

## APOPTOTIC EFFECT OF GOLD NANOPARTICLES ON HUMAN COLON CANCER CELL LINES CACO-2 BUT NOT ON THE NORMAL EPITHELIAL CELL LINES WISH

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### ABSTRACT

Colorectal cancer (CRC) is the third most common cancer worldwide. Consequently, the discovery of new strategies for therapeutic intervention remains a priority. The gold nanoparticles (AuNPs) have been discovered to exert cytotoxicity and induce apoptosis in some cancer cells. The aim of the present study was to investigate the effect of AuNPs on human colon cancer cell lines (Caco-2) and normal human epithelial cell lines (WISH) *in vitro*. The two cell lines were treated with different concentrations of AuNPs for 5 days and cytotoxicity of AuNPs was assessed. RT-PCR was used to analyze the expression profiles of pro- and anti-apoptotic genes. Tumor necrosis

factor alpha (TNF- $\alpha$ ) and interleukin-6 (IL-6) levels were measured in the culture supernatant using ELISA. Results revealed that AuNPs has a cytotoxic effect on Caco-2 cell lines at high and low concentrations, but has no cytotoxic effect on WISH cell lines. Upregulation of the pro-apoptotic gene (bax) was induced in AuNPs treated Caco-2 cell lines but not WISH cells. The levels of IL-6 secreted by Caco-2 cell line treated with AuNPs increased gradually up to 96 h then began to decline thereafter. On the other hand, the levels of TNF- $\alpha$  reached its peak after 24h then started to decrease gradually. In conclusion, AuNPs did not cause acute cytotoxicity for normal human epithelial cell lines (WISH), but they have the ability to induce apoptosis in human colon cancer cell lines (Caco-2).

**KEYWORDS:** Colon cancer, Gold nanoparticles, Apoptosis, TNF- $\alpha$ , Interleukin-6.

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## INTRODUCTION

Colorectal cancer is the abnormal growth of epithelial cells which form the lining of the colon or rectum.<sup>[1]</sup> These small growths (known as polyps) are often benign, although some have the potential to develop and become cancerous. Up to two thirds of colorectal polyps are pre-malignant and associated with a risk of colorectal cancer.<sup>[2]</sup>

The most common kind of polyp is called an adenomatous polyp or adenoma. Adenomas arise from glandular cells, which produce mucus to lubricate the colorectum.<sup>[1,3]</sup> Although all adenomas have the capacity to become cancerous, fewer than 10% are estimated to progress to invasive cancer.<sup>[1]</sup> An adenoma will evolve into cancer as much as it becomes larger.<sup>[4]</sup> Treatment of colon cancer depends on tumor type, the disease stage, age of patient, patient's level of health, and his attitude towards life.<sup>[3]</sup> Currently, most anti-cancer drugs in use are selected on their ability to induce apoptotic cell death so the use of nanoparticles for cancer therapy has been gaining popularity in recent years.<sup>[5]</sup>

Nanoparticles are of great scientific interest as they are considered a bridge between bulk materials and atomic or molecular structures.<sup>[6]</sup> Nanoparticles are characterized by the large surface area of the material, compared to that made by the small bulk of the material.<sup>[7]</sup> They have been increasingly used in biological and medical applications including clinical diagnostics, therapeutics development and drug delivery, as well as advanced imaging.<sup>[8]</sup>

Nanoparticles can target tumor cells by an accumulation and entrapment process, known as permeation and retention effect imposed by angiogenic vessels and improper lymphatic flow.<sup>[9]</sup> They can accumulate selectively inside the tumor cells, showing bright scattering at higher concentrations than the normal cells.<sup>[10,11]</sup>

Gold nanoparticles were recently investigated with regard to cytotoxicity and biocompatibility according to their interaction with cells.<sup>[12,13]</sup> Gold possess unique physical and chemical properties through its conjugation with a variety of drugs to serve as carrier for drug delivery. The potential to use AuNPs as a targeted drug delivery agent stems from the absence of any report on AuNP-induced cytotoxicity.<sup>[14]</sup> AuNPs used also as contrast agents for imaging enhancement, and for topical thermal therapy.<sup>[15]</sup>

Several pro-inflammatory cytokines released by innate and adaptive immune cells have been shown to regulate cancer cell growth and thereby contribute to tumor promotion and

progression. Among these, interleukin-6 (IL-6) seems to take a center stage in human cancer development.<sup>[16]</sup> IL-6 is produced by various cell types, mainly by monocytes and macrophages during acute and T cells during chronic inflammation.<sup>[17]</sup> An increased expression of IL-6 has been detected and associated with an unfavorable prognosis in patients with various types of cancer including both sporadic and colitis-associated CRC.<sup>[18]</sup> Experimental studies found an activation of important oncogenic pathways in cancer cells through IL-6.<sup>[16]</sup> As well, Tumor necrosis factor alpha (TNF- $\alpha$ ) is one of the cytokines that exhibit dual roles in tumorigenesis. TNF- $\alpha$  is cytotoxic to tumor cells under certain conditions; however, it also fuels tumor-promoting inflammation and angiogenesis.<sup>[19,20]</sup>

So, the aim of the present study was to examine the cytotoxic effects of gold nanoparticles on the viability of human colon cancer cell line (Caco-2) and normal human epithelial cell line (WISH). Also the effect of AuNPs on the expression of pro-apoptotic (bax and p53) and anti-apoptotic (bcl-2) genes as well as the induction of TNF- $\alpha$  and IL-6 cytokines were investigated.

## MATERIALS AND METHODS

### Cell line and cell culture

Human colon cancer cell line (Caco-2) and normal human epithelial cell line (WISH) were purchased from tissue culture department of the holding company for biological products and vaccines (VACSERA, 51 Wezaret EL Zeraa Street, Agouza, Giza). Caco-2 cell lines were cultured in RPMI 1640 media (Lonza, Co.) while WISH cell lines were cultured in DMEM media (Lonza, Co.) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Lonza, Co.), 2mM L-Glutamine (Biochrome, Co.), antibiotics (Penicillin 100IU/ml, Streptomycin 100 $\mu$ g/ml) (Biochrome, Co.) and 1mM Na.Pyruvate (Lonza, Co.) in T-75 culture flask (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and kept under standard culture conditions (95% humidified air incubator at 37C° and 5% CO<sub>2</sub>).

### Gold nanoparticles and Cisplatin

The Citrate capped-Gold Nanoparticles (AuNP<sub>s</sub>) were purchased from NanoTech (Egypt for Photo-Electronics). The average size of AuNPs measured by scanning Transmission electron microscopy (TEM) was 15 $\pm$ 5nm with uniform and fairly spherical shape. The peak absorption band obtained for stabilized gold nanoparticles was 522nm. AuNPs was characterized by liquid form (water soluble), pink in color and with 2.8mM concentration

(0.5mg/ml). Cisplatin (positive control, concentration of 1.0 mg/ml) was purchased from a commercial pharmacy.

