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In vitro and *in vivo* study of hazardous effects of Ag nanoparticles and Arginine-treated multi walled carbon nanotubes on blood cells: Application in hemodialysis membranes

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Abstract: One of the novel applications of the nanostructures is the modification and development of membranes for hemocompatibility of hemodialysis. The toxicity and hemocompatibility of Ag nanoparticles and arginine-treated multi-walled carbon nanotubes (MWNT-Arg) and possibility of their application in membrane technology are investigated here. MWNT-Arg is prepared by amidation reactions, followed by characterization by FTIR spectroscopy, Raman spectroscopy, and thermogravimetric analysis. The results showed a good hemocompatibility and the hemolytic rates in the presence of both MWNT-Arg and Ag nanoparticles. The hemolytic rate of Ag nanoparticles was lower than that of MWNT-Arg. *In vivo* study revealed that Ag nanoparticle and MWNT-Arg decreased Hematocrit and mean number of red blood cells (RBC) statistically at concentration of 100 $\mu g\ mL^{-1}$. The mean decrease of RBC and Hematocrit for Ag nanoparticles (18% for Hematocrit and 5.8 $\times$ 1,000,000/μL) was more than MWNT-Arg (20% for Hematocrit and 6 $\times$ 1000000/μL). In addition, MWNT-Arg and Ag nanoparticles had a direct influence on the White Blood Cell (WBC) drop. Regarding both nanostructures, although the number of WBC increased in initial concentration, it decreased significantly at the concentration of 100 $\mu g\ mL^{-1}$. It is worth mentioning that the toxicity of Ag nanoparticle on WBC was higher than that of MWNT-Arg. Because of potent antimicrobial activity and relative hemocompatibility, MWNT-Arg could be considered as a new candidate for biomedical applications in the future especially for hemodialysis membranes. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 103A: 2959–2965, 2015.

Key Words: Ag nanoperticle, MWNT-Arg, blood cells, hemocompatibility, toxicity

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INTRODUCTION

Nanotechnology is the manufacturing of various structures or organizations at the molecular size. This field covers both modern effort and concepts that are more advanced.[1,2] Nanostructures have shown many promising applications which attracted numerous researchers in different fields of science.[3–10] Unfortunately, the extensive use of nanostructures without considering their implications has posed a significant risk to environment, which directly has influenced plants, animals, and human.[11–15] Therefore, the investigation of nanostructures toxicity seems critical to not only understand their functional mechanisms but also decrease their harmful and negative impacts on human health.[16–18] It is obvious that nanostructures can penetrate human cells through inhalation, ingestion, dermal contact and injection.[19–22] They could be easily distributed into the blood circulation systems and from there to sensitive organs.[23,24] They also have shown side effects on blood cells.[25,26] Among a variety of available nanostructured materials, Ag nanoparticles and carbon nanotubes are the most widely utilized in industry.[27–30] Multi-walled carbon nanotubes (MWNT) are allotropes of carbon with a cylindrical nanostructure, which have unusual properties.[31] In particular, due to their extraordinary thermal conductivity and promising mechanical and electrical properties, carbon nanotubes find applications as additives to various structural materials.[32] Ag nanoparticle has well-known as an antimicrobial

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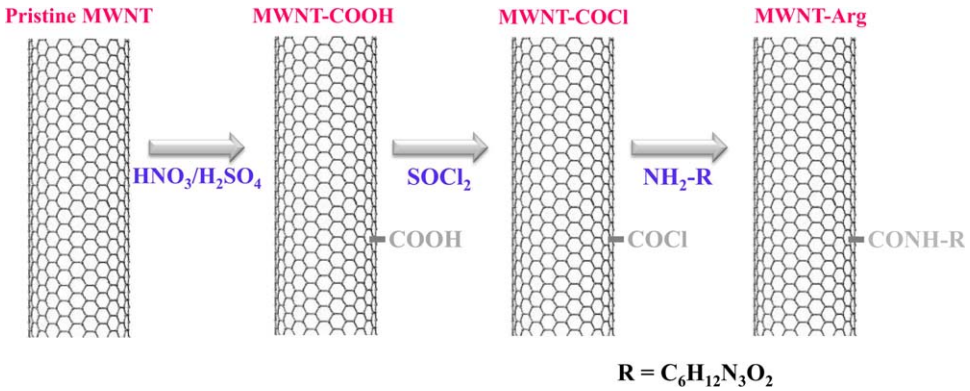

