Original research

The toxicity of selenium oxide nanoparticles conjugated with folic acid and antibody on the mononuclear cells from patients with chronic lymphoid leukemia and from normal persons

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http://jabs.eu5.org/

Received: June. 17, 2014
Accepted: June. 30, 2014

Vol. 1, No. 1, 2014, pages 12-29.

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Abstract

The aim of this study was to synthesize antibody-conjugated radioactive nanoparticles (ACRNPs) and folic acid-conjugated radioactive nanoparticles (FACRNPs). Also, their toxicity on the mononuclear cells from patients with chronic lymphoid leukemia (CLL) and from normal persons was evaluated. Also, the potential use of ACRNPs and FACRNPs was investigated to detect cancer cells. After synthesis of nanoparticles, blood samples from CLL patients and normal persons were obtained, and their mononuclear cells were isolated by Ficoll method. To evaluate cytotoxicity, serial concentrations of ACRNPs and FACRNPs were separately added to cancerous and normal mononuclear cells, incubated for 12 hours at 37 ºC, and then different assays including MTT, MTS, cell metabolic, and ATP assay were carried out. On the other hand, the mononuclear cells were captured by anti-CD20 antibody in the polystyrene tube, and then ACRNPs and FACRNPs were separately added to them. After washing, the radioactivity (counts per minute (CPM)) of each tube was read. Based on results, both ACRNPs and FACRNPs had higher toxicity on the cancerous cells than normal cells. There was a significant difference between CPM of cancerous cells and CPM of normal cells after treatment with ACRNPs or FACRNPs.

Keywords: Radioactive; Selenium oxide; Nanoparticles; Detection; Treatment; CLL

Introduction

Based on previous studies, radioactive atoms which bound to monoclonal antibodies or other ligands have been used in the radio-immunotherapy and radio-immunoassay [1-7]. In the brachy therapy, the radioactive microparticles such as ^{125}I, ^{103}Pd, and ^{90}Y are used for internal radiation [8-10]. It is fact that the size of intercellular fenestrations of tumor vasculature (150–300 nm) are smaller than the size of radioactive microparticles (50–100 μm), and this leads to high intravascular retention, high side effect, and low anticancer activity [11]. Recently, various studies have been declared that the use of radioactive nanoparticles (RNPs) can improve brachy therapy [12-14], and may be applied for detection of cancers. Wang et al. showed that β-emission of ^{186}Re integrated nanoliposomes had high therapeutic effects on the squamous cell carcinoma in the nude rat xenograft model [15]. Also, Zavaleta et al. worked on pharmacokinetics and biodistribution of ^{186}Re-biotin-nanoliposomes, and showed a 30% decrease of tumor volume compared with control [16]. Bouchat et al. showed that treatment of highly vascularized tumors could be done by RNPs [17]. Chanda et al. demonstrated the decrease of prostate cancer size by radioactive gold nanoparticles conjugated with epigallocatechin-gallate (EGCg) [18]. It must be mentioned that although these nanoparticles can kill cancer cells, but normal cells can kill, too. In this research, selenium oxide (ThO_2) nanoparticles were selected, because of low cost, low radioactivity, and high safety than other commercial radioactive materials. The aim of this study was to synthesis of antibody-conjugated radioactive nanoparticles (ACRNPs) and folic acid-conjugated radioactive nanoparticles (FACRNPs). Then, the cytotoxicity of these nanoparticles was investigated on the mononuclear cells from patients with chronic lymphoid leukemia (CML) and from normal persons. Also, the potential use of ACRNPs and FACRNPs was studied to detect cancer cells by counting of radioactivity. Now, there is no same article about ACRNPs and FACRNPs.

Materials and methods

Materials

ThO_2 nanoparticles were purchased from Zyst Fannafar Shargh Company, Iran. N-ethyl-N-(dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), carboxy polyethylene glycol (CPEG) with molecular weight 3,400 daltons, Hanks buffered salt solution (HBSS), 3-(4,5-Dimethylthiazol-2)-2,5diphenyltetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-tetrazolium (MTS), and CD20 antibody were obtained from Sigma-Aldrich Chemical Company, USA. Folic acid was sourced from Aburaihan Co., Iran. Alamar Blue and ATP kit were provided from Gibco Invitrogen, UK.

Synthesis of conjugated RNPs

PEGylation

Firstly, 100 mg of CPEG was dissolved in 10 mL of phosphate buffered saline (PBS) at pH 6.0. Then, one gram of ThO_2 nanoparticles was added to 10 mL of CPEG solution, shaken gently, and incubated for 24 hours at 37 ^{o}C. After incubation, ThO_2 nanoparticles were centrifuged at 10000 rpm for 15 minutes, the supernatant discarded, and then nanoparticle pellets were re-suspended in PBS. The final concentration of ThO_2 nanoparticles was 0.1g/mL.

