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Therapeutic effect of antimyeloma antibodies conjugated with gold nanoparticles on the growth of myeloma cell line

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Abstract
Nanobiotechnology is a field of biomedical application of nanosize system which is a rapidly developing area within nanotechnology among these nonmaterial, gold nanoparticles (AuNPs) which receive a significant attention due to their unique physical, chemical and biological properties. AuNPs and bio-conjugated AuNPs have been widely used in cancer treatment besides other application on cancer detection and diagnosis. In this study the potential therapeutic effects of polyclonal Antimyeloma antibody (AbMM) conjugated to AuNPs in comparison with naked (AbMM) or AuNPs alone in modulation of proliferation capacity in vitro and different stages of MM cell cycle have been evaluated besides evaluation of their effects on tumor growth delay. Effect of AuNPs , (AbMM) and (Nanogold -Antimyeloma Antibodies conjugate) (gold-AbMM) on growth of myeloma cells showed decreasing in multiple myeloma SP2OR (MM) viability with increasing dose of these treatments compared to that of control also a significant enhancement in the apoptosis after conjugation of Nanogold to the Antimyeloma was observed. The induction of apoptosis with gold-AbMM was significantly higher than the MM cells exposed to only AbMM or AuNPs. The study concluded that the efficacy of (gold-AbMM) on induced myeloma growth inhibition is better than that of individual AuNPs and AbMM.

Keywords: Myeloma Cell Line, Immunization, Antibody Conjugates, Gold Nanoparticles.

INTRODUCTION

Cancer is an abnormal growth of cells caused by multiple changes in gene expression leading to deregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity (Raymond, 2007).

Multiple myeloma is a progressive hematological disease. It is a cancer of the plasma cell, an important part of the immune system that produces antibodies to help fight infection and disease. .The term MM describes features of this disease it's found in multiple sites in bone Marrow (Myth) with accumulation of tumor (oma) cells. Under normal Condition plasma cells constitute about 1% of the cells in bone Marrow while in MM then cells is overproduced and may comprise of 10%. To 80% of Cells in bone marrow clinically. It is characterized by high level of parameters in blood and /or urine lytic bone lessons, anemia renal dysfunction (Mukherjee and Bhattacharya, 2008) elevated level of Many cytokines include (VEGF). Current treatments include Combination of chemotherapy and Stem cell transplantation treatment of chemotherapy includes using alkaylating agents and Thalidomide. However none of these therapeutic Modules is curative and in most patients the disease will need further therapy.

Due to the highly heterogeneous nature of the disease, the main challenge to cancer therapists today is to deliver drugs that can be specifically targeted to the different ''hallmarks'' of cancer growth.

Attempts to treat cancer with polyclonal antibodies, in the form of anti-tumor sera, date back to the 1880s (Currie *et al.,* 1972). Treatment of mice with allogeneic anti-tumor serum was shown to prevent development of a transplantable tumor. Furthermore, occasional successful treatment of human malignancies, such as melanoma and renal cell carcinoma, with anti-tumor sera derived from experimental animals or from humans, has been reported. Finally, intravenous administration of human IgG, pooled from normal donors, was shown to inhibit growth and limit the spread of tumor cells (Jonsson *et al.,* 2000).

Colloid gold nanoparticles are currently being developed as a possible drug delivery technology to fight cancerous solid tumors. The gold nanoparticles could be safely administrated and used to enhance the tumor dose during radiation therapy. Also, the use of gold nanoparticles seems more promising than earlier methods because of the high atomic number of gold and because nanoparticles can more easily penetrate the tumor vasculature. (Azzam and Morsy, 2008).

Biocompatible gold nanoparticles labeled with specific targeting molecules (antibodies-proteins) are playing incredible role in cancer treatment and diagnosis (Salata, 2004). In this paper we investigate the potential therapeutic effects of polyclonal Antimyeloma antibody (AbMM) conjugated to AuNPs in comparison with naked (AbMM) or AuNPs alone in modulation of proliferation capacity *in vitro* and different stages of MM cell cycle beside

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evaluation their effects on tumor growth delay in *vivo*.