FIGURE 1. Schematic diagram of functionalization procedure of MWNT with arginine. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

agent.[33,34] Functionalization of MWNT with different agents can improve their weak interaction with other materials. Arginine is one of the basic aminoacids that plays an important role in cell division, healing of wounds, removing ammonia from the body, immune function, and releasing of hormones. This aminoacid is metabolized in various organs such as kidney, intestines and liver without any side effects.[35,36] In agreement with our previous works,[37–39] the published have shown that the antimicrobial activity of aminoacid treated MWNT were higher than that of pristine sample, which showed a promising property of treated sample for biological applications. Despite there are useful properties associated with MWNT, the negative effect of MWNT and Ag nanoparticles on biological systems has opened a great concern.[40] Although, *in vitro* toxicity assessments of different nanomaterials being studied by different researchers, the *in vivo* toxicity evaluation has not yet been investigated especially on blood cells.

A significant amount of research has been applied on Ag nanoparticles while the MWNT seem to be more cost-effective, have lower cytotoxicity and so are more benign to the environment.[41] One of the main obstacles in blood filtration is clogging due to thrombus deposition, which limits the maximum filter life to 15–40 h during both hemodialysis and Continuous Renal Replacement Therapy (CRRT).[42–44] The membrane pores have to be sufficiently small to prevent protein loss from blood plasma, while the membrane surface should be carefully engineered to provide high membrane hemocompatibility and minimal thrombosis.[45] Membrane surface engineering through the alteration of surface chemistry and structure by incorporating of nanomaterials has gained a significant attention. Ag nanoparticles and MWNT are possibly the most important nanomaterials that used broadly in different areas of sciences.[33,34,46] Thus, the low toxicity of the nanostructured materials on blood cells is more important in hemodialysis.[47,48] Generally, nanoparticles are incorporated into the membrane matrix through the two well-known approaches of coating[49–52]and blending.[53–55] In the coating approach, the membrane is first casted and then the nanoparticles are deposited on the membrane surface. On the other hand, in the blending approach, the nanoparticles are added into the membrane casting solution and then the membrane is casted. Because of advantageous properties of arginine-functionalized MWNT, these nanostructures may be considered as agents for performance enhancement of hemodialysis and hemofiltration membranes if their hemocompatibilities are proved.

So, the present study aims in assessing the effect of arginine-functionalized MWNT and Ag nanoparticles on blood cells. In this study, the hemolysis activity of these nanostructures is also evaluated in the presence of *in vitro* analyses. The results of this study will be suitable for design of new hemodialysis membranes, which is the subject of our future publications.

EXPERIMENTS

Preparation of MWNT-arg

Functionalization process of MWNT with arginine (MWNT-Arg) is schematically shown in Figure 1. According to the technique adopted from previous research publications.[7,56] the MWNT carboxylation was performed. To obtain carboxylated MWNT (MWNT-COOH), the pristine sample was first sonicated in a mixture of nitric acid/sulfuric acid with a volume ratio of 1/3 for 6 h at 100°C in a closed vessel. The solution was cooled down to room temperature and then filtered by a PTFE-membrane. The cake like-filtrate was thoroughly washed with the pure water until a desired pH value of 6-7 was obtained. To generate acyl chloride groups (MWNT-COCl) in the main structure of MWNT, MWNT-COOH was stirred in the mixture of thionyl chloride and anhydrous DMF at 70°C for 24 h, and then cooled at room temperature. The resulted solution was filtered by a PTFE membrane while thoroughly washed by THF. Finally, the collected black filtrate was dried at 40°C under vacuum and anhydrous conditions. Then, it produced MWNT-COCl (100 mg) which was sonicated in a solution of arginine (200 mg) and DMA (20 mL) at 100°C for 24 h. The synthesized solution was then cooled and filtered through a PTFE membrane. To eliminate unreacted arginine, the filtrate black cake collected on the membrane was thoroughly washed by DMA, pure water, and THF, and then dried in an oven for 48 h.