Conjugation of antibody and folic acid with RNPs

To conjugate folic acid with CPEG-coated-ThO_2 nanoparticles, One mL of EDC at concentration of 50 mM, one mL of NHS at concentration of 60 mM, 5 mL of CPEG-coated-ThO_2 nanoparticles at concentration of 100 mg/mL, and one mL of folic acid at concentration of 0.2 mg/mL were mixed,

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and incubated at 37 ºC for 1 hour. To conjugate anti-CD20 antibody with CPEG-coated-ThO_2 nanoparticles, one mL of 50 mM EDC, one mL of 60 mM NHS, and 5 mL of 100 mg/mL CPEG-coated-ThO_2 nanoparticles, and one mL of 0.2 mg/mL anti-CD20 antibody were added, and incubated at 37 ºC for 1 hour. After incubation, the excess of EDC, NHS, antibody, and folic acid was removed by centrifugation, and the pellet was re-suspended in PBS buffer. Finally, serial concentrations (25, 50, 100, 200, and 400 µg/mL) of two conjugates were prepared in $RPMI_{1640}$.

To confirm the attachment of CPEG to ThO_2 nanoparticles, and conjugation of antibody and folic acid, Fourier transform infrared spectroscopy (FTIR) (ELICO, India) were used. The structure and distribution size of ACRNPs and FACRNPs were studied by scanning electron microscopy (SEM) (Hitachi, S-2400, Japan) and dynamic light scattering (DLS) (Malvern Instruments, Italy), respectively.

Cytotoxicity assays

MTT assay

Firstly, 100 µL of ACRNPs and FACRNPs at serial concentrations was separately added to 100 µL of leukocyte cell suspension (10^3 cells), and then incubated for 12 hours at 37 ºC. After incubation, cells were washed with HBSS to remove any nanoparticles, and 25 µL of 5 mg/mL MTT was added and incubated at 37 ºC for 5 hours. Then, 100 µL of 70% v/v isopropanol was added to each well, and optical density (OD) of each well was read by an ELISA reader (Novin Gostar Co., Iran) at 490 nm. Finally, all data were normalized to control, *i.e.*, the OD of each sample was divided to OD of negative control. The cells which were not exposed to nanoparticles considered as negative control.

MTS assay

Briefly, 100 µL of serial concentrations of ACRNPs and FACRNPs was separately added to 100 µL of leukocyte cell suspension (10^3 cells), and incubated for at 37 ºC 12 hours. After incubation and washing with HBSS, 25 µL of 5 mg/mL MTS was added and incubated at 37 ºC for 5 hours. Then, the OD of each well was read at 490 nm by ELISA reader. Same as MTT assay, all data were normalized to control, *i.e.*, the OD of each well was divided to OD of negative control.

Cell metabolic assay

Firstly, 100 µL of serial concentrations of ACRNPs and FACRNPs was separately added to 100 µL of leukocyte cell suspension (10^3 cells), incubated for at 37 ºC 12 hours, and washed with HBSS. Then, 100 µL of the Alamar Blue reagent was added to each well, and incubated for 4 hours at 37 °C. After incubation, the OD of each well was read at 590 nm by ELISA reader, and was normalized to control, *i.e.*, the OD of each well was divided to OD of negative control which was not treated with nanoparticles.

ATP assay

First, 100 µL of serial concentrations of ACRNPs and FACRNPs was separately added to 100 µL of leukocyte cell suspension (10^3 cells), incubated for at 37 ºC 12 hours, and washed with HBSS. Then, 100 µL of ATP determination reagent and 100 µL of $RPMI_{1640}$ were added, and incubated for 10 minutes at room temperature. In the final step, the OD of each well was read by a luminometer (model 9100-102, Turner Biosystems, UK), and then all data were normalized to control, *i.e.*, the OD of each well was divided to OD of negative control which was not treated with nanoparticles.

Detection of cancer cells

Briefly, polyethylene tubes were coated with anti-CD20 monoclonal antibody by overnight incubation at 4 ºC, and the unbound antibodies were removed by washing with PBS. Next, heparinized blood samples from patients with CML (n=10) and normal persons (n=10) were obtained. The leukocyte fraction of both CML and normal samples were isolated by Ficoll method [19], washed with PBS, and then one mL of $RPMI_{1640}$ was added to them (the final cell density was 1000 cells/mL). In the next step, 100 µL of both cancer and normal cell suspension was separately added to anti-CD20 coated tubes, incubated for 1 hour at 37 ºC, and unbound cells were washed three times by PBS. Then, one mL of ACRNPs and FACRNPs was separately added to tubes, and incubated at 37 ºC for 1 hour. All tubes were washed with HBSS, radioactivity of each tube was determined by beta counter (Biosystem, UK), and then the mean of counts per minute (CPM) of each tube was read. The cells which were not treated with any RNPs considered as negative control.

