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# Evaluating the blood toxicity of functionalized graphene-arginine with anticancer drug ginsenoside Rh2 in balb/c mouse model with breast cancer

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**Abstract**

**Background:** Gensenoside Rh2 is an anticancer drug with low toxicity and stability in the body. The aim of this study was to evaluate the blood toxicity of functionalized graphene-arginine with anticancer drug ginsenoside Rh2 in balb/c mouse model with breast cancer.

**Materials and Methods:** Graphene-Arginine (G-Arg) and Graphene-Arginine-ginsenoside Rh2 (G-Arg-Rh2) were synthesized using microwave method. For evaluation of blood toxicity, 32 mice with breast tumors were randomly divided into 4 groups: control (3mg/kg 6 mg / kg PBS sterile), group 1 (6 mg / kg ginsenoside), group 2 (3 mg / kg G-Arg), and group 3 (3 mg / kg G-Arg-Rh2). Treatment was done intravenously once every three days for 32 days. Finally, blood factors were also examined by sampling from the heart.

**Results:** Complete functionalization was proven by FTIR and Raman. Examination of blood factors showed that white blood cells had a very small increase. Anova test showed significant difference among four groups in term of WBC count ($p=0.016$). Pair sample T test showed that there was significant difference between control and group 1($p=0.036$) and control and group 2 ($p=0.036$). There was no significant difference between control and group 3 ($p=0.051$). Other blood factors had no significant difference among examined groups ($p>0.05$).

**Conclusion:** Based on results, after treatment with all designed nanostructures, only white blood cells had a very small increase and inflammatory reactions were statistically similar in all groups. This indicates the high efficiency of designed drug.

**Key words:** Gensenoside Rh2, balb/c mouse, Graphene-Arginine, Graphene-Arginine-ginsenoside Rh2, blood

## Introduction

The main challenge in cancer treatment is target the cancerous cells without any toxicity on normal cells and tissues (1). One of the ways to increase the effectiveness of treatment and reduce the side effects of chemotherapy is application of biocompatible carrier systems for delivery of chemotherapy drugs to cancer tissue (2). Another solution is development of new drugs with low toxicity instead of Chemotherapy drugs (3). Ginsenoside Rh2 (Rh2) is a glycosidic active substance belonged to 20 (S) - radiopannaxadiol saponin family (3). Rh2 has shown anti-cancer activity by inhibiting cell growth and inducing apoptosis in several cancer cells and low toxicity on normal cells (4, 5). Low oral bioavailability (due to its high hydrophobicity and rapid removal of plasma by glycosidase) has limited its use as a therapeutic compound (6). To overcome this dilemma, zare-zardini et al used graphene as drug delivery system for enhancement of stability and bioavailability of Rh2. In zare-zardini et al studies, the toxicity of functionalized graphene with Rh2 were evaluated on OVCAR3 ovarian cancer, MDA-MB breast cancer, human A375 melanoma, and human mesenchymal stem cell lines. These study showed that Gr-Arg-Rh2 has the highest cytotoxic effect on cancer cells compared to others designed

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nanostructures. The side effects of G-Arg was also weaker than non-functionalized graphene (7, 8). One of the most important aspects of drug toxicity, especially in the field of anticancer drugs, is the toxicity on blood cells (9). Therefore, the development and introduction of Gr-Arg-Rh2 as a therapeutic compound requires the study of its toxicity, especially blood toxicity. Therefore, this study was designed to evaluate the blood toxicity of G-Arg and G-Arg-Rh2 in a balb / c mouse model with breast cancer.

