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Preliminary Evaluation on the EMT6 Breast Tumor Inhibitory Activity of Carbon Nanotubes with Hyperthermia

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Abstract. Breast cancer is considered one of the most dangerous types of cancer with a high mortality rate among women worldwide. Potential alternative treatments such as hyperthermia (HT) often associated with poor specific heat distribution within the tumor microenvironment, thus reducing its anticancer potential. Owing to the fact that CNTs possessed high thermal conductivity, which could provide a uniform heat distribution, this study aims at exploring the combined effect of CNTs and HT in managing breast tumors. In this study, multiwalled CNTs were functionalized by acid washing protocol and validated by characterization analysis. Female Balb/c mice were inoculated with EMT6 breast cancer cells and then were intratumorally injected once with MWCNTs before being subjected to HT treatment for 3 consecutive days. Tumor progression and survival curves of mice were monitored. Our results demonstrated a successful functionalization of MWCNTs throughout dispersion, FTIR, TGA, XRD and FESEM analysis. Moreover, the combined-treated tumor showed complete eradication and prolonged survival median. The histological data revealed that combined treatment induced significant tumor necrosis post-treatment. Altogether, results from this preliminary study suggested that functionalized CNTs in combination with HT demonstrated a promising effect in eliminating EMT6 breast tumor.

INTRODUCTION

Recent years have recorded the emerging cases of breast cancer incidences worldwide. The Global Cancer Statistics, 2019 stated that breast cancer contributed to the primary cause of cancer death among women. In cancer oncology, conventional treatments available in the clinical setting play a major role as the main standard practice to treat breast cancer [1,2]. Unfortunately, treatments such as chemotherapy, radiotherapy, hormone therapy and surgery have been associated with systemic toxicity, caused adverse reactions, invasive, multidrug resistance cancer cells and tumor recurrence [3,4]. Moreover, surgery, which is the most practiced cancer treatment, caused traumatic injuries towards breast cancer patients [5]. Hence, alternative therapies have emerged due to the inefficiency of the standard treatments. Such therapies for example herbal therapy and photothermal therapy (PTT) trained the self-healing mechanism to fight against cancer [6].

Hyperthermia (HT), which is a part of the PTT procedure, works by the elevation of the body temperature higher than normal body temperature [7]. HT has been reported to generate promising outcomes with the involvement of both direct and indirect killing mechanisms in tumor microenvironment [8]. Besides, the HT procedure is non-invasive since the near-infrared radiation (NIR) laser is not being absorbed by normal tissue [9]. Despite its successful results in cancer therapy, HT experienced low efficacy condition since heat is difficult to distribute inside tumor tissue [10]. Hence, modified multiwalled-carbon nanotubes (MWCNTs) with high thermal stability and conductivity are used to overcome this limitation [11]. MWCNTs are expected to increase the heat retention time and thermal distribution with minimally invasive protocols besides their low toxicity properties. Therefore, our study aims to investigate the efficacy of oxidized MWCNTs with HT as an anti-breast cancer agent in the animal model.

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EXPERIMENTAL DETAILS

Pristine MWCNTs were purchased from Universiti Sains Malaysia, Malaysia. The acid washing procedure was performed to functionalize the MWCNTs by a mixture of hydrochloric acid and sulphuric acid. A series of washing process was done to stabilize the ox-MWCNTs. Then, the prepared ox-MWCNTs were characterized by colloidal dispersion test, FTIR analysis, TGA, XRD analysis and FESEM imaging. Sample preparation was done prior to each characterization analysis.

For the *in vivo* tumor inhibitory activity, the EMT6 breast cancer cells were inoculated on the right flank of the female Balb/c mice. All animal experiments were performed under the ethics approval of Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC), Malaysia (code 59/2019). After 7 days post-inoculation, mice were then subjected to MWCNTs injection and proceeded for localized hyperthermia treatment for 3 days. The temperature of the tumor was monitored and maintained at 43 °C. Then, tumor size was individually measured in each mouse using a digital caliper. Next, the survival median of mice from each treatment group was produced. For histological analysis, mice were sacrificed 10 days post-inoculation. Solid tumors were harvested, paraffin-fixed, sectioned and stained with H&E staining. Tissue sections were observed under light microscope. All statistical analysis was generated within GraphPad Prism 7 software.