Statistical analysis

All experiments were done three times, and all data are shown as mean ± standard deviation (SD). SPSS software was used for Statistical analysis, and P-values <0.05 which obtained by Student *t-test* were considered as significant difference.

Results

Characterization of conjugated RNPs

The SEM images of ACRNPs and FACRNPs are shown in Figure 1a and Figure 1b, respectively. As demonstrated, both of them were spherical, and had same size (near 50 nm). The FTIR spectrum of ThO_2 nanoparticles (I), folic acid (II), antibody (III), FACRNPs (IV), and ACRNPs (V) is shown in Figure 1c. As seen, FACRNPs and ACRNPs have the specific peaks of folic acid and antibody, respectively which confirmed the binding of folic acid and antibody to nanoparticles. The DLS

results are shown in Figure 1d and Figure 1e. As seen, the size distribution of ACRNPs and FACRNPs was approximately 40-100 nm.

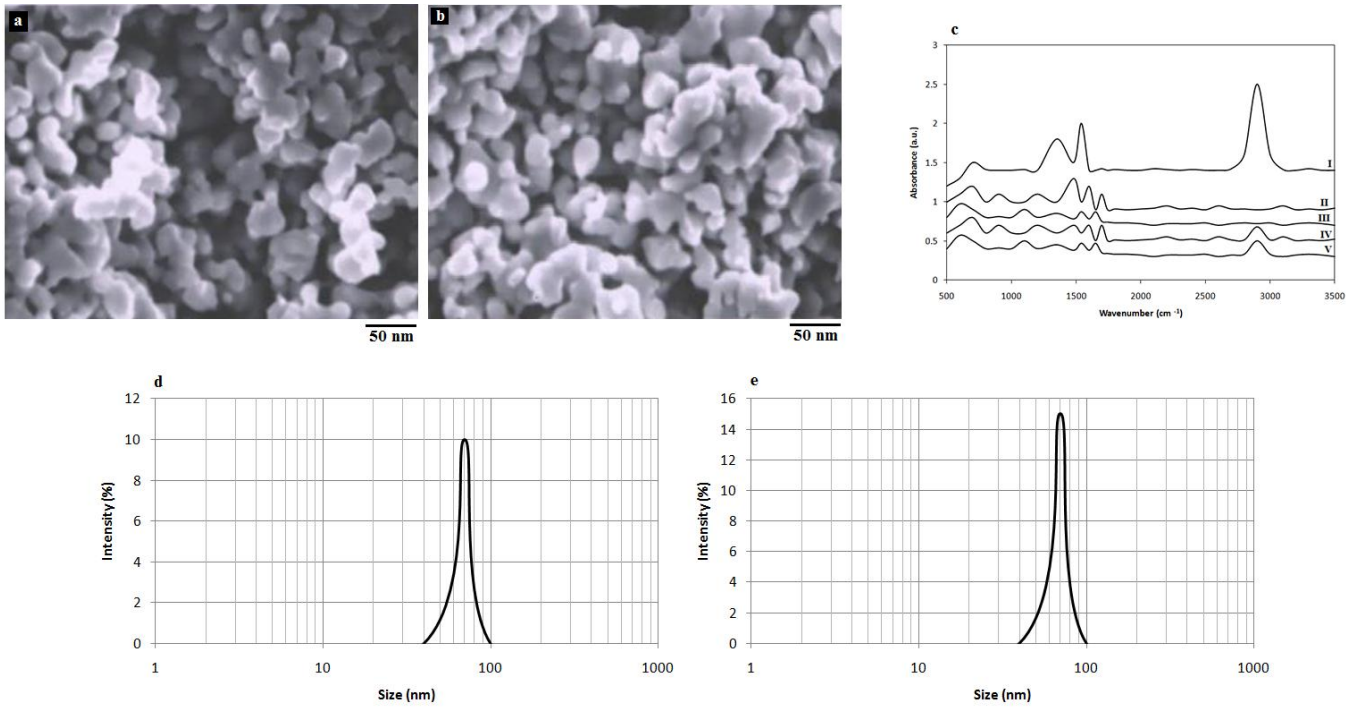

Figure 1. The SEM images of ACRNPs (a) and FACRNPs (b). The FTIR spectrum (c) of ThO_2 nanoprticles (I), folic acid (II), antibody (III), FACRNPs (IV), and ACRNPs (V). The DLS graph of ACRNPs (d) and FACRNPs (e).

Detection of cancer cells by radioactivity measurement

Figures 2a and Figure 2b show the CPM of tubes after treatment with ACRNPs and FACRNPs, respectively. This test obviously demonstrated that there was a direct relationship between CPM and concentration of ACRNPs and FACRNPs in both cancer and normal samples. Also, there was a significant difference between CPM of cancer and normal samples after treatment with ACRNPs or FACRNPs ($P<0.05$). But, no significant difference was observed between ACRNPs and FACRNPs ($P>0.05$).

