additionally, the zeta potential for the 0.22  $\mu\text{m}$  filter sterilized sample was determined as -29 mV while the zeta potential of the unfiltered sample was -28.1 mV indicating filtration did not have any impact on the electric charge around the surface of the particles.

To determine the stability of colloidal Pt at different pH values (1.2, 2.0, 3.0, 4.0, and 5.0), we have resuspended the colloidal Pt solution at 10 % (v/v) in deionized water and 2 g/L NaCl and adjusted the pH with HCl. As a control we used deionized water containing 2 g/L NaCl at pH 7.5. We observed that at pH 1.2, 2.0 and 3.0 the colloidal Pt underwent aggregation and precipitation, which was manifested in a dramatic increase in the hydrodynamic size of the colloidal particles at these pH values



**Fig. 2.** Characterization of colloidal platinum nanoparticles. Intensity particle size (radius in nm-r.nm) distribution, obtained by the Dynamic Light Scattering (DLS, Malverin Zeta Sizer, 135° backscatter), for unfiltered (A), 0.22  $\mu\text{m}$  (B), 0.45  $\mu\text{m}$  (C) filtered colloidal Pt samples are shown. The data were collected 10 times for each sample.

(Table 3). We also measured the zeta potential of the samples at the different pH values and observed a decrease in the zeta potential of the samples at the lower pH values.

Generation of a colloidal solution requires a high input of energy that is likely to push these systems far from equilibrium [30]. The colloidal structure consists of two continuous phases that can exhibit phase transitions manifested by sudden changes in parameters such as temperatures and/or concentrations [30]. It is possible that the colloidal system in the Pt solution got protonated at the lower pH values, which may have resulted in more positive charges on the particles and thereby aggregation and precipitation. This can also be seen from the zeta potential of the samples that changed from  $-28$  mV when incubated in water at pH 7.5 to significantly more positive values at the lower pHs. However, it is difficult to determine the precise mechanism of the aggregation at the critical pH value of 3 without having exact knowledge of the chemical composition of the surfactant found in the colloidal Pt solution; this data is unavailable to the public.

**Table 3.** Hydrodynamic size and zeta potential of colloidal Pt at different pH values. The pH of the solution was adjusted with HCl (except the control at pH 7.5). All solutions contained 2 g/L NaCl and all solutions were incubated for 2 h at 37 °C.

| pH  | Hydrodynamic size (nm) | Zeta potential (mV) |
|-----|------------------------|---------------------|
| 1.2 | 500 ± 59.2             | −12.9 ± 1.8         |
| 2.0 | 470.7 ± 58.9           | −11.3 ± 2.1         |
| 3.0 | 485.1 ± 60.8           | −15.2 ± 1.2         |
| 4.0 | 7.8 ± 0.3              | −17.4 ± 3.1         |
| 5.0 | 7.2 ± 0.5              | −12.0 ± 0.5         |
| 7.5 | 7.6 ± 0.4              | −28.7 ± 1.3         |

It is well known that the pH of the gastric fluid in healthy subjects is in the range of 1.3–2.5 during fasting [31]. However, after eating, the pH can increase to a range of 4.5–5.8. Around 1 h after eating, the pH of the stomach can decrease to less than 3.1 [31]. Thus, it is likely that if consumed with food rather than on an empty stomach, the colloidal Pt solution will be in an environment with a higher pH value at which it is more stable.

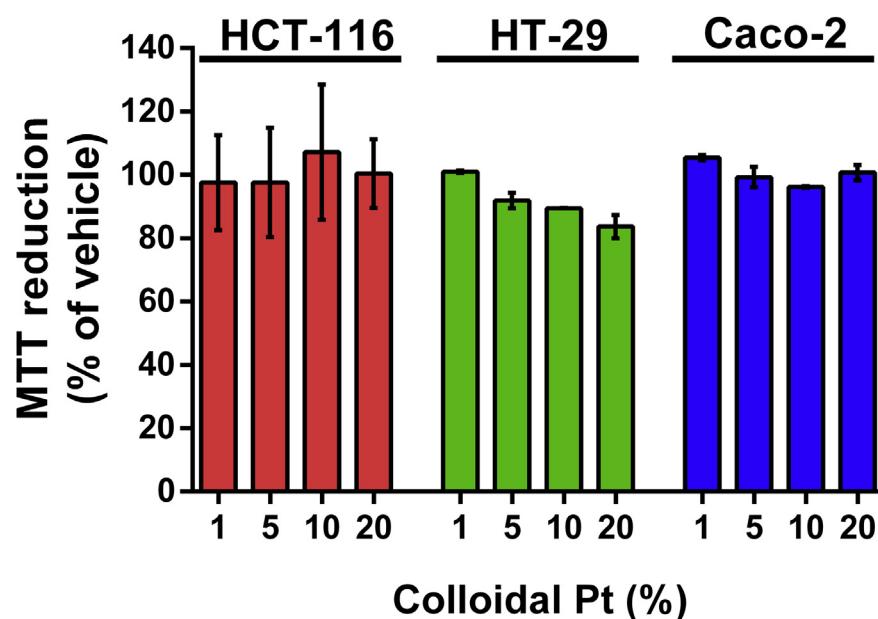
We also determined the pH of 1%–20% (v/v) colloidal Pt samples diluted in cell culture medium using a pH meter. Colloidal Pt itself was acidic (pH: 4.86), while the pH of the cell culture medium was 8.73. The pH of the cell culture medium containing colloidal was 8.73 for every dilution used, indicating that the presence of colloidal Pt did not change the pH of the culture medium.