## Materials and Methods

Similar to our previous study (7), G-Arg and G-Arg-Rh2 was synthesized by microwave technique and characterized by Raman and FTIR methods. Thirty-two healthy female Balb/c mice (same weight, 4-6 weeks) were purchased from the Pasteur Institute and kept in optimal laboratory conditions. The 4T1 cell line (purchased from the Pasto Institute of Tehran cell bank) was used to induce cancer in mice. This cell line was cultured in DMEM or Dulbecco's Modified Eagle's medium with 10% FBS. After trypsinization and washing with PBS buffer, cell count was performed and 500,000 cells per syringe of insulin were injected subcutaneously in the back of each mouse. As soon as the tumors became palpable, drug injections were started. For this purpose, the mice were randomly divided into 4 groups (8 mice in each group): control (3mg/kg 6 mg / kg PBS sterile), group 1 (6 mg / kg ginsenoside), group 2 (3 mg / kg G-Arg), and group 3 (3 mg / kg G-Arg-Rh2). Injections were performed every three days through caudal vein (intravenous injection). The drug injection was continued for 21 days, i.e. each mouse received 7 injections. After 21 days, blood was collected and examined. Kolomogorov-Smirnov statistical test was used to evaluate the normality of blood factors. The test used for statistical analysis of each blood factor is kruskal wallise. All data was displayed as mean ± SEM. Significant levels are considered at the level of 0.05.

## Results

FTIR and Raman techniques were used to characterize the synthesized nanostructures. The FTIR results showed that pure graphene lacked a functional group and had only vibrations related to the C-H bond and the hydroxyl group attached to pure graphene. The vibration of the C-N bond and NH-type amine were related to the presence of arginine in the structure of G-Arg and G-Arg-Rh2 with peaks at 1200 and 1560 cm-1, respectively. The esterification of the carboxyl group of arginine with the hydroxyl groups of ginsenoside Rh2 in the structure of G-Arg-Rh2 was proved by the strong peak at 1700 cm-1 (Figure 1). Examination of the Raman spectrum also proved correct functionalization. Pure graphene has no sharp D band. The Ram spectra of G-Arg and G-Arg-Rh2 have D and G bands in 1342 and 1575 cm-1. This spectrum has a strong D band and a high ID/IG ratio, which confirms the structural deformation by functionalization. Structural deformation is manifested by the activation of arginine and ginsenoside Rh2, with the appearance of a strong peak in 1342 cm-1 (Figure 2). The results of the study of blood factors are reported in Table I. As shown in this table, there is a significant difference only in white blood cells. There was no significant difference in other blood factors. Anova test showed significant difference among four groups in term of WBC count (p=0.016). Pair sample T test showed that there was significant difference between control and group 1(p=0.036) and control and group 2 (p=0.036). There was no significant difference between control and group 3 (p=0.051). Other blood factors had no significant difference among examined



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groups (p>0.05). The mean of white blood cells in the control group was very high. This increase is due to the body's inflammatory response to cancer condition. In the G-Arg-Rh2-treated group, the lowest increase in white blood cells was observed. The use of G-Arg-Rh2 reduced the need of production of higher levels of the white blood cells and the inflammatory reactions. The reduction of inflammatory reactions along with the use of the drug indicates the positive effect of the drug. In general, the reduction of erythrocytes and platelets has been observed in control group due to cancer. According to the mean platelets, G-Arg and G-Arg-Rh2 have reduced this complication, respectively.

*Table I: Statistical analysis of blood factors. Significant levels are considered at the level of 0.05*