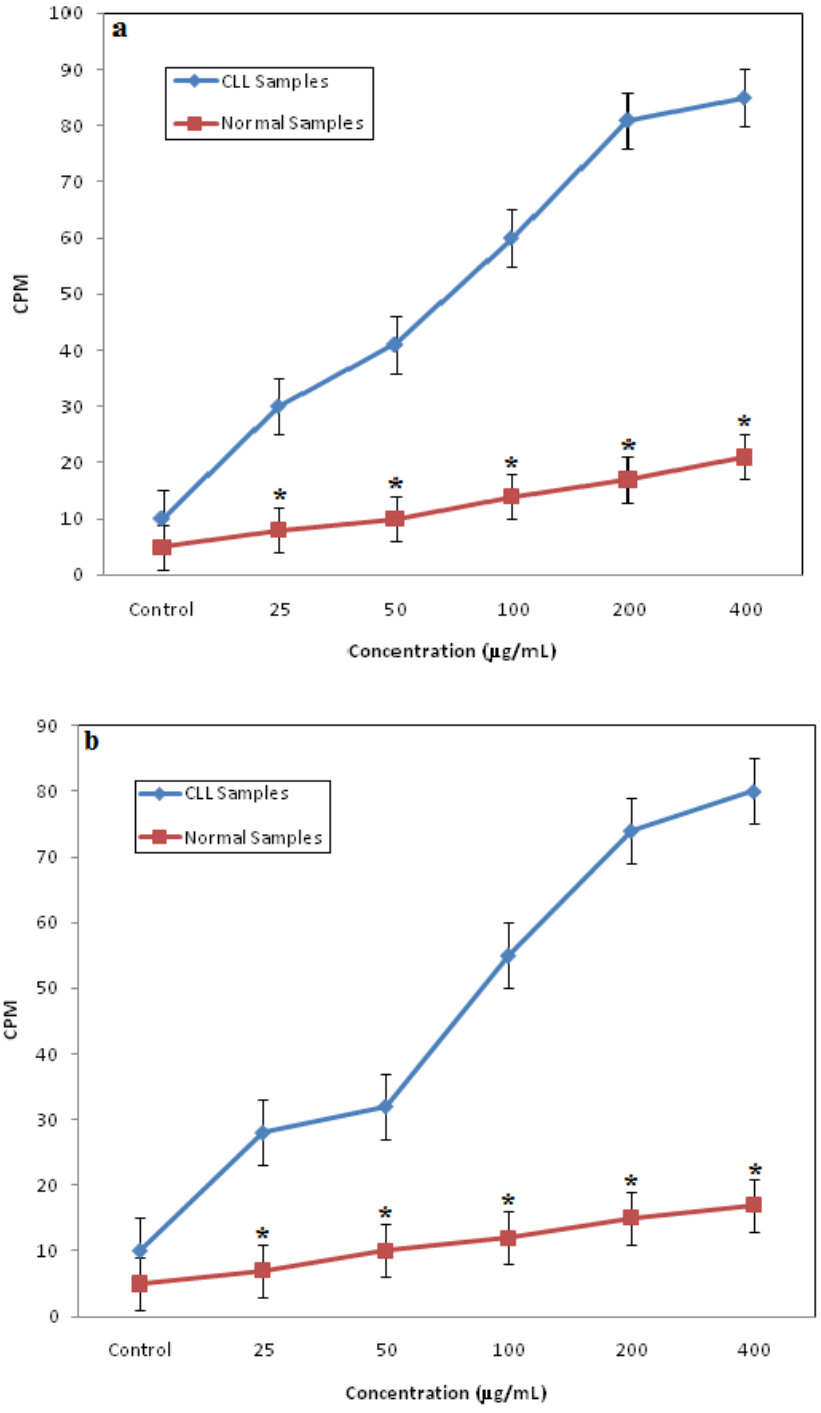

Figure 2. The CPM of tubes after treatment with ACRNPs (a) and FACRNPs (b). * P<0.05 compared with CLL samples at the same concentration.

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Cytotoxicity of ACRNPs and FACRNPs

To evaluate toxicity, different experiments were carried out. The results of MTT, MTS, Alamar Blue, and ATP assay are shown in Figure 3, Figure 4, Figure 5, and Figure 6, respectively. Each figure has two parts: (a) and (b), where (a) is the toxicity of ACRNPs on the cancer and normal cells, and (b) is the toxicity of FACRNPs on the cancer and normal cells. In case of all tests, there was an inverse relationship between concentration of ACRNPs or FACRNPs and their toxicity. There was a significant difference between toxicity of ACRNPs or FACRNPs on the cancer and normal samples ($P<0.05$). We did not observe significant difference between toxicity of ACRNPs and FACRNPs ($P>0.05$).