### 3.2. Effect of colloidal platinum on cell viability

In order to assess the effect of the colloidal platinum on cell viability, an MTT assay was carried out with HCT-116, Caco-2 and HT-29 cell lines. 20% (v/v) colloidal Pt (corresponding to 80 ng/μl colloidal Pt) was the maximum concentration that was used since more than 20% (v/v) vehicle (dH<sub>2</sub>O) in the control wells decreased the number of viable cells. Cell viability remained unaffected at all doses of colloidal Pt in three different CRC cell line models (Fig. 3). Attempts to concentrate the colloidal Pt using a Speedvac (Thermo Scientific) to achieve higher treatment concentrations resulted in a loss of the colloidal nature of the solution.

Pt based drugs such as cisplatin, oxaliplatin and carboplatin are widely used as chemotherapy drugs [32]. Although Pt as a metal is highly inert, these drugs contain Pt (II) that is known to be highly active as it can accept two electrons [33]. The primary mechanism of action of these compounds includes formation of adducts with DNA that create bulky lesions and therefore inhibit transcription by RNA polymerase II. If the damage is extensive and cannot be repaired by DNA repair pathways,

the cell undergoes apoptosis [34]. Colloidal Pt, consisting of a colloidal dispersion of the metal, needs to be stabilized to prevent aggregation; this in turn involves capping of the active sites, leading to lower reactivity [33]. Additionally, colloidal metal solutions by definition have neutral metal atoms; although based on the isoelectric point of platinum (2.8), the solution at pH 4.9 is likely to have a negative charge [35]. Li et al. reported that colloidal Pt showed considerable toxicity towards the esophageal squamous cell carcinoma cell line KYSE-70, which was further enhanced in the presence of gamma irradiation [9]. These authors used 0, 50 and 100 ppm colloidal Pt, with nearly 50% reduction in cell viability and approximately 40% increase in apoptosis reported with 100 ppm colloidal Pt when compared to untreated cells. In the current study, we have used 80 ppm colloidal Pt as the maximum concentration but have observed minimal cell death in three different epithelial cancer cell lines. The surface reactivity of platinum nanoparticles is dependent on size; larger particles with lower work function or those particles with enhanced O<sub>2</sub> chemisorption show greater catalytic activity [36]. On the other hand, in biological systems, colloidal Pt with smaller particle sizes (around 3 nm) were reported to show greater toxicity [7]. In the current study, the hydrodynamic size of the colloidal particles was between 7 nm and 8 nm. It is possible that the size of the colloidal particles used in the current study was not toxic to the cells.

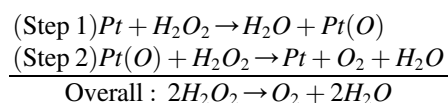


**Fig. 3.** Effect of colloidal platinum on cell viability. HCT-116, Caco-2 and HT-29 cells were treated with 1%–20% (v/v) colloidal Pt for 48 h, then MTT assay was carried out. Percent viable cells normalized to their corresponding vehicle treated control cells are shown. The experiment was carried out with at least 2 independent biological replicates, each containing 8 technical replicates.

### 3.3. Effect of colloidal Pt on the generation of Reactive Oxidative Species (ROS)

Colloidal Pt has been previously reported to protect from oxidative stress [11]. To determine whether the same could be seen in the CRC cell lines used, a Dihydroethidium (DHE) assay was used. DHE is a membrane permeable dye used extensively for monitoring superoxide production [37]. Caco-2 cells were first treated with 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 1 h to induce ROS production; some of the cells were co-treated with 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and 20% (v/v) colloidal Pt for 1 h, followed by the DHE assay. We observed low ROS production in Caco-2 cells treated with colloidal Pt sample or vehicle control (Fig. 4, 17.5% DHE stained cells), showing that colloidal Pt by itself did not result in ROS production and oxidative stress. More importantly, we observed a nearly 50% decrease in ROS production (Fig. 4, 23.6% DHE stained cells) in Caco-2 cells co-treated with colloidal Pt and  $\text{H}_2\text{O}_2$  as compared to cells treated with  $\text{H}_2\text{O}_2$  alone (Fig. 4, 78.4% DHE stained cells). This suggests that treatment of cells with colloidal Pt may relieve  $\text{H}_2\text{O}_2$  triggered oxidative stress.