Hemolysis assay (*in vitro*)

For hemolysis investigation, anticoagulated blood was prepared from 20 mL healthy rat's blood. Then, Red Blood Cells

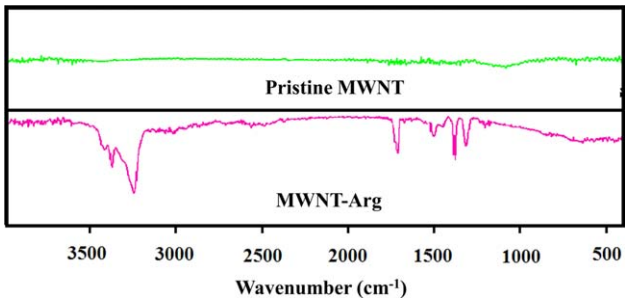

FIGURE 2. FTIR spectra of pristine and treated MWNT with arginine. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(RBC) were isolated by centrifugation and washed and diluted via sterile phosphate buffered saline (PBS). Hemolysis was assessed according to a method introduced by the present authors.[57,58] Briefly, the serial dilution of nanostructures and the prepared RBC were first mixed. After incubation at 37°C for 60 min and centrifugation for 15 min, the absorbance of supernatant (the concentration of free hemoglobin in the supernatant) was measured at 545 nm. Normal saline and distilled water were used as the negative and positive controls, respectively.

Assessment of toxicity

The serial dilution of Ag nanoparticles and the treated MWNT with arginine (20, 40, 80, and 100 mg) were prepared. For assessment of toxicity, 80 rats (40 for assessment of MWNT and 40 for Ag nanoparticles) were treated by different concentrations of nanostructures. For this, 40 rats were divided into five groups (four sample groups and one control group). About 1 mL of the mixture of serial dilution of Ag nanoparticle as well as MWNT were intraperitoneally injected to the four sample groups and control groups were received 1 mL of physiological saline. This protocol was repeated for 2 weeks. Then, the blood samples were prepared from all groups and finally hematocrit, white and RBC were calculated by a cell counter device (model Horiba, France). All of the results were collected and comprised. Also, the statistical analysis was carried out by SPSS software.

TABLE I. FTIR Interpretation of the MWNT-Arg

Type of Functionalized MWNT	Peak (cm^{-1})	Interpretation
MWNT-Arg		
	3300–3400	O—H and N—H stretching vibration (secondary amines)
	3239	N—H stretching vibration of primary amine
	2994	C—H stretching vibration
	1700	C═O stretching vibration (amide bond)
	1488	—NH bending vibration
	1366	C—N stretching vibration
	1298	C—O stretching vibration of carboxylic acids

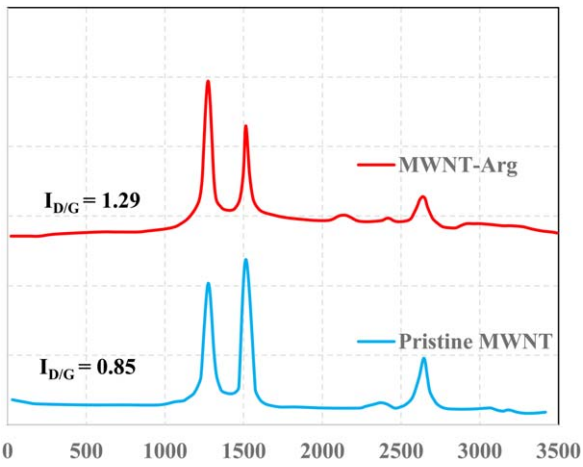

FIGURE 3. Raman spectra of pristine and treated MWNT with arginine. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS

Functionalization results

To evaluate functionalization of MWNT, TGA analysis, Raman and FTIR spectroscopy were applied.