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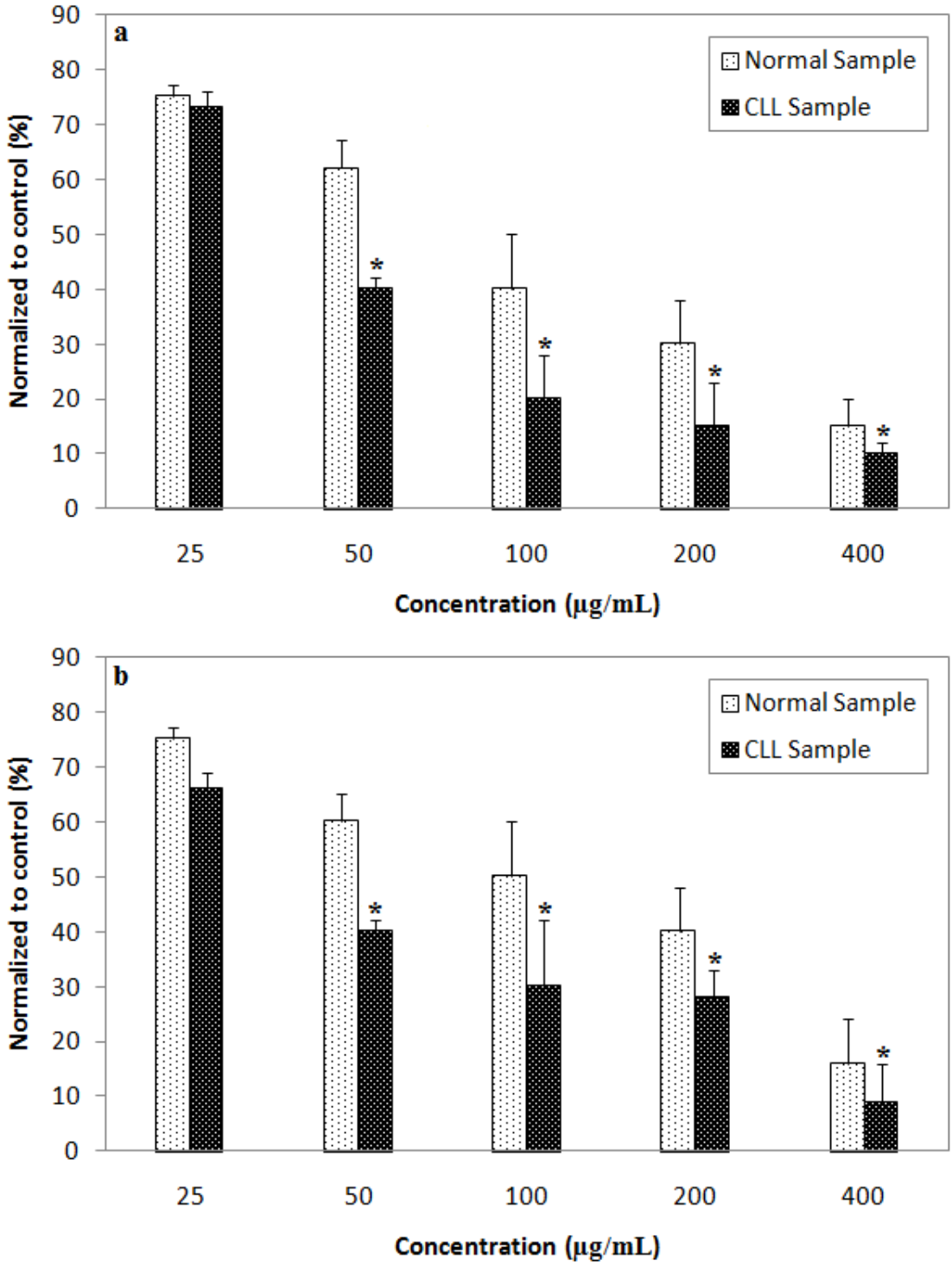

Figure 3. The toxicity of ACRNPs (a) and FACRNPs (b) obtained by MTT assay. * P<0.05 compared with normal samples at the same concentration.