The decomposition reaction  $\text{H}_2\text{O}_2$  with Pt as a catalyst was reported to be a two-step process as shown below:

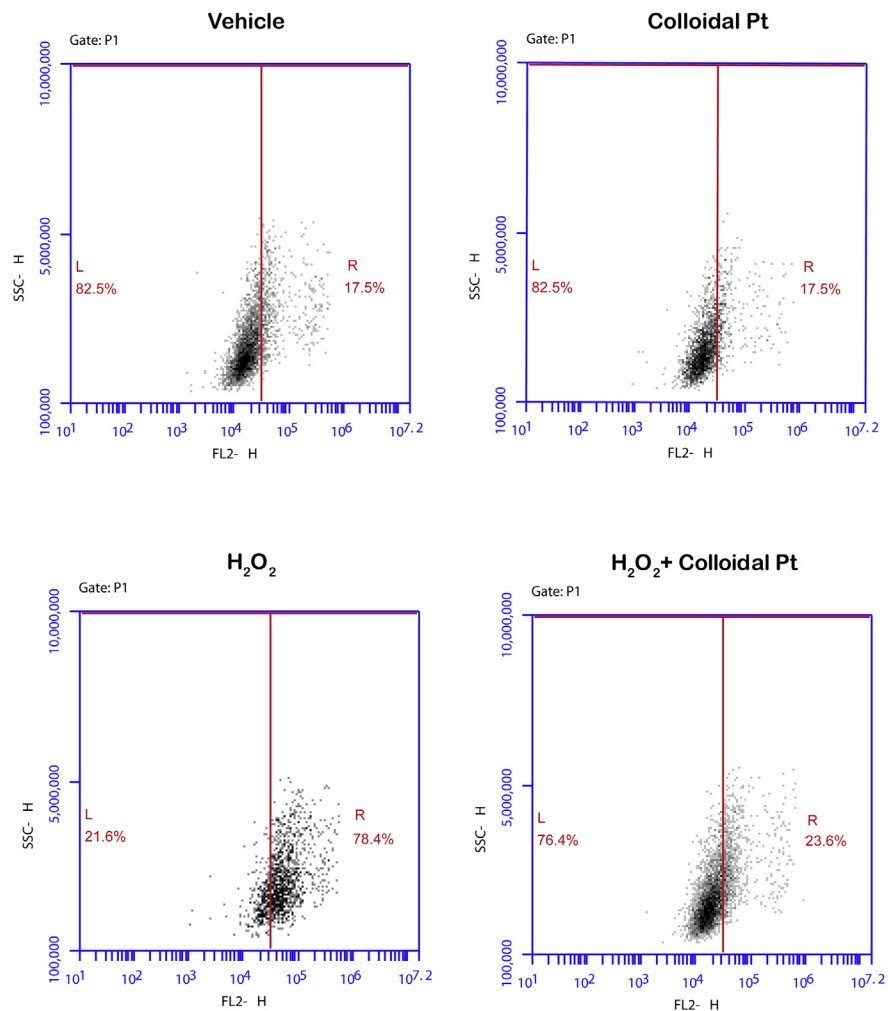


The first and rate determining step includes adsorption of  $\text{H}_2\text{O}_2$  on oxide-free Pt and the decomposition of  $\text{H}_2\text{O}_2$  into water and oxidized Pt(O) (Step 1). The next step (Step 2) is initiated due to the instability of the oxidized Pt, which promotes the decomposition of  $\text{H}_2\text{O}_2$  further into  $\text{H}_2\text{O}$  and  $\text{O}_2$  [36].

In the current study, a mixture of colloidal Pt and  $\text{H}_2\text{O}_2$  led to the formation of bubbles in the culture medium indicating that the steps described above were most likely occurring. Thus, the decomposition of  $\text{H}_2\text{O}_2$  into water and oxygen ameliorated  $\text{H}_2\text{O}_2$  mediated ROS formation in the cells as shown in Fig. 4.