MATERIALS AND METHODS

1. Preparation of Colloidal Nanogold (Gold Nanoparticles (AuNPs) of 20 nm Diameter):This type of AuNPs was prepared according to (Azzam e*t al.,* 2009).
2. Production of Antimyeloma Antibodies: Production of Antimyeloma Antibodies was carried out by using seven healthy female mature White New-Zealand rabbits, 2-3 Kg. They were immunized with myeloma cells SP2OR to raise Antimyeloma antibodies according to (Hoppe *et al.,* 1971).
3. Preparation of Nanogold-Antimyeloma Antibodies conjugate: Nanogold - Antimyeloma Antibodies conjugate prepared according to method described by (Geoghegan and Ackerman, 1977).

Antitumor Test:
In-vitro

The antitumor activity for the compounds under investigation was carried out on myeloma cell line SP2OR compared to control. The method used is the trypan blue exclusion test of Mclimans *et al.* (1957). As following: Myeloma cells were cultured in the culture medium. a three 24 well plates was prepared as follows 1 ml of myeloma cells was added into the first row of the plate and mixed well the contents of the wells then serial dilution was performed between wells of the first row and the wells of other rows after that 1ml of media was added into all wells of the plate and the plate mixed well.

The colloid Nanogold, Antimyeloma antibodies and their conjugate serum were diluted in culture media to prepare following doses (5, 10, 15, 20 and 25mg/ml) and (5, 10, 15, 20 and 25µg/ml) for colloidal gold nanoparticles, (Au-AbMM) and AbMM respectively each dose added to each plate and incubated at 37ºc for 24 hours and the total count and viability were calculated according to (Mclimans *et al.,* 1957). The effective dose then added to another new plate and incubated at 37ºc for 6 days and the total count and viability were calculated daily colloidal gold nanoparticles, AbMM and there conjugate.

In-vivo
First: Induction of tumor in mice (ascites)

A line of myeloma cell (SP2OR) was used in the induction of ascites described by (Harlow and David, 1988). Treatment was started study after development of measurable tumor.

Second: Effect of treatment on tumor bearing mice:

This experiment was carried out using 5 groups of mice each comprise six mice. The first group was the positive control group, second control (without any treatment), the mice of the third group were injected i.p with 2.5 mg/kg.b.wt/day of (colloidal solution of gold nanoparticles in size around 20 nm) (BarathManiKanth *et al.,* 2010) and the mice of the fourth group were injected i.p with 2.5 µg/kg.b.wt/day of (serum containing Antimyeloma antibodies) and the mice of the fourth group were injected i.p with 2.5 mg/kg.b.wt/day of (Nanogold - Antimyeloma Antibodies conjugate) kept in cages for recommended times (1, 2, 3, 4, 5 and 6 day). Ascites fluid was withdrawn under aseptic conditions (ultraviolet laminar flow system) from the peritoneal cavity of ascites bearing mice at the last day and then undergoes cell cycle analysis and apoptosis detection.

RESULTS
1.Transmission electron microscope (TEM) measurements

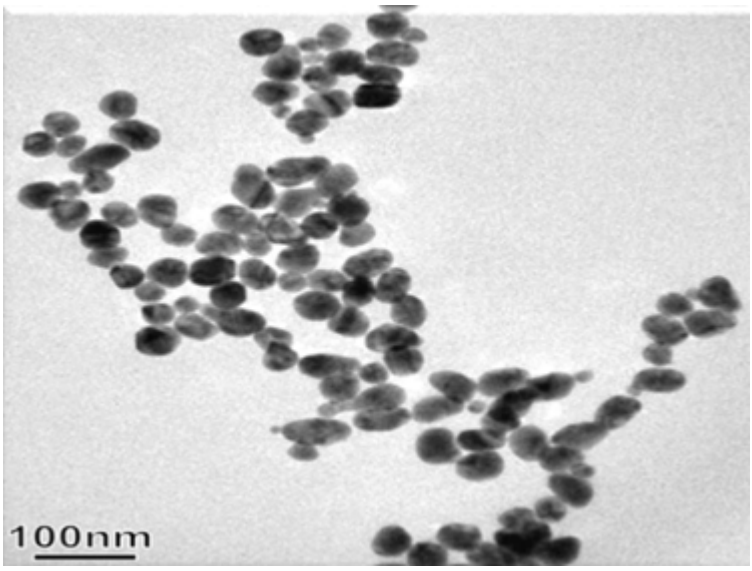

Fig 1. Gold nanoparticles (AuNPs) deposited onto a copper grid for TEM imaging.
From a statistical analysis counting 178 AuNPs a mean particle size of 26±7 nm was determined.