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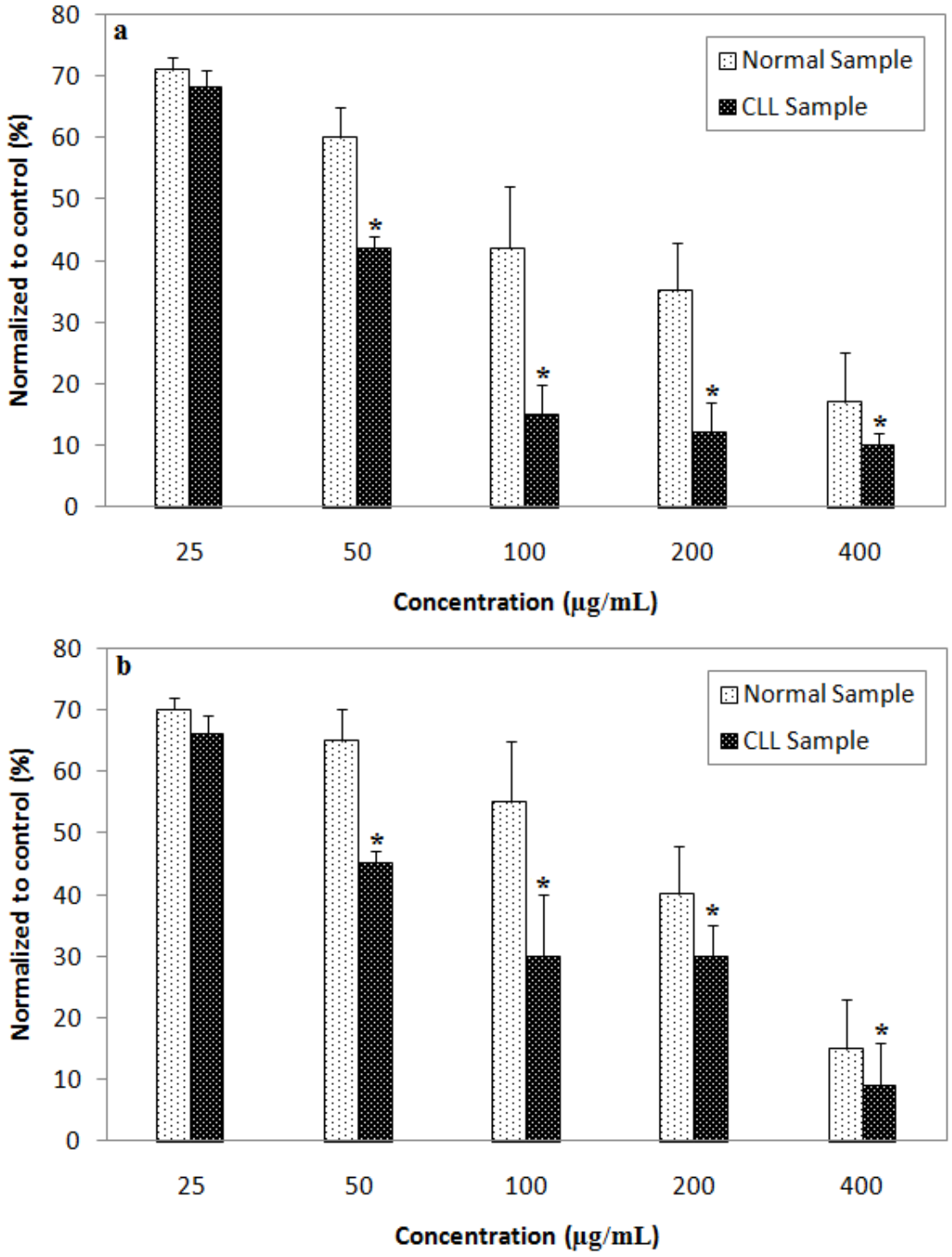

Figure 4. The toxicity of ACRNPs (a) and FACRNPs (b) obtained by MTS assay. * P<0.05 compared with normal samples at the same concentration.