### 3.4. Effect of ROS generators with colloidal platinum nanoparticles on cellular viability

We next wanted to determine whether amelioration of oxidative stress in the presence of colloidal Pt could also affect cell proliferation. For this, we either co-treated Caco-2 cells with colloidal Pt and 2, 2-azobis (2-amidinopropane) dihydrochloride (AAPH) or with colloidal Pt and  $\text{H}_2\text{O}_2$ .  $\text{H}_2\text{O}_2$  is a source of reactive oxygen species (ROS). ROS are chemically-reactive molecules containing oxygen, including superoxide anions or singlet oxygen and their production is catalyzed



**Fig. 4.** Effect of colloidal Pt nanoparticles on the production of reactive oxidative species (ROS). Caco-2 cells were treated with only 20% (v/v) colloidal Pt or only 500  $\mu$ M  $H_2O_2$ , or with a combination of 500  $\mu$ M  $H_2O_2$  and 20% (v/v) colloidal Pt for 1 h. 20% (v/v)  $dH_2O$  treatment was used as vehicle. The production of ROS was determined by the DHE staining using flow cytometry. Representative flow cytometry dot plots of two independent experiments are shown.

by oxidative enzymes in cell membranes, mitochondria, peroxisomes and endoplasmic reticulum [38].

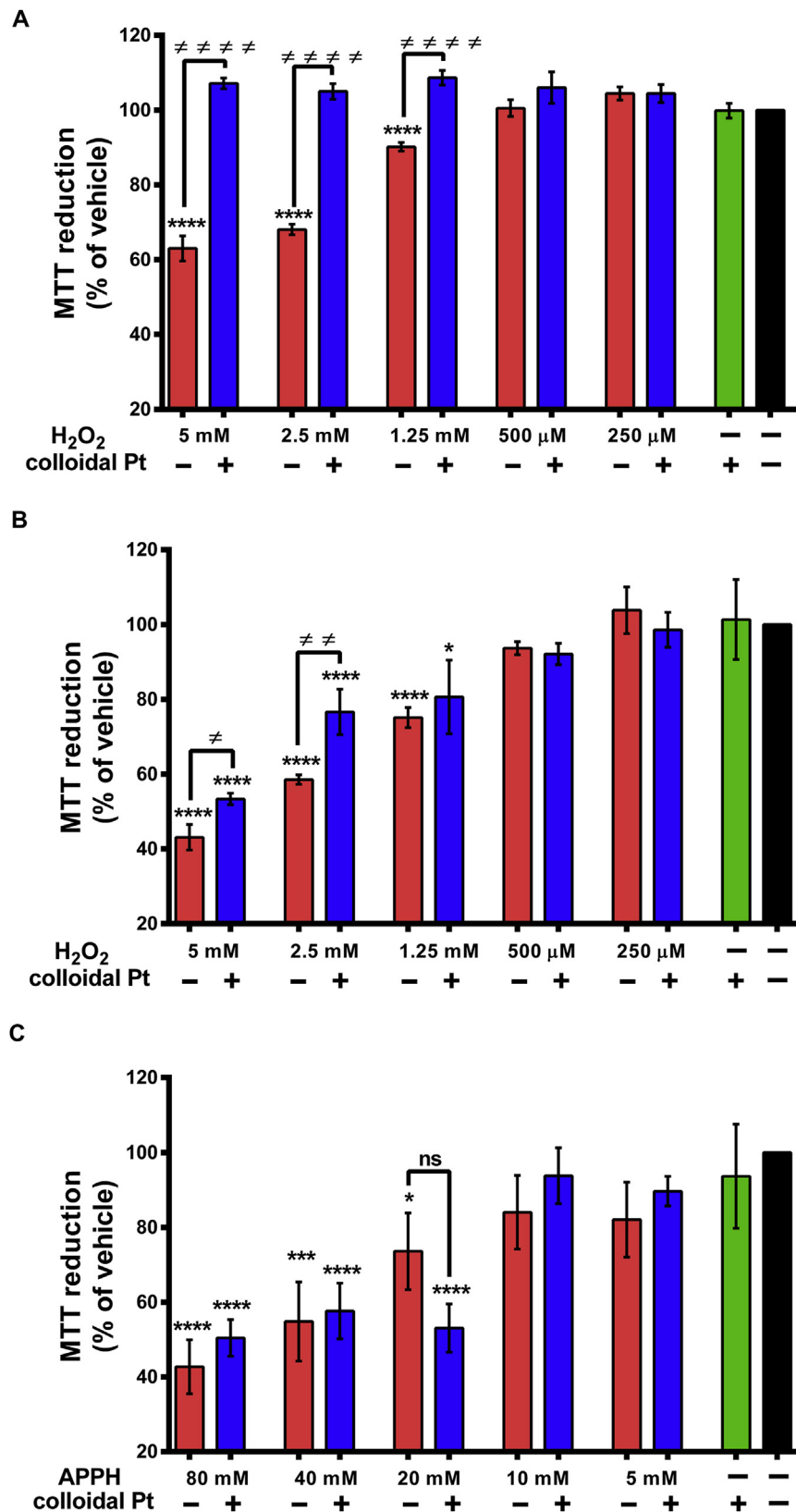
To determine whether pretreatment of Caco-2 cells with colloidal Pt could protect against  $H_2O_2$  induced cell death, Caco-2 cells were co-treated with 20% (v/v) colloidal Pt and  $H_2O_2$  (250  $\mu$ M–5 mM) for 1 h. In another experimental set-up, the cells were treated with 20% (v/v) colloidal Pt for 24 h first; then, colloidal Pt was removed, the cells were washed and treated with  $H_2O_2$  (250  $\mu$ M–5 mM) for 1 h and assayed for cell viability. As can be seen in Fig. 5A and B,  $H_2O_2$  treatment of Caco-2 cells resulted in nearly 50% cell death as expected. However, co-treatment of Caco-2 cells with  $H_2O_2$  and colloidal Pt resulted in a complete recovery