### **Human colon cancer cell lines (Caco-2) and normal human epithelial cell lines (WISH) cytotoxicity assay using Sulphorhodamine B**

SRB assay measure drug-induced cytotoxicity even at large-scale application.<sup>[21]</sup> Sulphorhodamine B (SRB) is a bright pink Aminoxanthine dye with two sulfonic groups. Under mild acidic conditions, SRB binds dye to basic amino acid residues in Trichloro acetic acid (TCA) fixed cells to provide a sensitive index of cellular protein content. The monolayer cell cultures of Caco-2 and WISH cell lines were separately trypsinized (0.25% trypsin with 0.025% EDTA), then suspended in fresh RPMI-1640 and DMEM media respectively, supplemented with 10% FBS at a final cell density of  $10 \times 10^4$  cell/ml. Cells were seeded in a tissue culture 96-well flat bottom plate and kept under standard culture conditions (37°C, 95% humidified air and 5% CO<sub>2</sub>). After 24 hours the growth medium was discarded and a 100µl of serum free media were added directly to the cells containing serial dilutions of the AuNPs and Cisplatin (12.5µg/ml-0.024µg/ml) each one in quadruplicate and the plates were then incubated for 72 hours with microscopic examination was carried out and observations recorded every 24 hour After an incubation period, media was discarded and cell monolayers were fixed with 10% trichloroacetic acid (TCA) (wt/vol) for one hour at +4°C then washed by distilled water for three times to remove TCA completely from cells. Then the plates were stained with 0.4% SRB dissolved in 1.0% acetic acid for 10 minutes at room temperature (70µl/well). After which the excess dye is removed by washing repeatedly with 1% (vol/vol) acetic acid. The plates were then air - dried. The protein-bound dye is dissolved in 10mM Tris base solution for optical density (O.D) determination at 570nm using a microplate reader.

### **Treatment of human colon cancer cell lines (Caco-2) and normal human epithelial cell lines (WISH) with gold nanoparticles (AuNPs)**

A total of twenty 75cm<sup>2</sup> flasks of Caco-2 cells were treated with 0.048 and 0.39µg/100µl AuNP<sub>s</sub> and Cisplatin in serum free RPMI-1640 media for 24, 48, 72, 96 and 120 hours. Also a total of ten 75cm<sup>2</sup> flasks of WISH cells were treated with 0.048µg/100µl AuNP<sub>s</sub> and Cisplatin in serum free DMEM media for 24, 48, 72, 96 and 120 hours. At the end of each respective sampling time (24, 48, 72, 96 and 120hrs) cells were harvested by trypsinization and centrifugation. After two washes with ice-cold PBS, cells were further used for RNA

extraction while supernatant was kept in -80 for measuring tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-6 (IL-6) cytokines by ELISA.

### Measurement of tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-6 (IL-6) cytokines using ELISA

Human IL-6 and TNF- $\alpha$  ELISA Kits (Boster immunoleader, USA) were used for quantitative detection of human IL-6 and TNF- $\alpha$  in cell culture supernatants according to the manufacturer's protocol.<sup>[18,19]</sup> Color reaction developed was measured as OD units at 450 nm. The concentration of each cytokines was determined by using standard curve constructed with kit's standards and was expressed in pg/ml. The minimum detectable concentration of the IL-6 and TNF- $\alpha$  ELISA kit was less than 2 pg/ml.

### RNA extraction

RNA extracted using RNA-spin™ Total RNA Extraction Kit which is designed for rapid isolation of total RNA from cells and the procedure was performed according to pamphlet.<sup>[22]</sup>

### Preparation of cDNA

DNA was synthesized complementary to expressed pro-apoptotic genes (p53 and bax) and anti-apoptotic genes (bcl-2).

Maxime RT PreMix Kit was the product in which every component for cDNA synthesis was mixed in each tube for 1 rxn PCR. The cDNA synthesis reaction was performed according to the manufacturer's protocol and using PCR machine (Table 1).

**Table 1: cDNA synthesis reaction.**

Reaction step	Temp.	Time
cDNA synthesis	45 °C	60 min
RTase inactivation step	95 °C	5 min

### Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed using iNtRON's Maxime PCR PreMix Kit, according to manufacturer's protocol.<sup>[22]</sup>

### Gel-Electrophoresis

The gel was prepared by dissolving 0.9 gm of agarose powder (Biotools, Co.) to prepare 1.5% gel of 60 ml in boiling TBE-buffer solution (0.89M Tris-Borate 0.02M EDTA) (Lonza, Co.), then 2 $\mu$ l of Ethidium Bromide (ICN, Co.) was added to facilitate visualization of DNA

after electrophoresis and letting it to cool to approximately 45 °C and poured into a casting tray which serves as a mold. Finally photographed the gel in U.V apparatus to examine bands.

**Table 2: primer sequences of genes.**

Gene	Primer sequence	Primer length	RT/PCR product
<b>GAPDH</b>	forward 5'- GAAGGTGAAGGTCGGAGTCA - 3'	20	226bp
	reverse 5'-GAAGATGGTGTATGGGATTTC-3'	20	
<b>BAX</b>	forward 5'- CCGCCGTGGACACAGAC-3'	17	133bp
	reverse 5'-CAGAAAACATGTCAGCTGCCA-3	21	
<b>BCL-2</b>	forward 5'-TCCGATCAGGAAGGCTAGAGT- 3'	21	108bp
	reverse 5'-TCGGTCTCCTAAAAGCAGGC-3'	20	
<b>P53</b>	forward 5'- CAGCCAAGTCTGTGACTTGACGTAC-3'	26	226bp
	reverse 5'- CTATGTGCGAAAAGTGTCTCTGTCATC-3'	26	