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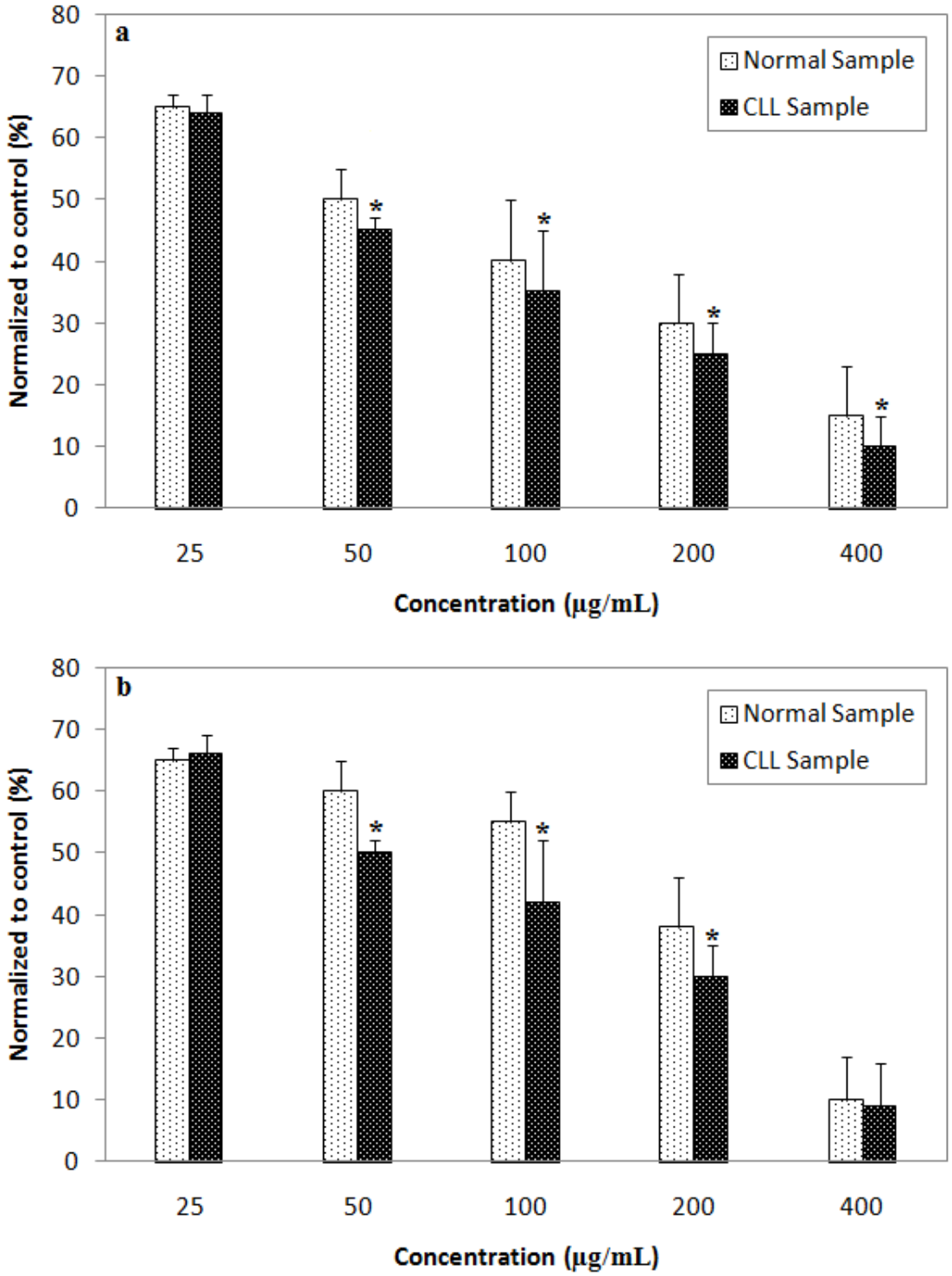

Figure 5. The toxicity of ACRNPs (a) and FACRNPs (b) obtained by Alamar Blue assay. * P<0.05 compared with normal samples at the same concentration.

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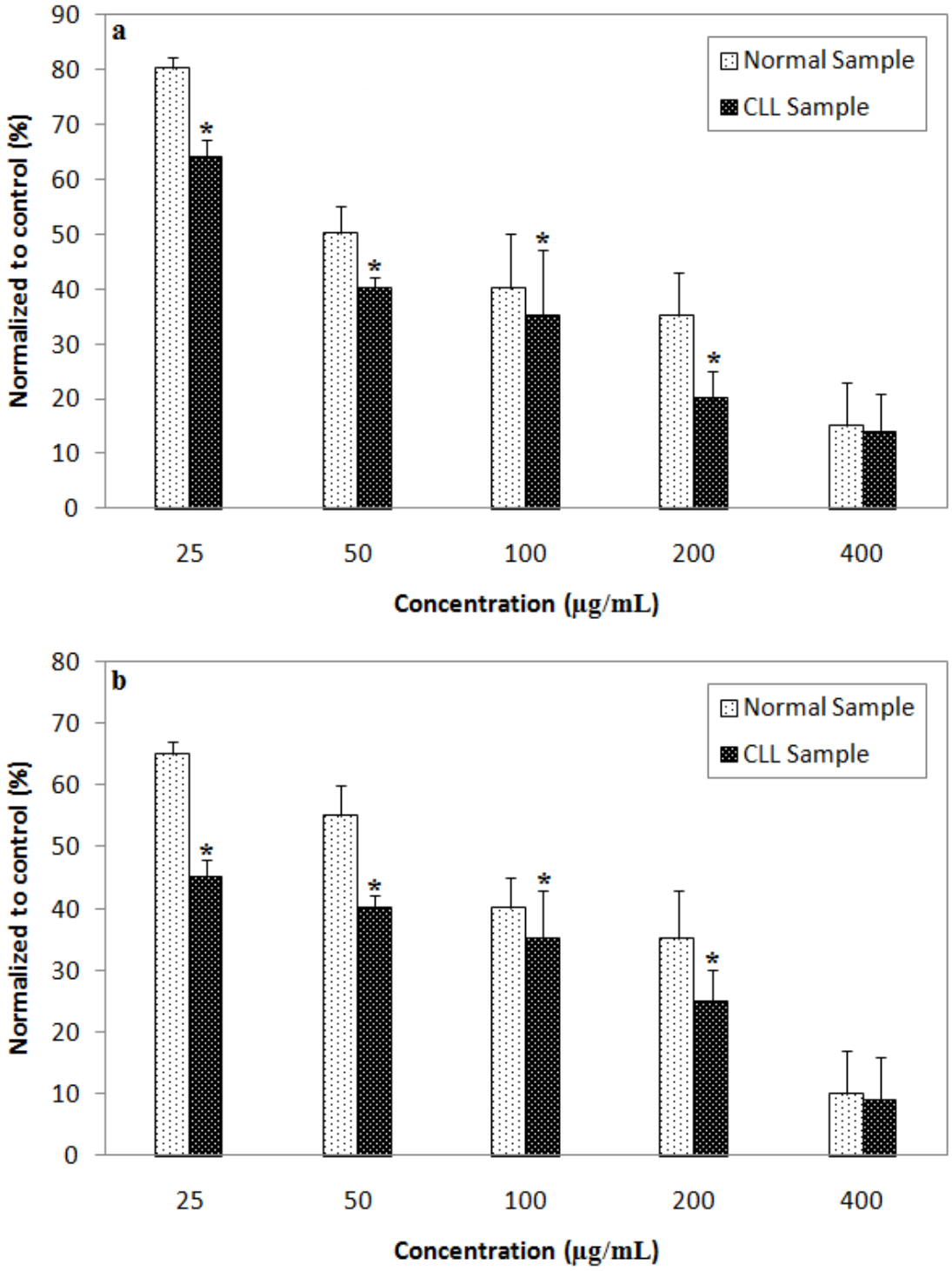