in cellular viability (Fig. 5A). This was most likely due to the chemical decomposition of  $\text{H}_2\text{O}_2$  into  $\text{O}_2$  and  $\text{H}_2\text{O}$  in the presence of Pt. Pre-treatment with colloidal Pt followed by  $\text{H}_2\text{O}_2$  treatment was also found as protective against  $\text{H}_2\text{O}_2$  mediated cytotoxicity but to a lesser extent than colloidal Pt and  $\text{H}_2\text{O}_2$  co-treatment (Fig. 5B). Collectively, these results suggest that colloidal Pt could reduce  $\text{H}_2\text{O}_2$ -mediated cytotoxicity in target cells; more importantly, cellular viability was preserved even when the cells were pretreated with colloidal Pt followed by treatment with only with  $\text{H}_2\text{O}_2$ , suggesting that colloidal Pt could inhibit oxidative stress related cell death. To explain the mechanism behind the observation that pre-treatment of cells with colloidal Pt could protect from  $\text{H}_2\text{O}_2$  induced cell death, it has been suggested that Pt can act as a superoxide dismutase/catalase mimetic [39, 40] whereby it can reduce the formation of superoxides and reduce the expression and activity of apoptosis related proteins such as caspase 3 and Bid [40].

Oxidative stress can result in cellular and structural damage that may affect membrane, proteins, lipids and DNA; thereby affecting cellular proliferation and gene expression [41]. Most of biological sources of  $\text{H}_2\text{O}_2$  demand the spontaneous or catalytic breakdown of superoxide anions ( $\text{O}^\cdot$ ) which is produced by the partial reduction of oxygen during aerobic respiration.  $\text{H}_2\text{O}_2$  can also be produced extracellularly for instance by the immunoglobulin G-catalyzed oxidation of water, by receptor/ligand interactions (for example in response to growth factors), and by phagocytic immune cells [42] and can enter cells by simple or facilitated diffusion [43].  $\text{H}_2\text{O}_2$  is involved in normal cellular metabolism at low concentrations as a metabolite from non-enzymatic and superoxide dismutase-catalyzed reactions and under normal conditions it is rapidly neutralized in tissues by anti-oxidant defense systems [41]. It is one of the key molecules in the redox cycle in which it can act as a messenger to carry the redox signal to the target site [41]. Additionally, it can be converted to reactive hydroxyl radicals ( $\text{HO}^\bullet$ ) with Fenton reaction, which then can react with biological molecules to accept a hydrogen atom. When iron salt containing foods are ingested frequently, it can lead to the formation of hydroxyl radicals via the Fenton reaction. In this case, the main target organ is the gastrointestinal (GI) tract where iron absorption primarily occurs. Moreover, it has been shown that high concentrations of  $\text{H}_2\text{O}_2$  can affect cell membrane dynamics and disrupt membrane permeability, which can result in cell death eventually [41, 44]. Besides, Rao et al. showed that  $\text{H}_2\text{O}_2$  induces impairment of the intestinal epithelial barrier function by disrupting paracellular junctional complexes [45]. Therefore, protective action of colloidal Pt against detrimental effects of  $\text{H}_2\text{O}_2$  may contribute to the proper maintenance of tissues.

The recommended dose for colloidal Pt from the vendor's webpage (platinsai) is 5 mL per day (<https://www.londoh.co.jp/en/products/sai/index.html>). Based on the concentration of Pt in colloidal Pt (400 ppm), the amount of Pt recommended per day in colloidal form is about 2 mg. In an average individual with 5 L of blood,





this would amount to a plasma concentration of around 0.4 ppm, assuming 100% absorption in the gut. In the current study, up to 80 ppm colloidal Pt was used, which is nearly 200 times higher than the recommended amount of colloidal Pt. The concentration of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in blood and plasma is unclear. Forman et al. have stated that human blood  $\text{H}_2\text{O}_2$  levels range from a possible low of 0.25  $\mu\text{M}$  to a probable normal range of 1–5  $\mu\text{M}$  [46]. In the current manuscript, we used a range of 5–0.25 mM of  $\text{H}_2\text{O}_2$  and observed that concentrations of 1.25 mM and higher were highly toxic to the cells, which is 250 folds higher than the normal range of  $\text{H}_2\text{O}_2$  in blood. Therefore, when compared to estimated physiological ranges, both colloidal Pt and  $\text{H}_2\text{O}_2$  were used in excess in this study. Nonetheless, the ratio of the Pt amount and  $\text{H}_2\text{O}_2$  concentration are comparable (human body: 2 mg Pt/5  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ; experimental, *in vitro*: 400 mg Pt/1250  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ).