### Statistical analysis

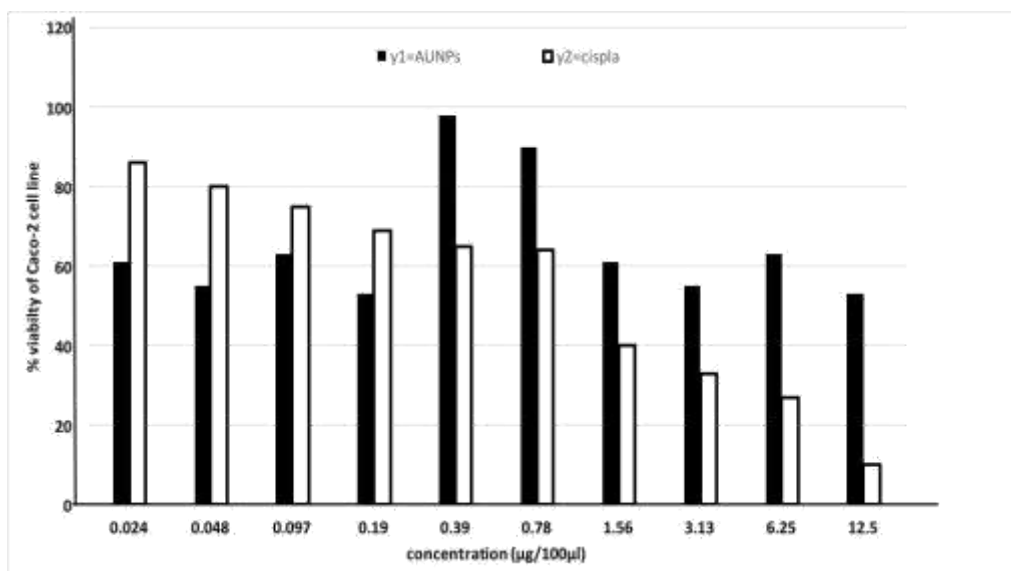
Statistical analysis was performed using Graph Pad Prism software. Paired Student's t test was used to compare between different treatments. ANOVA followed by Tukey-Kramer test as post ANOVA to compare different activation time points. The data obtained were represented as mean  $\pm$  S.D. Results with a P value  $<0.05$  were considered significant.

## RESULTS

In this study the effect of gold nanoparticles (AuNPs) was investigated on Caco-2 and WISH cell lines as models of human colon cancer cell line and normal human epithelial cell line respectively. Cisplatin was used as positive control.

### Cytotoxicity of gold nanoparticles (AuNPs) on human colon cancer cell line (Caco-2)

Figure 1a shows the cell viability variation of Caco-2 cell line exposed to 2 fold dilutions of AuNP<sub>s</sub> and Cisplatin for 72h. Caco-2 cell line treated with AuNP<sub>s</sub> showed high viability 98% and 90% at concentrations (0.39 and 0.78 $\mu$ g/100 $\mu$ l) respectively, while there was depletion in cell viability at low concentrations (0.024, 0.048, 0.097 and 0.19 $\mu$ g/100 $\mu$ l) by percent changes of 61%, 55%, 63%, and 53% respectively as well as at high concentrations (1.56, 3.13, 6.25 and 12.5  $\mu$ g/100 $\mu$ l) by percent changes of 72%, 58%, 55% and 35% respectively. For Caco-2 cell line treated with cisplatin there was increase in viability by 86%, 80%, 75%, 69%, 65% and 64% at concentrations (0.024, 0.048, 0.097, 0.195, 0.39 and 0.78 $\mu$ g/100 $\mu$ l) respectively, while there was depletion in cell viability at high concentrations (1.56, 3.125, 6.25 and 12.5  $\mu$ g/100 $\mu$ l) by percent change of 40%, 33%, 27% and 10% respectively.



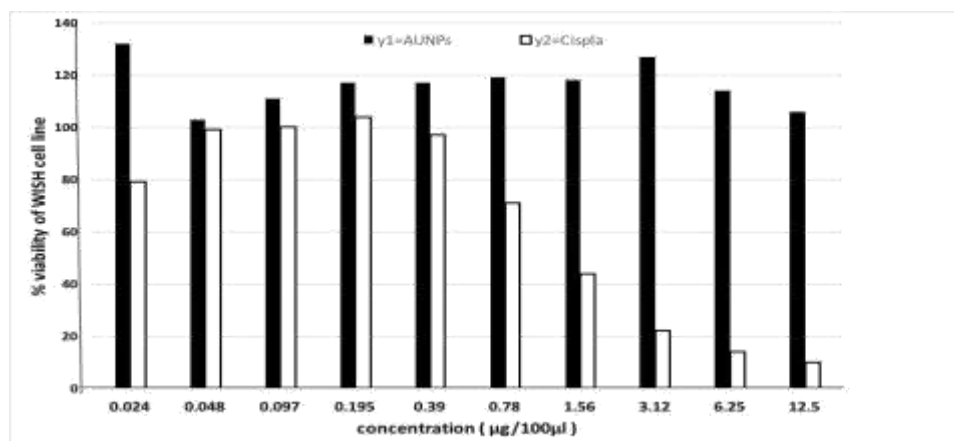
**Fig. 1(a): Cytotoxicity of AUNPs and Cisplatin on Caco-2 cell line after 72hr of treatment.**

#### **Cytotoxicity of gold nanoparticles (AuNPs) and cisplatin on normal human epithelial cell lines (WISH) cell lines**

Figure 1b shows the cell viability variation of WISH cell lines exposed to 2 folds dilutions of AuNP<sub>s</sub> and Cisplatin for 72h. WISH cell lines treated with AuNP<sub>s</sub> showed high viability at all concentrations (0.024, 0.048, 0.079, 0.195, 0.39, 0.78, 1.56, 3.12, 6.25 and 12.5µg/100µl) by percent changes of 132%, 103%, 111%, 117%, 117%, 119%, 118%, 127%, 114% and 106.% respectively.

For WISH cell lines treated with cisplatin there was increase in viability 79%, 99%, 100%, 104%, 97% and 71% at low concentrations (0.024, 0.048, 0.079, 0.19, 0.39 and 0.78µg/100µl) respectively, while there was depletion in cell viability at high concentration (1.56, 3.13, 6.25 and 12.5µg/100µl) by percent change of 44%, 22%, 14% and 10% respectively.

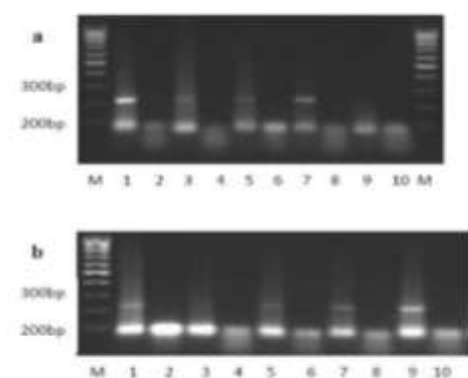




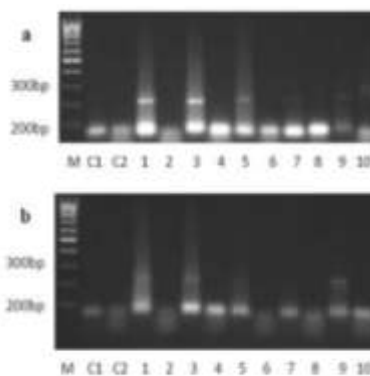
**Fig. 1(b): Cytotoxicity of AuNPs and Cisplatin on WISH cell lines after 72h of treatment.**

### Effect of gold nanoparticles (AuNP<sub>s</sub>) on expression level of pro- and anti-apoptotic genes

A single band with predicted size 226bp was GAPDH, bcl-2 band with size 108bp, bax band with size 133bp and p53 band with size 292bp, the molecular-weight size marker was in the form of a 100bp RNA ladder. Caco-2 and WISH cell lines treated with AuNP<sub>s</sub> or Cisplatin were positive for Bcl-2 mRNA expression at all time intervals and at all concentrations (Fig. 2, 3 and 4). p53 mRNA was expressed in Caco2 and WISH cell lines treated with 0.048µg/100µl AuNP<sub>s</sub> (Fig. 2a and 4a). After treatment with 0.048 and 0.39µg/100µl of AuNP<sub>s</sub> for 72, 96 and 120h Caco-2 cell lines expressed bax (Fig. 2a and 3a), while in case of treatment with 0.048µg/100µl of Cisplatin the bax gene expressed after 48h (Fig. 2b) while at the higher concentration of Cisplatin (0.39µg/100µl) it was expressed after 24h (Fig. 3b). WISH.