RESULTS AND DISCUSSION

Prior to the *in vivo* animal anticancer evaluation, p-MWCNTs require specific a modification to improvise their dispersibility and reduce their toxicity. Pristine MWCNTs have been reported to cause pulmonary toxicity and have poor stability inside a polar environment [12]. Since MWCNTs will be administered intratumorally, modification is essential to prevent them from coagulating in the blood capillaries while preventing them from being engulfed by the macrophages [13]. In characterization studies, ox-MWCNTs displayed higher dispersibility conditions as compared to p-MWCNTS since they were stable inside the aqueous solution for more than 20 days, as shown in Figure 1 (a). Besides, the FTIR analysis (Figure 1(b)) indicated strong and broad peaks of hydroxyl and carboxylic functional groups, which indicated the abundance presence of oxygen decorating the surface of ox-MWCNTs [14]. Moreover, the TGA and XRD analysis both supported the successful modification of p-MWCNTs since the thermal degradation of ox-MWCNTs is greater compared to p-MWCNTs, which indicated the combustion of oxygenated-functional groups on the MWCNTs surface as shown in Figure 1 (c). Meanwhile, the XRD pattern suggested that an increase in the ox-MWCNTs peak indicates the formation of loosely ordered MWCNTs floss after acid-washed while the structure is preserved despite the acid functionalization process as displayed in Figure 1 (d).

On the other hand, FESEM imaging was performed to validate the structural changes post-modification. As shown in Figure 2, p-MWCNTs often appeared in long and fluffy tubular structures while ox-MWCNTs have smoother and shorter tubular structure [15]. Furthermore, clumps were typically observed in p-MWCNTs, which indicated higher impurities compared to ox-MWCNTs. In addition, modified MWCNTs showed cut tubular ends that facilitated the decoration of functional groups at the open end during the acid cutting process [16]. On the same token, the surface of ox-MWCNTs showed a groovy structure that indicated the cut-opened surface of the MWCNTs after modification.

For preliminary tumor inhibitory activity, localized HT treatment was applied at 43 ^{o}C for 30 minutes. The NIR radiation at 750nm wavelength was used with a power density of 1.5 W/cm^2. Intratumoral injection of ox-MWCNTs was performed for the combined therapy before HT treatment was applied for 10 minutes. The results in Figure 3 show that ox-MWCNTs in combination with HT treatment (3d) and were able to eradicate tumor tissue completely in all mice within 40 days. On the other hand, the tumor size of untreated mice (3a) and CNT-treated mice (3b) increased over time until they exceeded the maximum allowable size of the tumor, which is 150 mm^2. Meanwhile, 3 tumors of HT-treated mice (3c) displayed complete eradication while the others showed the increased in size. On the same token, the survival curve analysis indicated 100% survival of mice in the combined treatment group since all mice were tumor-free while half of the HT-treated mice survived from breast cancer as shown in Figure 3(e). Meanwhile, the histological analysis of tumor sections in Figure 4 revealed that untreated and CNT-treated tumor have normal cells architecture with the intact cytoplasm and dense nuclei. However, HT-treated and combined-treated tumor experienced cells necrosis due to the heat induction. These events can be observed by the shrinkage in the size of nuclei as well as the dissociation of cytoplasmic structure of the breast cancer cells. Therefore, this study indicates that ox-MWCNTs may increase the efficacy of HT treatment *in vivo*. Furthermore, the result suggested that combined treatment is more efficient compared to HT alone since temperature elevation is faster with the presence of ox-MWCNTs in tumor microenvironment plus shorter irradiation time was applied thus reducing the laser exposure period.