We next wanted to determine whether colloidal Pt would have a similar cytoprotective effect on cells treated with a different source of ROS such as AAPH. However, when we treated Caco-2 cells with both AAPH and colloidal Pt, we did not observe any recovery in cell viability compared to AAPH treated cells alone (Fig. 5C). These observations may have resulted from differences in the mode of action of  $\text{H}_2\text{O}_2$  and AAPH. AAPH, a water soluble compound, is responsible for the generation of alkyl radical ( $\text{R}^\bullet$ ), which in the presence of oxygen is converted to the corresponding peroxy radicals ( $\text{ROO}^\bullet$ ). AAPH therefore can induce lipid peroxidation, which may then lead to formation of different hydroperoxides depending on reaction conditions [47]. Additionally, these peroxy radicals can result in further oxidization of polyunsaturated fatty acid (PUFA) molecules, causing new chain reactions to produce more radicals [48]. On the whole, because of the sophisticated nature of ROS biology including different expression profiles of oxidative versus anti-oxidative enzymes, their subcellular locations, and the types of the substrates for these enzymes may have resulted in different results in these assays.

### 3.5. Caco-2 cell line model of intestinal barrier

The gastrointestinal barrier is formed of a single layer of cells connected by junctional proteins that plays an important role in the protection of the internal environment from many ingested toxins, food contaminants and bacteria; the barrier also

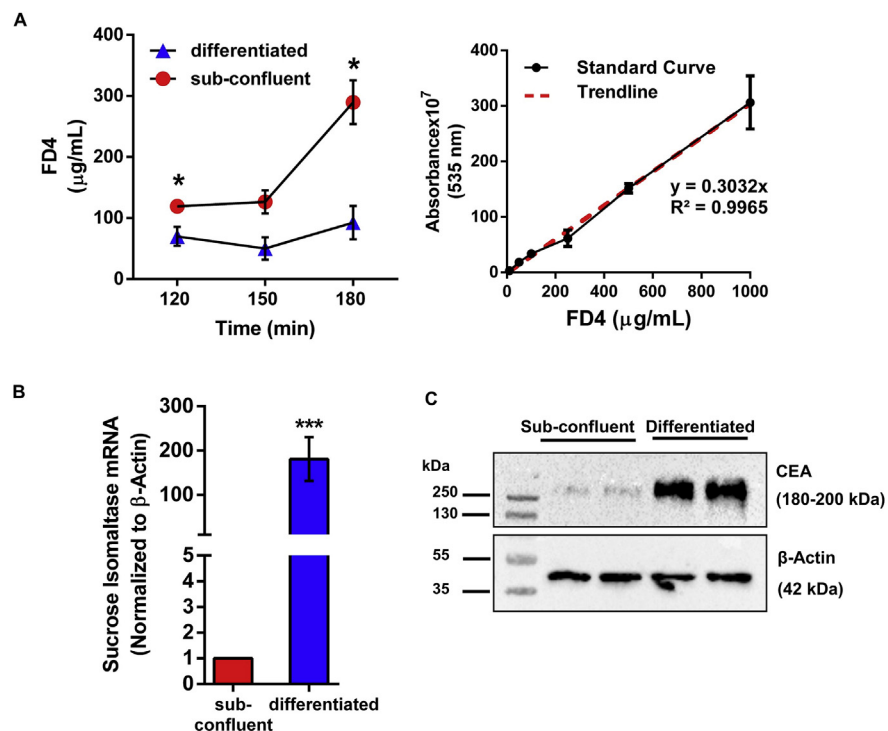
**Fig. 5.** Effect of ROS generators with colloidal platinum nanoparticles on cell viability. A. Caco-2 cells were co-treated with 20% (v/v) colloidal Pt and 250  $\mu\text{M}$ –5 mM  $\text{H}_2\text{O}_2$  for 1 h, B. Caco-2 cells were treated first with 20% (v/v) colloidal Pt for 24 h, then colloidal Pt was removed and cells were washed and then treated with 250  $\mu\text{M}$ –5 mM  $\text{H}_2\text{O}_2$  for 1 h, C. Caco-2 cells were co-treated with 20% (v/v) colloidal Pt and 5 mM–80 mM 2, 2-azobis (2-amidinopropane) dihydrochloride (AAPH) for 1 h, then MTT assay was carried out. The experiment was carried out with at least two independent biological replicates, each containing 8 technical replicates. The statistical analyses were performed using t-test. For the statistical analysis, “\*” stands for comparing with “vehicle”, “≠” stands for comparing with the corresponding treatment.