| Analytic variables | Group I[a] | Group II[b] | Group III[c] | Group IV[d] | P-value |
|---|---|---|---|---|---|
| **White blood cell ($10^3$/µL)** | 36 ±10.64 | 48.27 ±13.24 | 50.64 ±19.53 | 47 ±30 | Pt=0.01<br>$P^{a-d}$ =0.036<br>$P^{b-d}$ =0.036<br>$P^{c-d}$ =0.051 |
| **Neutrophil** | 78.5 ± 5.22 | 77.21 ±2.58 | 83.26 ±2.94 | 45.75 ±2.15 | Pt=0.072<br>$P^{a-d}$ =0.296<br>$P^{b-d}$ =0.602<br>$P^{c-d}$ =0.699 |
| **Lymphocyte** | 14.01 ±4.33 | 11.63 ±1.20 | 10.7 ±1.96 | 36.9±0.3 | Pt=0.132<br>$P^{a-d}$ =0.037<br>$P^{b-d}$ =0.036<br>$P^{c-d}$ =0.053 |
| **Monocyte** | 3.01±0.54 | 6.2±1.06 | 2.68±0.95 | 6.5±1.1 | Pt=0.039<br>$P^{a-d}$ =0.037<br>$P^{b-d}$ =0.148<br>$P^{c-d}$ =0.053 |
| **Red blood cell ($10^6$/ µL)** | 7.01 ± 0.41 | 6.61 ± 0.32 | 6.69±0.37 | 7.6 ±0.46 | Pt=0.28<br>$P^{a-d}$ =0.296<br>$P^{b-d}$ =0.192<br>$P^{c-d}$ =0.699 |
| **Hemoglobin (g/dL)** | 10.85 ± 0.29 | 10.77 ± 0.51 | 10.76 ± 0.81 | 11.55 ±0.76 | Pt=0.93<br>$P^{a-d}$ =0.690<br>$P^{b-d}$ =0.295<br>$P^{c-d}$ =0.329 |
| **Hematocrit (%)** | 35.47± 2.13 | 31.35 ± 1.53 | 32.08 ±1.81 | 36.2± 2.2 | Pt=0.8<br>$P^{a-d}$ =0.602<br>$P^{b-d}$ =0.192<br>$P^{c-d}$ =0.409 |
| **platelet count ($10^3$/µL)** | 705.5±103.36 | 837.75±112.29 | 695.6±110.41 | 528±117.54 | Pt=0.36<br>$P^{a-d}$ =0.433<br>$P^{b-d}$ =0.192<br>$P^{c-d}$ =0.439 |

Group I; Gr-Arg-Rh2, Group II; Gr-Arg, Group III; Rh2, Group IV; Control



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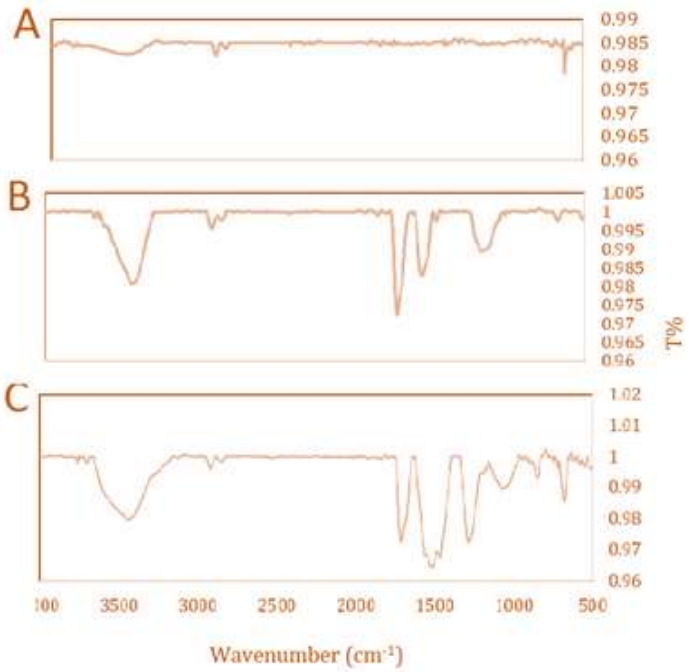


Figure 1. The FTIR analysis of pure graphene (A), G-Arg(B), and G-Arg-Rh2 (C)