**Fig [2 (a, b)]**



**Fig [3 (a, b)]**

**Fig. 2 and 3: Expression of anti-apoptotic gene (bcl-2 at 108bp) and pro-apoptotic genes (bax at 133bp and p53 at 292) in Caco-2 cell lines treated with 0.048µg/100µl and 0.39 µg/100µl of AuNPs (a) and Cisplatin (b) respectively.**



- (1, 2): Expression of bcl-2/GAPDH and bax/p53 after 24h.  
 (3, 4): Expression of bcl-2/GAPDH and bax/p53 after 48h.  
 (5, 6): Expression of bcl-2/GAPDH and bax/p53 after 72h.  
 (7, 8): Expression of bcl-2/GAPDH and bax/p53 after 96h.  
 (9, 10): Expression of bcl-2/GAPDH and bax/p53 after 120h.

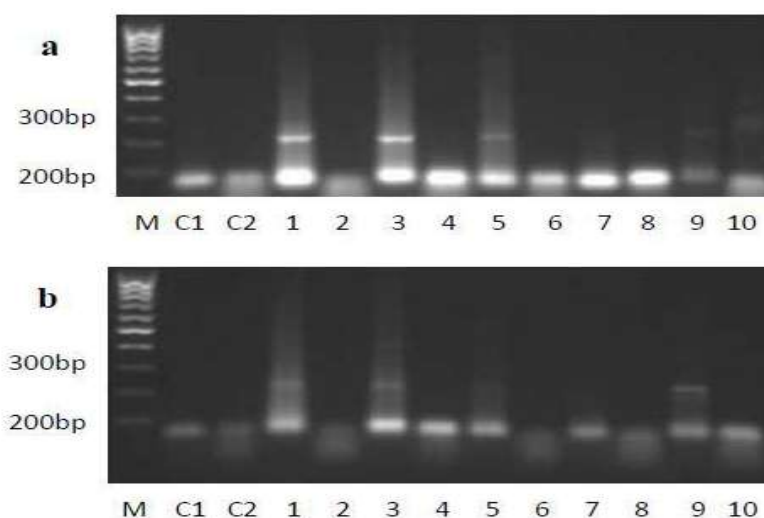
M: marker.

C1: Expression of bcl-2/GAPDH in untreated cells.

C2: Expression of bax/p53 in untreated cells.

GAPDH represent positive control which predicted at 226bp.

Cell lines treated with 0.048 $\mu$ g/100 $\mu$ l of AuNP<sub>s</sub> bax mRNA was detected after 48, 72, 96 and 120h of treatment (Fig. 4a) but it was only expressed after 48h of treatment with 0.048 $\mu$ g/100 $\mu$ l of Cisplatin (Fig. 4b).



**Fig. 4: Expression of anti-apoptotic genes (bcl-2 at 108bp) and pro-apoptotic genes (bax at 133bp and p53 at 292) in WISH cell lines treated with 0.048 $\mu$ g/100 $\mu$ l of AuNPs (a) and Cisplatin (b).**

- (1, 2): Expression of bcl-2/GAPDH and bax/p53 after 24h.  
 (3, 4): Expression of bcl-2/GAPDH and bax/p53 after 48h.  
 (5, 6): Expression of bcl-2/GAPDH and bax/p53 after 72h.  
 (7, 8): Expression of bcl-2/GAPDH and bax/p53 after 96h.  
 (9, 10): Expression of bcl-2/GAPDH and bax/p53 after 120h.

M: marker.

C1: Expression of bcl-2/GAPDH in untreated cells.

C2: Expression of bax/p53 in untreated cells.

GAPDH represent positive control which predicted at 226bp.

### **Estimation of IL-6 levels secreted by human colon cancer cell lines (Caco-2) in response to gold nanoparticles (AuNP<sub>s</sub>) treatment**

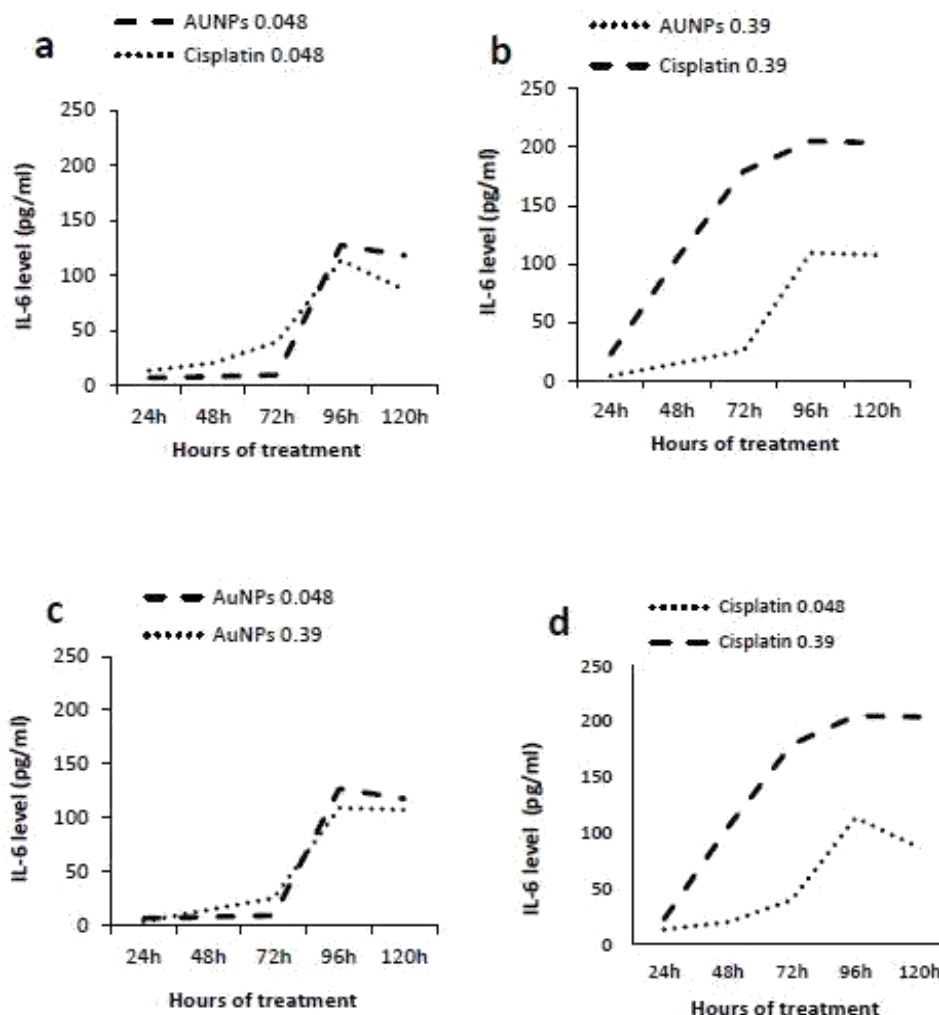
The level of IL-6 released from Caco-2 cell lines stimulated with AuNP<sub>s</sub> (0.048µg/100µl) showed significant changes ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h as compared to untreated cells. However, Caco-2 cell lines stimulated with Cisplatin (0.048µg/100µl) released significant level of IL-6 ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h as compared to untreated cells. Comparing IL-6 levels released from Caco-2 cell lines stimulated with AuNP<sub>s</sub> (0.048µg/100µl) to the corresponding concentration of Cisplatin there were significant changes ( $P < 0.05$ ) after 24h, 48h, 72h and 120h while it showed non-significant change ( $P=0.065$ ) at 96h (Fig. 5.a).