helps in the rapid absorption of electrolytes and water via paracellular movement [49]. The Caco-2 cell line is a very well established *in vitro* model, which when spontaneously differentiated, mimics small intestinal cells in which the monolayer can show brush border microvilli, tight junctions, and dome formation [27]. In the current study, we differentiated Caco-2 cells on Transwells for 10 days to establish a polarized differentiated cells layer containing an apical and a basolateral side. Intestinal barrier formation was confirmed by determining the flux of 4 kDa FITC-dextran (FD). As expected, there was a significant decrease in the permeability of FD in differentiated cells because of the barrier formation when compared with the sub-confluent cells (Fig. 6A). The flux was determined with the help of a standard curve generated with known amounts of FD4 (Fig. 6A, right panel).

To further establish the differentiation of the cells (and thereby the establishment of a barrier) we determined the expression of sucrose isomaltase (SI), an enzyme that is highly expressed in small-intestinal enterocyte-like cells [50]. High expression of SI is a marker of differentiation in Caco-2 cells [22]. Human carcinoembryonic antigen (CEA), another differentiation marker for Caco-2 cells, is a cell surface glycoprotein and an important intercellular adhesion molecule [51]. As compared with the proliferating cell control (sub-confluent cells), Caco-2 cells differentiated in the Transwells exhibited significantly higher expression of both SI and CEA (Fig. 6B and C; Supplementary Fig. 1).

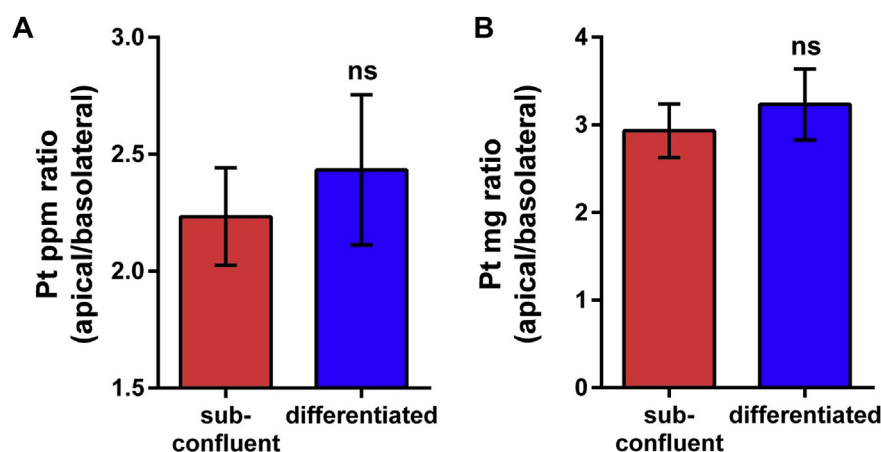
### 3.6. Effect of colloidal Pt nanoparticles on Caco-2 intestinal barrier model

After establishing the 3D *in vitro* intestinal barrier in Transwells, we next wanted to determine whether the colloidal Pt nanoparticles could easily cross the barrier. This data is important in establishing whether colloidal Pt can be used as a health supplement. Complete medium containing 20% (v/v) colloidal Pt was added to the upper chamber (representing the apical border of the membrane) of both differentiated and sub-confluent state cells. After 3 h, media from both the upper chamber and the lower chamber (representing the basolateral pole) were collected and subjected to ICP-MS to determine the Pt levels. The Pt ratio determined in the apical to basolateral regions was slightly higher in the differentiated cells compared to proliferating cells, although the difference was not statistically significant (Fig. 7A and B). Based on these experiments it is possible to conclude that colloidal Pt is capable of crossing the intestinal barrier. Passage of drugs/supplements across the intestinal barrier is a determinant of oral bioavailability [52]. Particles can pass the intestinal barrier either through transcytosis or in a paracellular manner. Transcytosis can be via endocytosis, an energy dependent process that includes clathrin-mediated endocytosis, caveolae mediated endocytosis, macropinocytosis and phagocytosis, or via energy independent non-endocytic pathways [53]. Previous studies have shown that