Fourier transform infrared spectroscopy. FTIR spectra of pristine and functionalized MWNT with arginine (MWNT-Arg) are presented in Figure 2. As could be seen, MWNT-Arg provides various peaks at different wavenumbers, while FTIR spectrum of pristine MWNT showed no cue of functional groups. For MWNT-Arg, the list of peaks in FTIR spectrum and their interpretations were detailed in Table I. As compared with Kumar and Rai results,[59] pure arginine have bands at 3151, 2928, 2842, 1680, 1574, and 1464 cm^{-1}, which are respectively corresponded to the NH_2 stretching, asymmetric stretching of CH_3, symmetric stretch of CH_3, out-of-plane bending of NH_2, stretching vibration of C═O and asymmetric bending of CH_3. A majority of peaks with some shifts are obvious in MWNT-Arg. Also, the O—H stretching vibration at 3300–3400 cm^{-1} can be related to the carboxylation phase. The C═O stretching vibration (amide bond) has shown a peak at 1700 cm^{-1}, which resulted from amidation reaction. According to the results, it was obvious that the peak of carboxyl groups was sharp, which could have resulted by arginine molecules attached to the surface of MWNT and/or oxidation treatment with nitric and sulfuric acids.[7]

Raman spectroscopy. Raman spectroscopy is commonly employed to characterize different functional groups. This technique can also estimate the degree of covalent functionalization.

The Raman results of pristine sample and MWNT-Arg are presented in Figure 3. The spectra of pristine sample and functionalized MWNT illustrate D, G, and 2D bands at 1274, 1513, and 2653 cm^{-1} respectively, which are related to the main structure of MWNT. The I_D/I_G bands are considered as a ratio of sp^3 carbon to sp^2 carbon.[7,59] In the field of functionalization, enhancement of I_D/I_G ratio depicts the

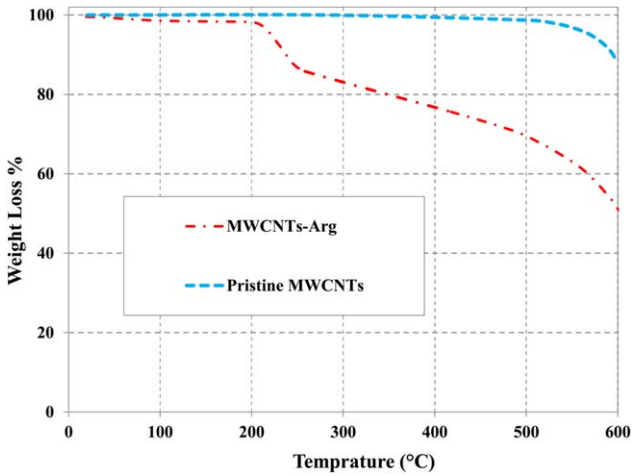

FIGURE 4. TGA curves of pristine and treated MWNT with arginine. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

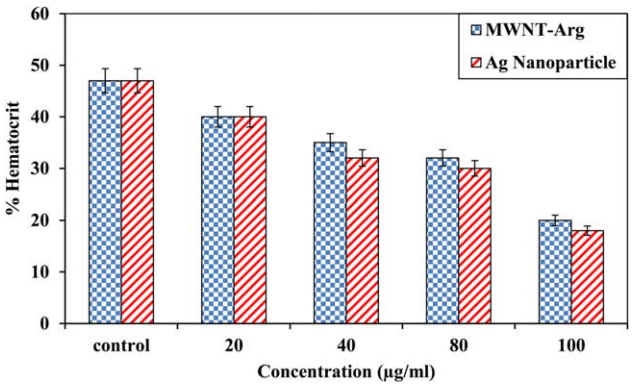

FIGURE 5. Effect of different concentrations of MWNT-Arg and Ag nanoparticles on hematocrit. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

more extent of covalent groups on the MWNT structure.[60] As can be seen, the MWNT-Arg shows higher enhancement of I_D/I_G ratio than that of pristine MWNT.

The G band is commonly related to the motion in opposite pattern of two adjacent carbon atoms in the graphitic sheet, which imply on the crystalline graphitic carbon in MWNT structure. Meanwhile, the D band is related to the disordered carbon, which resulted from adding functional groups to the main backbone.[37]

According to the results of FTIR and Raman spectroscopy, functionalization of MWNT with arginine was confirmed.