Similarly, Caco-2 cell lines stimulated with AuNP<sub>s</sub> (0.39µg/100µl) produced significant level of IL-6 ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h as compared to untreated cells. As well, Caco-2 cell lines stimulated with Cisplatin (0.39µg/100µl) produced significant level of IL-6 ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h as compared to untreated cells.

Comparing IL-6 levels released from Caco-2 cell lines stimulated with AuNP<sub>s</sub> (0.39µg/100µl) to the corresponding concentration of Cisplatin, significant changes ( $P < 0.05$ ) were observed after 24h, 48h, 72h, 96h and 120h (Fig. 5.b).

Comparing IL-6 levels released from Caco-2 cell lines stimulated with AuNP<sub>s</sub> (0.048µg/100µl) to the other concentration (0.39µg/100µl) showed significant changes ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h (Fig. 5.c).

Comparing IL-6 levels released from Caco-2 cell lines stimulated with Cisplatin (0.048µg/100µl) to the other concentration (0.39µg/100µl) showed significant changes ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h (Fig. 5.d). The differences among all time intervals was significant as analyzed by ANOVA.



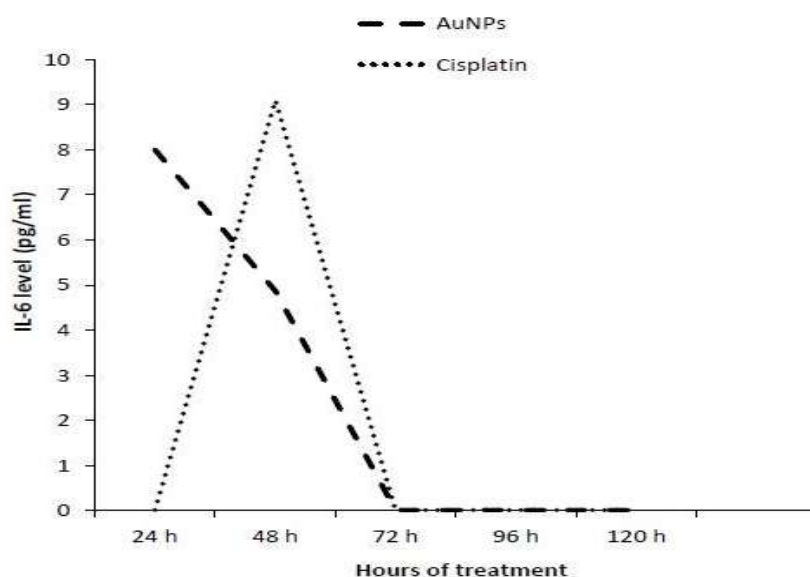
**Fig. 5:** Levels of IL-6 secreted by Caco-2 cell lines in response to AuNPs and Cisplatin treatment. (a) Concentration of (0.048 $\mu$ g/100 $\mu$ l) AuNPs and Cisplatin, (b) Concentration of (0.39 $\mu$ g/ $\mu$ l) AuNPs and Cisplatin, (c) Concentration of AuNPs (0.048 and 0.39 $\mu$ g/100 $\mu$ l) and (d) Concentration of Cisplatin (0.048 and 0.39 $\mu$ g/100 $\mu$ l).

Data represented as % to untreated cells

#### Estimation of IL-6 levels secreted by normal human epithelial cell lines (WISH) in response to gold nanoparticles (AuNPs) treatment

In response to treatment with 0.048 $\mu$ g/100 $\mu$ l AuNPs, WISH cell lines produced significant level of IL-6 ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h as compared to untreated cells. Similarly, WISH cell lines stimulated with 0.048 $\mu$ g/100 $\mu$ l cisplatin produced significant level of IL-6 ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h as compared to untreated cells. Comparing IL-6 levels released from WISH cell lines stimulated with 0.048 $\mu$ g/100 $\mu$ l AuNPs to the corresponding concentration of 0.048 $\mu$ g/100 $\mu$ l Cisplatin showed significant changes

( $P < 0.05$ ) after 24h and but after 72h, 96h and 120h there was a non-significant level of IL-6 (Fig. 6). The differences among all time intervals was significant as analyzed by ANOVA. ( $P = 1.00$ ).