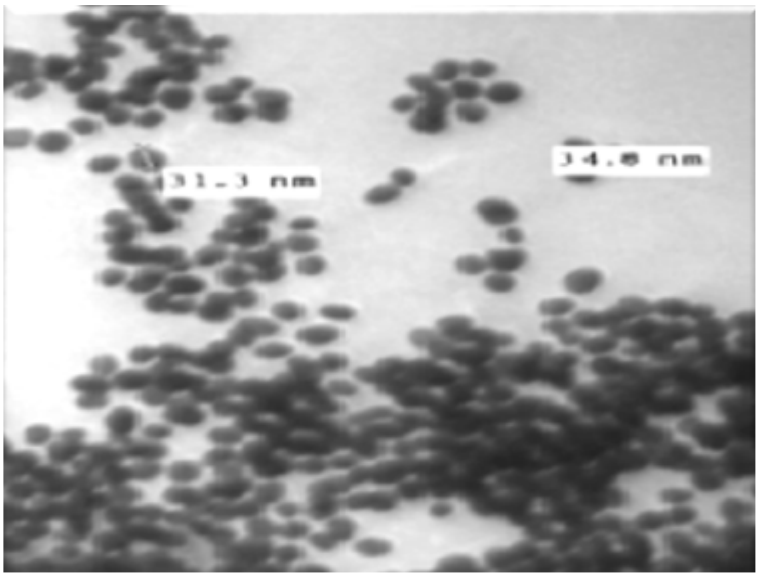

Fig 2. Gold nanoparticles conjugate AbMM deposited onto a copper grid for TEM imaging.
From a statistical analysis counting 178 AuNPs a mean particle size of 32±3 nm was determined.

Effect of colloid nanogold on growth of myeloma cells

Table 1. Effect of variable doses of colloid Nanogold on growth of myeloma cell line SP2OR compared to control after 24 hours (mean/ n=3).

Treatment	Nanogold variable doses (mg/ml)					
	Control	5	10	15	20	25
Total count	124	20	17	15	11	10
Dead count	10	10	12	10	8	8
Viable count	114	10	5	5	3	2
Total count per ml×10^4	26	10	8	7	5	5
Viability (%)	92	50	29	33	27	20

It was observed that total count per ml and viability% decreased with increasing dose of Nanogold colloid compared to that of control. It was observed that at 25 mg/ml the total count per ml reached 5 and viability% became 20% after 24 hours.

Effect of Antimyeloma antibodies on growth of myeloma cells.

Table 2. Effect of variable doses of Antimyeloma antibodies serum on growth of myeloma cell line SP2OR compared to control after 24 hours.

Treatment	AbMM doses (mg/ml)					
	Control	5	10	15	20	25
Total count	124	25	25	21	17	10
Dead count	10	17	19	15	12	7
Viable count	114	8	6	6	5	3
Total count per ml×10^4	26	12	12	10	8	5
Viability (%)	92	32	24	28	29	30

It was observed that total count per ml and viability% decreased with increasing dose of Antimyeloma antibodies serum compared to that of control. It was observed that at 25 mg/ml the total count per ml reached 5and viability% became 30% after 24 hours.

Effect of variable doses of (gold-AbMM) on growth of myeloma cells.

Table 3. Effect of variable doses of (gold-AbMM)) on growth of myeloma cell line SP2OR compared to control after 24 hours.

Treatment	(gold-AbMM) doses (mg/ml)					
	Control	5	10	15	20	25
Total count	124	20	12	10	9	7
Dead count	10	17	10	8	9	7
Viable count	114	3	2	2	0	0
Total count per ml ml×10^4	26	10	6	5	4	3
Viability (%)	92	15	16	20	0	0

It was observed that total count per ml and viability% decreased with increasing dose of (gold-AbMM) compared to that of control. It was observed that at 25µg/ml the total count per ml reached 3 and viability% reached Zero after 24 hours.