Thermogravimetric analysis. Figure 4 shows the curves of thermogravimetric analysis (TGA) for pristine MWNT and treated MWNT with arginine. It could be seen that the pristine MWNT curve illustrates no weight loss up to 500°C, which could be attributed to the decomposing temperature of graphitic structures. On the other hand, the treated sample elucidated three weight losses in the temperature range of 0–600°C. The first weight loss in the temperature range of 0–200°C could be related to the unreacted carboxyl and/or acyl chloride groups as well as small pieces of MWNT resulted from cutting in carboxylation step. Also, the second weight loss occurred in the temperature range of 200–250°C, which was associated with arginine as an unstable organic part on the structure of MWNT. The third weight loss in the temperature range of 300–600°C illustrated the decomposition temperature of graphitic structures of MWNT in air. The lower decomposition range (around 300°C) can be attributed to the defected, short, cut MWNT.

Hematological results

Equation (1) was used for the determination of hemolysis activity of nanostructurs:

$$\text{Hemolytic rate}(\%) = \frac{A-B}{C-B} \times 100 \qquad (1)$$

where A is the absorbance values of the nanostructures, B and C are the absorbance values of negative and positive control groups respectively.

As can be seen in Table II, the hemolytic rates of both MWNT-Arg and Ag nanoparticles were lower than the standard value (5%). Also, the hemolytic rate of Ag nanoparticles (value of 3.04%) was lower than that of MWNT-Arg (3.28%); but, this difference isn't significant. The results showed that Ag nanoparticle and MWNT-Arg can decreas the Hematocrit and average number of RBC. According to the results of both samples and control group, there was a drop in blood cells in the presence of both nanostructures samples, which was statistically considerable in the group treated by 100 µg mL^{-1}. The reported results are shown in Figures 5 and 6. These figures illustrated the effects of different concentrations of nanostructures on Hematocrit and the number of RBC. As shown in these figures, there is a linear relationship between the increase of dose and the reduction of Hematocrit and the number of RBC.

Also, the comparison between Ag nanoparticles and MWNT-Arg have demonstrated that the mean decrease of RBC and Hematocrit in groups treated by Ag nanoparticles

TABLE II. Hemolytic Rates of the MWNT-Arg and Ag Nanoparticle

Samples	Absorbance 1	Absorbance 2	Absorbance 3	The Mean of Absorbance	Hemolytic Rate (%)
Negative control	0.010	0.010	0.011	0.0103	-
Positive control	0.32	0.31	0.29	0.306	-
Ag nanoparticles	0.020	0.019	0.019	0.0193	3.04
MWNT-Arg	0.021	0.019	0.020	0.020	3.28

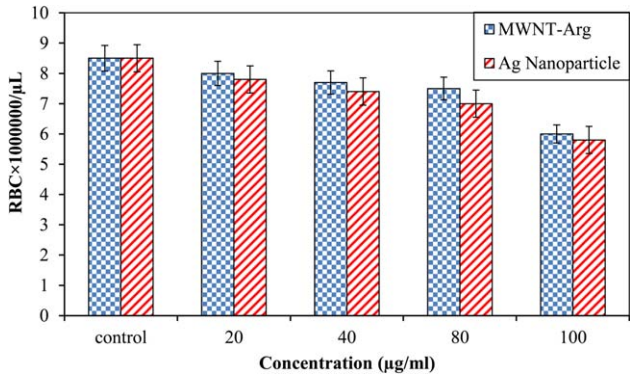

FIGURE 6. Effect of different concentrations of MWNT-Arg and Ag nanoparticle on RBC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

were more than those of the Arg-treated MWNT, which confirmed higher toxicity of Ag nanoparticle than MWNT-Arg.

For the average number of white blood cells (WBC), there was a non-linear relationship between increase of dose and decrease of WBC. The data showed that although the average number of WBC increased in the concentrations of 20, 40, and 80 µg mL^{-1} of Ag nanoparticle and MWNT-Arg, it decreased in the concentration of 100 µg mL^{-1} (Fig. 7). Ag nanoparticles had more adverse and/or negative effects on WBC as compared to MWNT-Arg.

DISCUSSION

In the current study, the effect of various concentrations of Ag nanoparticles and Arg-functionalized MWNT on rat RBC, WBC, and hematocrit were evaluated.