**Fig. 6:** Levels of IL-6 secreted by WISH cell lines in response to AuNPs and Cisplatin treatment.

Data represented as % to untreated cells

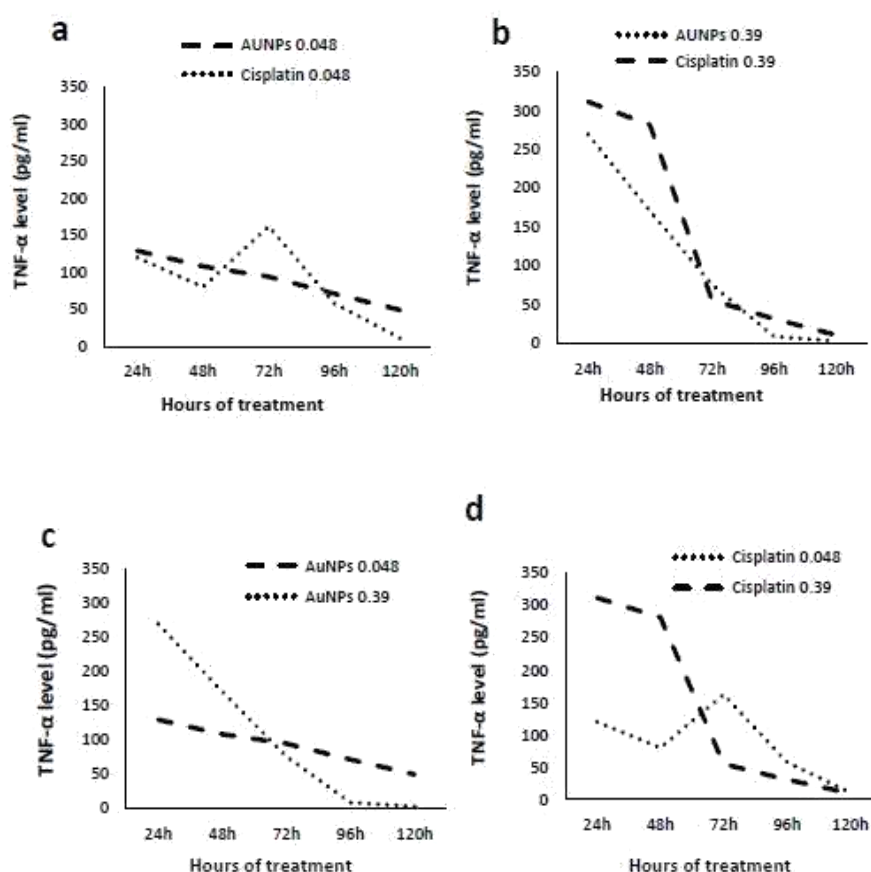
#### Estimation of TNF- $\alpha$ levels secreted by human colon cancer cell lines (Caco-2) in response to gold nanoparticles (AuNP<sub>s</sub>) treatment

The level of TNF- $\alpha$  released from Caco-2 cell lines stimulated with AuNP<sub>s</sub> (0.048 $\mu$ g/100 $\mu$ l) showed significant changes ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h as compared to untreated cells. However, Caco-2 cell lines stimulated with cisplatin (0.048 $\mu$ g/100 $\mu$ l) released significant level of TNF- $\alpha$  after 24h, 48h, 72h, 96h and 120h compared to untreated cells. Comparing TNF- $\alpha$  levels released from Caco-2 cell lines stimulated with AuNP<sub>s</sub> (0.048 $\mu$ g/100 $\mu$ l) to the corresponding concentration of Cisplatin showed significant changes ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h (Fig. 7a). Similarly, Caco-2 cell lines stimulated with AuNP<sub>s</sub> (0.39 $\mu$ g/100 $\mu$ l) produced significant level of TNF- $\alpha$  ( $P < 0.05$ ) after 24h, 48h, 72h and 96h as compared to untreated cells but after 120h there was a non-significant level of TNF- $\alpha$  as compared to untreated cells.

As well, Caco-2 cell lines stimulated with cisplatin (0.39 $\mu$ g/100 $\mu$ l) produced significant level of TNF- $\alpha$  ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h as compared to untreated cells.

Comparing TNF- $\alpha$  levels released from Caco-2 cell lines stimulated with AuNP<sub>s</sub> (0.39 $\mu$ g/100 $\mu$ l) to the corresponding concentration of Cisplatin showed significant changes ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h (Fig. 7b).

Comparing TNF- $\alpha$  levels released from Caco-2 cell lines stimulated with AuNP<sub>s</sub> (0.048 $\mu$ g/100 $\mu$ l) to the other concentration (0.39 $\mu$ g/100 $\mu$ l) showed significant changes ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h (Fig. 7c). Comparing TNF- $\alpha$  levels released from Caco-2 cell lines stimulated with cisplatin (0.048 $\mu$ g/100 $\mu$ l) to the other concentration (0.39 $\mu$ g/100 $\mu$ l) showed significant changes ( $P < 0.05$ ) after 24h, 48h, 72h and 96h while it showed non-significant change ( $P = 0.288$ ) at 120h (Fig. 7d). The differences among all time intervals was significant as analyzed by ANOVA.



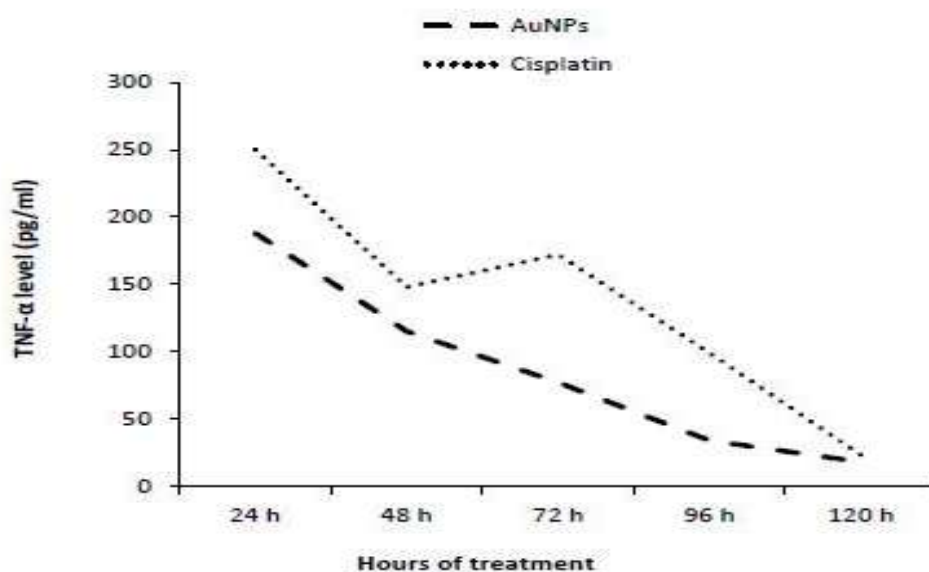
**Fig. 7:** levels of TNF- $\alpha$  secreted by Caco-2 cell lines in response to AuNPs and Cisplatin treatment.(a) Concentration of (0.048 $\mu$ g/100 $\mu$ l) AuNPs and Cisplatin, (b) Concentration of (0.39 $\mu$ g/100 $\mu$ l) AuNPs and Cisplatin, (c) Concentration of AuNPs (0.048 and 0.39 $\mu$ g/100 $\mu$ l) and(d) Concentration of Cisplatin (0.048 and 0.39 $\mu$ g/100 $\mu$ l).

Data represented as % to untreated cells.

**Estimation of TNF- $\alpha$  levels secreted by normal human epithelial cell lines (WISH) in response to gold nanoparticles (AuNP<sub>s</sub>) treatment**

In response to the treatment by 0.048 $\mu$ g/100 $\mu$ l AuNP<sub>s</sub>, the WISH cell lines produced significant level of TNF- $\alpha$  ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h as compared to untreated cells. Similarly, WISH cell lines stimulated with 0.048 $\mu$ g/100 $\mu$ l Cisplatin produced significant level of TNF- $\alpha$  ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h as compared to untreated cells.