Flow cytometer analysis

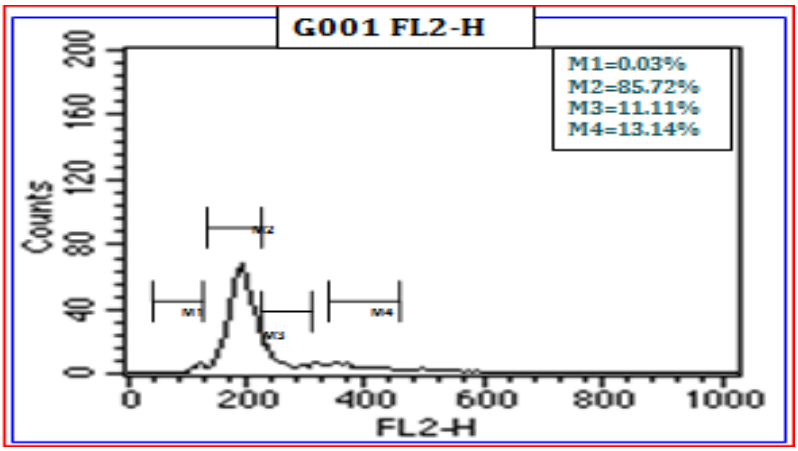

Fig 3. Flow cytometer analysis of cell cycle profile of myeloma cell line (SP2OR) without treatment.

M1=apoptosis, M2=G0/G1 phase [G0 (quiescence state), G1 (GAP1 phase)], M3= S phase [Synthetic phase], M4=G2/M phase [G2 (GAP1 phase), M (Mitosis)], It observed that the number of cells in M1 (apoptosis) was 0.03%, M2 (G0/G1 phase) =85.72%, M3 (S phase) =11.11%, M4 (G2/M phase) =13.14%

It observed that nanogold has remarkable effect on myeloma growth compared to the Antimyeloma Abs and nanogold has more effect after conjugation with Antimyeloma Ab on the growth and viability of myeloma cells.

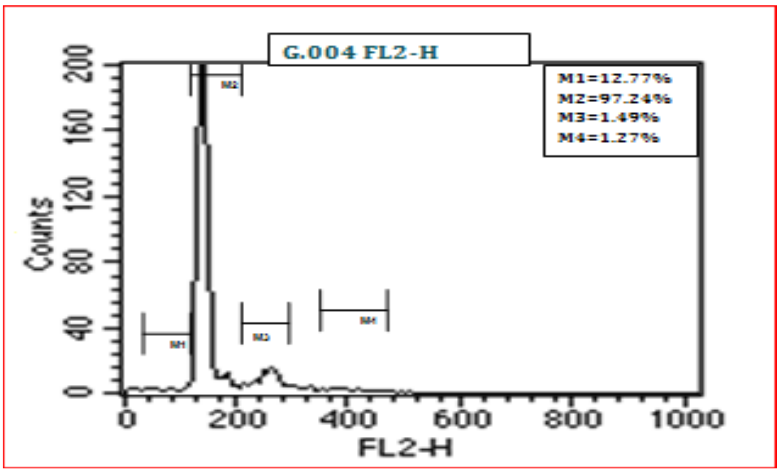

Fig 4. Flow cytometer analysis of cell cycle profile of myeloma cell- line (SP2OR) after (Nanogold treatment). It observed that the number of cells in M1 (apoptosis) was 12.77%, M2 (G0/G1 phase) =97.24%, M3 (S phase) =1.49%, M4 (G2/M phase) =1.27%.

Table 4. Effective dose of AuNPs, (AbMM) and (gold-AbMM) and their effect on growth myeloma cell line SP2OR compared to control over six days (mean±SD)