Figure 6. The toxicity of ACRNPs (a) and FACRNPs (b) obtained by ATP assay. * $P<0.05$ compared with normal samples at the same concentration.

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Discussion

In this study, ACRNPs and FACRNPs were synthesized, and their toxicity on the cancer and normal leukocytes was studied. In this research, ThO_2 nanoparticles which extracted from grid of gas lamp were used because of low cost, low radioactivity, and high safety than other commercial radioactive materials. But, it must be mentioned that different radioactive isotopes can be used for medical application [11, 17, 18]. To synthesize ACRNPs and FACRNPs, ThO_2 nanoparticles covered with CPEG, activated by EDC/NHS, and then reacted with antibody and folic acid. This method of conjugation was introduced in the previous studies [20, 21]. This research clearly showed that the use of ACRNPs and FACRNPs led to more increase of CPM in cancer samples than normal samples, but no difference was seen between CPM of ACRNPs and FACRNPs. On the other hand, this research demonstrated that both ACRNPs and FACRNPs had higher toxicity on the cancer samples than normal samples. As demonstrated, in both radioactivity and toxicity experiments, the results were dose-dependent. This means that the highest concentration of ACRNPs and FACRNPs has the highest CPM and toxicity.

To date, there is no data on the toxicity of ThO_2 nanoparticles or conjugated ThO_2 nanoparticles. For the first time, this study declared that conjugated ThO_2 nanoparticles had higher toxicity on the CLL cancer cells than normal cells. The authors hypothesize that the high level of folic acid receptor and CD20 on the CLL cancerous cells may be the reason of this difference. We explain that folic acid receptor and CD20 which over expressed on CLL cancer cells attach to ACRNPs and FACRNPs. This phenomenon is higher in the cancer samples than normal samples which have less folic acid receptor and CD20. The folate receptor is a glycosylphosphatidylinositol (GPI)-anchored, high-affinity membrane folate binding protein which over expressed in a wide variety of human tumors. Since normal tissue distribution of folate receptor is highly restricted, making it a useful

marker for targeted drug delivery. According to previous studies, folic acid is potentially superior to antibodies as a targeting ligand because of its small size, lack of immunogenicity, ready availability, and defined conjugation chemistry [22-24]. It must be mentioned that CD20 is expressed from late pro-B cells through memory cells, and it is found that CD20 is over expressed in B-cell lymphomas, hairy cell leukemia, B-cell chronic lymphocytic leukemia, and melanoma cancer stem cells [25, 26].

For the first time, this study presented a new radioactive nanoparticle for detection and treatment purposes. Previously, other RNPs have been used by other researchers. For example, Chanda et al. worked on gum Arabic glycoprotein-functionalized radioactive gold nanoparticles for targeting and destroying tumor cells [18]. They showed that these nanoparticles had significant therapeutic efficacy. In the other study, Bouchat et al. demonstrated that $^{90}Y_2O_3$ RNPs can be applied for treatment of vascularized tumors. They indicated that normal and tumoral cells were not equally sensitive to radiation [17]. At the University of Missouri, researchers showed that radioactive gold nanoparticles conjugated with epigallocatechin-gallate (EGCg) could treat prostate cancer cells [18]. Recently, some studies declared that the use of RNPs could improve brachy therapy efficacy [12-14]. According to these studies, RNPs are an ideal choice for detection and treatment of cancers in future. But their biocompatibility, tolerance, and toxicity must be evaluated at in vivo and in clinical trials.

Conclusion

This study shows that ThO_2 RNPs conjugated with folic acid and antibody have higher toxicity on the CLL cancer cells than normal cells, and may be used for treatment of cancer cells in future.

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