Comparing TNF- $\alpha$  levels released from WISH cell lines stimulated with 0.048 $\mu$ g/100 $\mu$ l AuNP<sub>s</sub> to the corresponding concentration of 0.048 $\mu$ g/100 $\mu$ l Cisplatin showed significant changes ( $P < 0.05$ ) after 24h, 48h, 72h, 96h and 120h (Fig. 8). The differences among all time intervals was significant as analyzed by ANOVA.



**Fig. 8:** levels of TNF- $\alpha$  secreted by WISH cell lines in response to AuNPs and Cisplatin treatment.

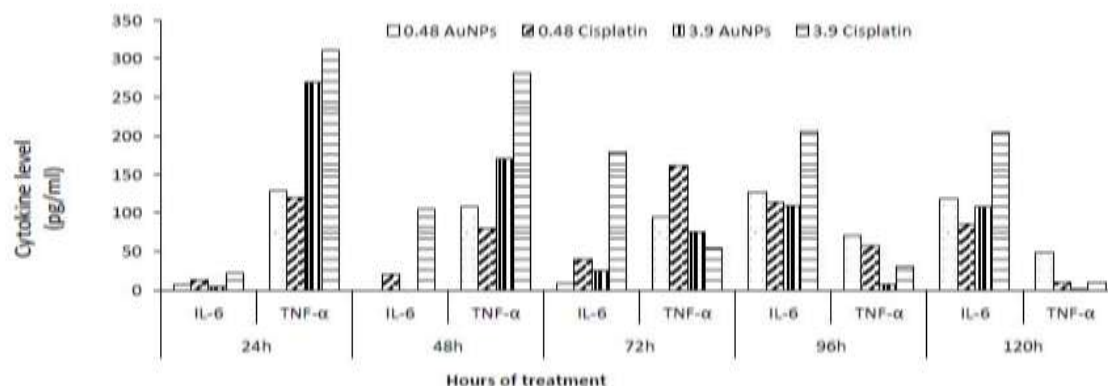
Data represented as % to untreated cells.

**Comparing the levels of TNF- $\alpha$  and IL-6 secreted by human colon cancer cell lines (Caco-2) and normal human epithelial cell lines (WISH) in response to gold nanoparticles (AuNPs) treatment**

Through the comparison between TNF- $\alpha$  and IL-6 secreted by Caco-2 cell lines in response to AuNP<sub>s</sub> treatment, it was noticed that TNF- $\alpha$  reached its peak after 24h then started to

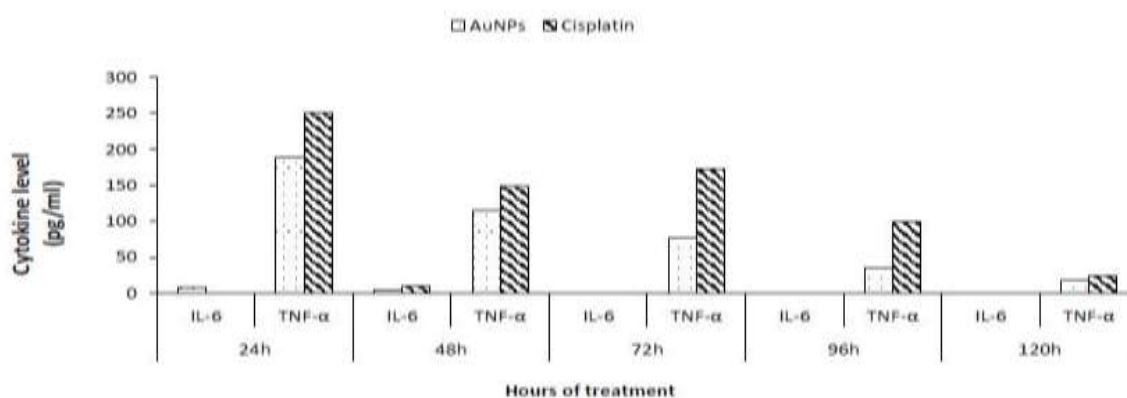
diminish by time until 120h recorded its lowest level. In the contrast, IL-6 had its lowest level after 24h then gradually increased to reach its peak after 120h (Fig. 9a).

In case of WISH cell lines, the levels of TNF- $\alpha$  also reached its peak after 24h then decreased, while IL-6 showed no detectable levels through treatment (Fig. 9b).



**Fig. 9(a): Comparison between TNF- $\alpha$  and IL-6 levels secreted by Caco-2 cell lines in response to AuNPs treatment.**

Data represented as % to untreated cells.



**Fig. 9(b): Comparison between TNF- $\alpha$  and IL-6 levels secreted by WISH cell lines in response to AuNPs treatment.**

Data represented as % to untreated cell.

## DISCUSSION

Cytotoxic effects of AuNP<sub>s</sub> at the cellular level appeared as inhibition of Caco-2 cell growth and colony forming efficiency was decreased, in response to treatment with smaller sizes of AuNP<sub>s</sub> (5 nm). This cytotoxic effect of AuNP<sub>s</sub> was observed only at relatively high levels of



exposure (200  $\mu\text{M}$  and 300  $\mu\text{M}$ ), with the most evident inhibitory effect detected at concentration of 300 $\mu\text{M}$  at 72 h<sup>[22]</sup> which complies with this study results.

According to cytotoxicity assay, at the moderate concentrations (0.39 and 0.78 $\mu\text{g}/100\mu\text{l}$ ) AuNP<sub>s</sub> showed less toxic effects on Caco-2 cell line, while, it became more toxic at lower and higher concentrations. And cisplatin (as a positive control) showed less toxic effects on Caco-2 cell line at lower concentrations while it became more toxic at higher concentrations. Meanwhile, in case of normal epithelial cell line (WISH) the AuNP<sub>s</sub> was non-toxic at all concentrations used in the current study while cisplatin was toxic at high concentrations only. There was no significant dead cells were observed in cells treated with AuNP<sub>s</sub>. This may indicate that AuNP<sub>s</sub> had no effect or very slight cell cytotoxic effect compared to cisplatin on normal cells.

Selim and Hendi<sup>[23]</sup> reported that gold nanoparticles produce significant cytotoxicity to breast cancer cells (MCF-7) in a dose-dependent manner with the concentration range of 25- 200  $\mu\text{g}/\text{mL}$ . Furthermore, quantitative real-time PCR analysis displayed that mRNA levels involved in the apoptosis was altered by gold nanoparticles. These data reported that gold nanoparticles may induce apoptosis in MCF-7 cells via p53, bax/bcl-2 and casapase pathways. This *in vitro* study showed the induction of apoptosis by gold nanoparticles recommended further investigation to determine if *in vivo* exposure consequences may exist for gold nanoparticles application.

In agreement with the result, Lan, *et al.*<sup>[24]</sup> found that the high dose of AuNP<sub>s</sub> was cytotoxic to NPC cells as it reduces cell viability in a concentration dependent manner. More than half of nasopharyngeal cancer cells have been killed after treatment with 100mM AuNP<sub>s</sub> for three days.