**Fig. 6.** *In vitro* Caco-2 cell line model of intestinal barrier. A. Caco-2 cells were grown on Transwells and differentiated for 10 days to form an intestinal barrier. Intestinal barrier formation was confirmed by determining the flux of FITC-dextran (FD) (on the left). Serially diluted FD was used to establish a standard curve (on the right). In all of the experiments, proliferating state sub-confluent cells were used as controls. Two independent biological replicates each with three technical replicates were used in this assay. B. qRT-PCR analysis of Sucrose Isomaltase (SI) mRNA levels was shown in differentiated versus proliferating (sub-confluent) cells. β-Actin mRNA levels were used for normalization. t-test was used to compare the means. C. Western blot analysis of carcinoembryonic antigen (CEA) is shown. β-Actin served as a loading control. For full non adjusted images of the Western blots please see Supplementary Fig. 1.

colloidal gold nanoparticles could be transcytosed by microfold (M) cells but could not cross the Caco-2 model of intestinal epithelial barrier in a paracellular manner [54]. Lipid nanoparticles have been shown to be transported across a Caco-2 barrier within 4 h preferentially by clathrin mediated endocytosis [52]. In the current study it appears that colloidal Pt could easily pass the intestinal barrier. It is possible that the particles were engulfed by the cells and then transported to the basolateral surface, ensuring oral bioavailability of the nanoparticles. After entry, most nanoparticles are trafficked along the endolysosomal pathway involving proteins such as Rab7a, Rab9a and Lamp1 which guide the vesicles to the lysosome for degradation or storage [55]. A portion of the endocytosed nanoparticles can also be trafficked for transcytosis for exit from the cells, most likely involving vesicle proteins such as Rab27a that is defined as an exocytotic marker [55]. While many nanoparticles are trafficked in this manner through the cells, future studies will indicate whether colloidal Pt is also trafficked by the same mechanism.



**Fig. 7.** Effect of colloidal Pt on Caco-2 intestinal barrier model. Caco-2 cells were differentiated on Transwells for 10 days after which complete medium containing 20% (v/v) colloidal Pt was added to the upper chamber of the Transwells in both differentiated and proliferating (sub-confluent) cells. After 3 h, the medium was sampled from both the apical and the basolateral regions (upper and lower chambers of the Transwell respectively) and subjected to ICP-MS to determine the Pt levels. Calculations were carried out according to a calibration curve shown in Fig. 1. The amount of Pt was determined as parts per million (ppm) and mg and the ratio of the Pt determined in the apical/basolateral sides are shown in the graphs. This assay was conducted as three independent biological replicates. t-test was used as the statistical method.

## 4. Conclusions

Nanoparticles (NPs) have one dimension that measures less than 100 nm at least. The properties of many conventional metals can change when formed from nanoparticles [56]. In particular, colloidal Pt NPs can possess a broad range of properties that make them convenient for many practical applications in fields such as nano-catalysts, electrical conductivity, optics, and for specific biomedical purposes such as implant coatings, imaging agents, diagnostics, drug delivery systems [57, 58]. Pt NPs are usually used in the form of colloid or suspension in a fluid [59]. A colloid is technically defined as a stable dispersion of particles in a fluid medium [60]. In this work, we investigated the cytotoxic effect as well as protective effects of colloidal Pt on oxidative stress.

We first analyzed the effect of colloidal Pt on cellular viability and found that colloidal Pt was not toxic to three different epithelial cell lines originating from the colon. Next, we examined the cellular response to oxidants in the presence of colloidal Pt. We found that colloidal Pt notably decreased  $H_2O_2$  triggered ROS production in Caco-2 human epithelial colorectal adenocarcinoma cells. Moreover, our results indicate that colloidal Pt can protect epithelial cells from oxidative stress induced cell death irrespective of whether it was co-treated or pre-treated with  $H_2O_2$ . On the other hand, we did not observe any protective effect of colloidal Pt against AAPH induced oxidative stress mediated toxicity in Caco-2 cells. In addition to these features, as a measure of oral bioavailability, we also asked if colloidal Pt

was able to pass through the intestinal barrier. Using a Transwell model of intestinal barrier generated from differentiated Caco-2 cells, we demonstrated that colloidal Pt could effectively cross intestinal epithelial barrier.  $H_2O_2$  is known to disrupt the intestinal barrier. Disruption of this barrier facilitates translocation of harmful substances and pathogens to the bloodstream by enhancing intestinal permeability. An extensive number of pathologies and diseases, such as infections with intestinal pathogens, inflammatory bowel disease, irritable bowel syndrome, obesity, celiac disease, non-celiac gluten sensitivity, and food allergies, is associated with a dysfunctional intestinal barrier [61]. Therefore, it can be concluded that because of its anti-oxidant and intestinal barrier crossing functions without any cytotoxic effects, colloidal Pt has the potential to help protect and maintain intestinal health.

## Declarations

### Author contribution statement

Sinem Tunçer: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Melis Çolakoğlu: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sinem Ulasan, Gulay Ertas: Performed the experiments; Analyzed and interpreted the data.

Çimen Karasu: Conceived and designed the experiments.

Sreeparna Banerjee: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

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