Days	Parameters measured	control	MM cell line treated with Nanogold (25mg/ml)	MM cell line treated with AbMM (25µg/ml)	MM cell line treated with(gold-AbMM) (25mg/ml
1	Total count	106 ±2.6	52±2.1	57±1.9	40±.98
	Dead count	4±0.9	30±0.7	35±3	29±.65
	Viable count	102±3.1	22±1.3	22±0.43	11±1.1
	Total count per ml×10⁴	53±4.2	26±0.8	28.5±2.1	20±1.4
	Viability (%)	96±1.7	42.31±2.9	38.60±3.2	27.25±1.6
2	Total count	127±4.6	34±1.7	42±0.3	21±2.6
	Dead count	91±0.7	21±0.7	25±0.9	18±0.96
	Viable count	118±3.9	13±0.8	17±1.1	5±0.4
	Total count per ml×10⁴	58±1.5	17±0.5	21±0.8	10±1.3
	Viability (%)	92±1.3	38.24±1.6	40.48±1.8	23.33±2.1
3	Total count	128±6.8	10±0.4	27±0.78	11±0.9
	Dead count	16±0.9	8±0.3	20±0.3	9±0.67
	Viable count	112±6.3	2±0.05	7±0.1	2±0.01
	Total count per ml×10⁴	64±3.9	5±0.1	13±2.4	5.±.43
	Viability (%)	87.5±1.1	20.00±1.1	25.93±3.1	18.18±1
4	Total count	168±11.8	10±0.9	12±0.98	7±0.21
	Dead count	40±0.6	9±0.9	10±0.9	7±0.21
	Viable count	128±12.8	1	2±0.01	0
	Total count per ml×10⁴	84±4.5	2±1.9	6±0.4	3±1.5
	Viability (%)	76±1.8	10±0.9	16±1.8	0
5	Total count	177±14.5	1±0.5	6±0.8	3±0.1
	Dead count	41±1.4	1±0.5	5±0.1	3±0.1
	Viable count	129±13	0	1±0.05	0
	Total count per ml×10⁴	88±7.9	1±0.2	3±0.22	1±0.2
	Viability (%)	73±1.8	0	16±0.87	0
6	Total count	257±7.9	3±0.1	6±0.1	3 ± 0.2
	Dead count	71±3.4	3±0.1	6±0.1	3 ± 0.2
	Viable count	186±8.4	0	0	0
	Total count per ml×10⁴	128±3.8	1 ± 0.08	3±0.05	1±0.3
	Viability (%)	72±2	0	0	0

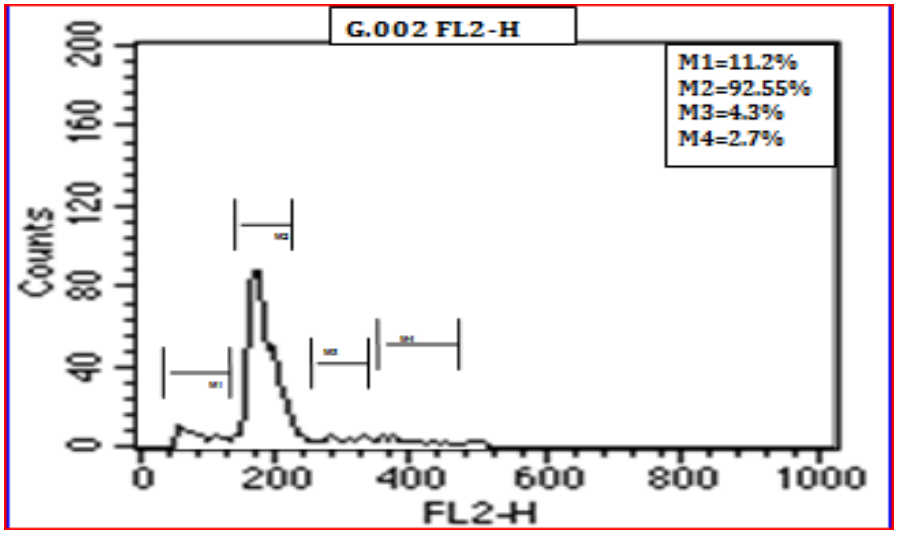

Fig 5. Flowcytometer analysis of cell cycle profile of myeloma cell line (SP2OR) after (Antimyeloma antibodies serum) treatment. It observed that the number of cells in M1 (apoptosis) was 11.2%, M2 (G0/G1 phase) =92.55%, M3 (S phase) =4.3%, M4 (G2/M phase) =2.7%.