Arnida, *et al.*<sup>[25]</sup> observed that gold nanoparticles were taken up by human prostate cancer cells but do not cause severe toxicity. The uptake of gold nanoparticles was size dependent.

Particles with core diameters of 30 and 50nm were taken up by the cells to a higher extent compared with larger particles.

In the current study results of the pro- and anti-apoptotic genes showed that there was increase in the expression of bax mRNA in Caco-2 and WISH cell lines treated with AuNP<sub>s</sub>

or cisplatin, while p53 mRNA was only expressed in Caco-2 and WISH cell line treated with 0.048 $\mu$ g/100 $\mu$ l AuNP<sub>s</sub>.

Complement to recent results, Selim and Hendi,<sup>[23]</sup> analyzed the mRNA expression levels of five apoptosis associated genes; p53, bax, bcl-2, caspase-3 and caspase-9 in response to gold nanoparticles exposure in MCF-7 cells. Quantitative real-time PCR results showed that gold nanoparticles up-regulated mRNA level of cell cycle checkpoint protein p53 and pro-apoptotic bax while expression of anti-apoptotic bcl-2 was down-regulated in cells exposed to gold nanoparticles.

p53 is a transcriptional modulator of bax gene which promotes apoptosis<sup>[26]</sup> and a p53 negative response element has been identified in the Bcl-2 gene.<sup>[27]</sup> Based on these findings, Iimura, *et al.*<sup>[28]</sup> postulated that bax induced apoptosis could be operative in inflamed colonic mucosa in ulcerative colitis (UC).

Molecular biomarkers related to AuNP<sub>s</sub> exposures were identified by changes in RNA expression levels. Broad range of responses which potentially mediate inhibition of cellular growth and other functions of Caco-2 cells in response to nano gold, were identified. These cellular processes affected by exposure to 5 nm AuNP<sub>s</sub> include, for the early time point examined (24 h), down regulation of genes involved in regulation of transcription (transcriptional factors), biogenesis of RNA and GTP binding among others. At the later time point studied (72 h), more dramatic changes in differentially expressed gene expression patterns evoked by 5 nm AuNP<sub>s</sub> were detected.<sup>[22]</sup>

Tumor necrosis factor alpha (TNF- $\alpha$ ) exhibits both tumor-promoting and tumor-inhibitory properties, depending on the experimental context within which the conclusions are made.

The results showed that TNF- $\alpha$  reached its peak after 24h and started to decrease gradually reaching its lowest level after 120h in response to Caco-2 cell line treated with AuNP<sub>s</sub>, so as for the WISH cell line treated with AuNP<sub>s</sub> levels of TNF- $\alpha$  recorded its peak after 24h and lowest level after 120h.

There is evidence that chronic synthesis of low amounts of TNF- $\alpha$  within a tumor microenvironment promotes tumor growth and favors angiogenesis, whereas higher doses can induce necrosis of tumor cells, stimulate antitumor immunity, and trigger vascular

collapse.<sup>[29]</sup> It is now accepted that chronic inflammation is a major risk factor for carcinogenesis, and emerging evidence shows that TNF- $\alpha$  has key roles in this process.<sup>[30]</sup>

IL-6 is a multifunctional cytokine that was originally characterized as a regulator of immune and inflammatory responses; however, elevated expression of IL-6 has been detected in multiple epithelial tumors<sup>[31]</sup> Given the reported involvement of IL-6 and its downstream targets in the regulation of cell proliferation, survival, and metabolism, it is not surprising that IL-6 signaling has also been implicated in tumorigenesis.<sup>[32]</sup> However, the nature of IL-6's involvement in cancer has been quite controversial, as dichotomous roles for IL-6 in both tumor-promoting and -suppressive activities have been reported. For example, IL-6 signaling has been linked to both pro- and anti-apoptotic activity in breast cancer cells.<sup>[33,34]</sup>

In this study, the levels of IL-6 secreted by Caco-2 cell line was increased gradually up to its highest level after 120h of treatment with AuNP<sub>s</sub>, while in WISH cell line treated with AuNP<sub>s</sub>, there was no detected levels of IL-6 after all times of treatment.

Multiple studies have documented high IL-6 levels in the serum of patients with certain carcinomas (i.e., breast, lung and lymphoma) and have correlated high IL-6 levels with a poor clinical prognosis. These data imply an oncogenic role for IL-6; however, lacking is an understanding of the mechanisms governing IL-6 production in tumors and the biological role of this cytokine in tumorigenesis.

The results of another *in vitro* study on murine macrophages has suggested that 60nm AuNP<sub>s</sub> are not cytotoxic or do not elicit pro-inflammatory IL-6 or TNF- $\alpha$  responses<sup>[35]</sup> that back up our results. On the other hand, Yen, *et al.*<sup>[36]</sup> found that both silver and gold nanoparticles enter the cells but that only the AuNP<sub>s</sub> (especially those with smaller diameter) upregulate the expression of pro-inflammatory genes (IL-1, IL-6, TNF- $\alpha$ ). The authors speculated that part of the negatively charged AuNP<sub>s</sub> may adsorb serum protein and enter cells via the more complicated endocytotic pathway, resulting in higher cytotoxicity and immunological response of AuNP<sub>s</sub> than silver nanoparticles.

In previous studies, a significant inflammatory response has been observed in the release of TNF- $\alpha$  and IL-1b after 24h of exposure to 15nm Ag nanoparticles, but there was no detectable level of IL-6 upon exposure to silver nanoparticles.<sup>[37]</sup> Park and Park<sup>[38]</sup> noticed that a single treatment of silica nanoparticles (50 mg/kg, ip) activates peritoneal macrophages

and increases blood levels of IL-1b and TNF- $\alpha$ ; mRNA expressions of inflammation-related genes were also elevated in the cultured peritoneal macrophages harvested from the treated mice. A single intra-tracheal instillation of platinum and silver nanoparticles causes progressive increase in pro-inflammatory cytokines (IL-1, IL-6, TNF- $\alpha$ ) by day 28 post-instillation.<sup>[39,40]</sup> Intra-gastric administration of titanium dioxide nanoparticles has been shown to increase significantly the mRNA expression of several inflammatory cytokines in mouse hepatocytes.<sup>[41]</sup> The above literature clearly indicates that AuNP<sub>s</sub> are comparatively safer and more biocompatible than other nanomaterials.

In conclusion, although it was confirmed that AuNP<sub>s</sub> do not cause acute cytotoxicity for normal epithelial cell lines (WISH), it had shown that in colon cancer cell lines (Caco-2) the AuNP<sub>s</sub> have been able to induce apoptosis. These results predict a new era of targeting therapies and avoid the negative side effects of other therapies in the market that affects both normal and tumor cells.

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