Functionalization process of MWNT with arginine (MWNT-Arg) is performed by the addition of carboxyl groups on the surface of MWNT and then formation of acyl chloride linkages, which provided a bridge for amidation reaction between amine groups of arginine and carboxylic acid sites. The functionalization process details were completely described in the previous studies.[37–39]

The results showed that the RBC have well tolerated the different concentrations of the two nanostructures in comparison with the positive group. However, comparison with Ag nanoparticles, MWNT-Arg had a lower hemolytic rate. A similar study showed that N-MWNT and MWNT were more hemocompatibility. They mentioned that the interaction of N-containing functional groups with blood tissues have shown a positive effect on hemocompatibility.[61] In this study, addition of basic aminoacid (arginine) may have the similar effect.

From the results, both of the nanostructures have toxicity on blood cells, but the overall toxicity of Ag nanoparticle was more than Ag-MWNT to some extent. The toxicity of different nanostructures was proven by different researcher groups.[40,62] The toxicity of Ag nanoparticles has been shown on different cells in many publications such as human lung cells, human macrophages, and human mesenchymal stem cells and so forth.[63–65] Previous study illustrated that the high concentration of Ag nanoparticles has toxicity on blood mononuclear cells.[66] MWNT has also shown side effect on the different parts of life. MWNT has different toxicity such as inhalation toxicity, genotoxicity and so forth.[67–69] Ag nanoparticle is one of the nanostructures used in different parts of life especially as the antimicrobial agent.[33,34] Toxicity of CNTs have been widely investigated by different groups and showed that their toxicity is related to composition, length, diameter and sizes.[70,71] Pristine CNT has minimum cytotoxicity at higher concentrations (both *in vivo* and *in vitro*). On the other hand, functionalized CNT by different compounds reduce toxicity in addition to enhancement of their biological activity.[72,73] Meng et al. showed that modification of MWNT surface can reduce their negative effects on human RBC.[74] Addition of carboxyl and amine groups on the surface of pristine MWNT can improve the hemotoxicity and hemocompatibility of these nanostructures.[75] In another study, it was shown that because of the contribution of N-containing functional groups to cell tissues, N-MWNT have the highest cell-adhesion strength, cell viability, cell proliferation in comparison to the pristine MWNT. So, this functionalization enhances good cytocompatibility of MWNT.[61] Moreover, the recent results have shown that the functionalization of MWNT by Arg enhances their antimicrobial activity significantly.[38] According to the results, functionalizations of MWNT by Arg improve their compatibility with blood cells and antimicrobial activity. In this study, results showed that funtionalized MWNT with arginine has higher hemocompatibility than Ag nanoparticles. On the other hand, previous results[38] showed that the addition of arginine on surface of MWNT enhance their antimicrobial activities on different Gram positive and Gram negative bacteria as well as fungal pathogens. So, arginine funtionalized MWNT may be the promising and effective biomedical material which could be selected as a good alternative for Ag nanoparticles for biological applications in the future, particularly as the substrates for the inhibition of infectious diseases. In addition, it is possible to modify and enhance the performance of the hemodialysis membranes by the addition of arginine funtionalized MWNT in the membranes.

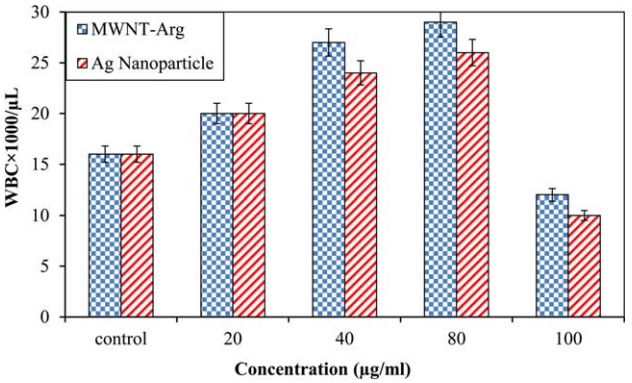

FIGURE 7. Effect of different concentrations of MWNT-Arg and Ag nanoparticle on WBC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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