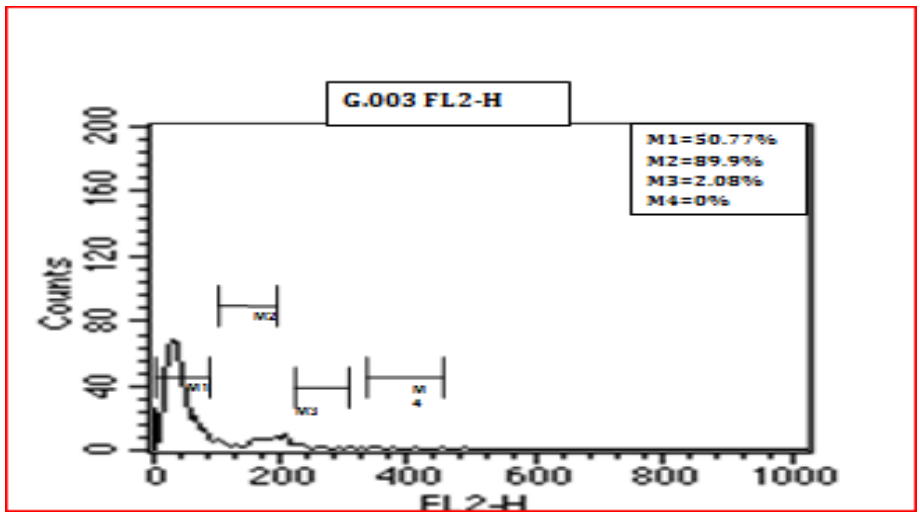

Fig 6. Flow cytometer analysis of cell cycle profile of myeloma cell line (SP2OR) after (Conjugated nanogold - Ab treatment).It observed that the number of cells in M1 (apoptosis) was 50.77%, M2 (G0/G1 phase) =89.9%, M3 (S phase) =2.08%, M4 (G2/M phase) =0%.

DISCUSSION

Synthesis of gold nanoparticles and their conjugate was occurred according to standard wet chemical methods as mention before and characterization occurred by TEM measurements. The TEM micrographs showed spherical gold nanoparticles of approximately 26±7 nm and spherical gold nanoparticles conjugated AbMM of approximately 32±3 nm size (figures 1, 2).

1. Effect of antimyeloma antibodies on the growth of myeloma

The results obtained indicated that T.C/ml and viability (%) decreased by increasing treatment concentration compared to that of control group and this illustrated that Antimyeloma polyclonal antibodies induced myeloma growth inhibition through its cytotoxicity to cells via two immune mechanisms include antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (Ben-Kasusa *et al.,* 2007).

2. Effect of colloid nanogold on the growth of myeloma

The results obtained indicated that T.C/ml and viability (%) decreased by increasing treatment concentration compared to that of control group and this illustrated that Colloidal gold nanoparticles induced myeloma growth inhibition through its cytotoxicity to cells via inhibit the function of heparin binding growth factors such as VEGF165, BFGF and posses anti-angiogenic properties (Bhattacharya *et al* ., 2007).

3. Effect of nanogold-antimyeloma antibodies conjugate) on the growth of myeloma

The results obtained indicated that T.C/ml and viability (%) decreased by increasing treatment concentration compared to that of control group and this illustrated that (Nanogold - Antimyeloma Antibodies conjugate) induced myeloma growth inhibition through its cytotoxicity to cells via double efficiently work of colloid Nanogold and anti-MM Abs. (Bhattacharya *et al*., 2004).

4. Influence of effective dose of colloid nanogold, antimyeloma antibody and their conjugate on growth myeloma cell line SP2OR compared to control over six days.

The results obtained indicated that Nanogold has remarkable effect on myeloma growth compared to the Antimyeloma Abs and Nanogold has more effect after conjugation with Antimyeloma Abs on the growth and viability of myeloma cells. The recorded data are in keeping with (Mukherjee *et al.,* 2007).

5. Cell cycle analysis:

In this study, a cell cycle analysis was performed for MM cells separated from ascites collected from treated and untreated mice the results indicated that AuNPs delayed the cell cycle progression as induced cell cycle arrest in G2/M phase and S phase , the recorded study proved that Nanogold remarkably induces apoptosis compared with AbMM treatment .The remarkable effect of Nanogold on MM cell cycle proliferation is due to anti_angioneogenic properties of Nanogold moreover multiple myeloma secrets Number of growth Factor like VEGF,bfGF which Nanogold can inhibit leading to the inhibition of cell proliferation . Gold-treated multiple myeloma cells show cell-cycle arrest in the G1-phase (the phase when chromosomes prepare for replication) by up-regulation of cell-cycle cyclin-dependent Kinase inhibitor proteins p21 and p27 and this lead to induction of apoptosis.

The recorded study observed a significant enhancement in the apoptosis after conjugation of Nanogold to the Antimyeloma. The induction of apoptosis with gold-AbMM was significantly higher than the MM cells exposed to only AbMM or GNP and this due to significant down regulation of anti-apoptotic proteins and exhibited PARP cleavage showed by the gold-AbMM treated cells (Mukherjee *et al*., 2007).

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