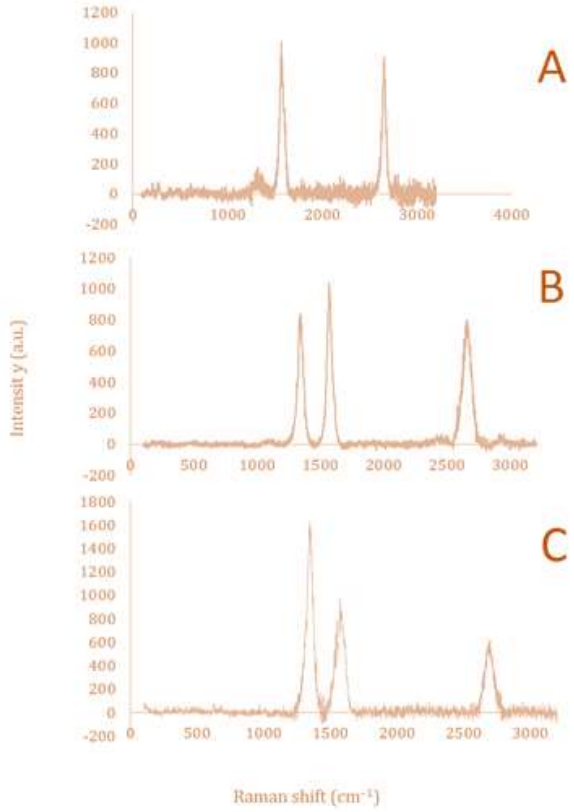


Figure 2. The Raman analysis of pure graphene (A), G-Arg (B), and G-Arg-Rh2 (C)

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**Discussion**

In our previous studies, we synthesized anticancer drug based on Rh2 and functionalized graphene with Arg. This designed drug showed potent anticancer activities against pediatric and adult cancer such as ALL and breast cancer (7, 8). The most important body fluids is blood. This fluid contain important cells: red blood cells (RBCs), white blood cells (WBCs), platelets, and etc (10). Thus, for completion of related test, the toxicity of G-Arg-Rh2, G-Arg, and Rh2 on blood factors was evaluated by treatment of mice with mentioned compounds. Our results showed that the use of G-Arg-Rh2 caused a much smaller increase in WBCs, indicating less involvement of inflammatory reactions and high efficacy along with drug use. Also, in the use of this drug, no negative effect was observed on the number of RBCs. Reduction of the amount of RBCs, hematocrit, and hemoglobin observed in all four groups is related to anemia of chronic diseases caused by cancer (11, 12). Since this reduction is in all 4 groups in the same range. It reduction is not a side effect of the drug. Platelet counts were much closer to normal in the G-Arg, G-Arg-Rh2, and Rh2, respectively. This results indicated that G-Arg and G-Arg-Rh2 reduced the risk of cancer-induced platelet decline. Based on other results obtained from previous studies, the positive effects of G-Arg-Rh2 were greater than G-Arg (7, 8). Shin et al, similar to our study, showed that the 13-week treatment of rat with a type of ginsenoside (UG0712) had no significant effect on hematological and biochemical serum parameters (13). The difference between Shin et al study and our study is significant effect of Rh2 on WBC in our study. As graphene nanostructures is commonly used through intravenous administration, biological fluids, i.e. serum and plasma expose to these nanostructures (14). So, change of hematological components must be considered. Graphene can interact with serum biomolecules by various methods such as p–p stacking, electrostatic, electrostatic, and hydrogen bonding (15). p–p stacking have important role in this interaction, especially in pure graphene. Functionalization of graphene with Arg and Rh2 led to reduction of p–p stacking. This can cause a decrease in blood toxicity of G-Arg and G-Arg-Rh2. Investigations showed that hemocompatiblity or graphne can be increased by functionalization with different agents (8, 14, 16, 17). Our study also showed that G-Arg-Rh2 had lower effect on blood parameters than G-Arg. Chowdhury et al showed that functionalization of graphene with dextran lead to its hemocompatibility without significant side effects on platelet activation and blood cell hemolysis (18). This results was similar to our study. In our study, functionalization with Arg and Rh2 lead to similar data. Singh et al showed that modification of graphene with amine can enhance the hemocompatibilty. Induction of positive charge by NH2 did not induce hemolysis and stimulatory effect on platelets (19). In our study, we also induced positive charge on surface of graphene by adding Arg. Cancer can induce lymphocytes activation due to simulation of immune system (20). In our study, the highest level of lymphocytes was observed in control group. In other groups, lymphocytes levels were significantly lower than control group. This data similar to other related articles.

## Conclusion

Based on results, after treatment with all designed nanostructures, only white blood cells had a very small increase and inflammatory reactions were statistically similar in all groups. This indicates the high efficiency of designed drug.

## Conflict of interest

There is no conflict of interest.



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