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Anti-inflammatory activity of prednisolone drug loaded on carbon nanotubes

Shafq AL-AZZAWI [1*], Asmaa HAMMADI [1], Dhafir MASHETA [1]

[1] College of Pharmacy, University of Babylon, Hilla, 51002, Iraq

[*] Corresponding Author. E-mail: phar.shafaq.kadhim@uobabylon.edu.iq (S.A.); Tel. +09647831349452.

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ABSTRACT: The activity of many anti-inflammatory drugs is limited due to inadequate tissue penetration and targeting efficiency and side effects of free drug. A properly designed delivery system can enhance therapeutic activity by overcoming these obstacles. This study aims to load prednisolone drug molecules on carbon nanotubes carrier (CNT) for improving its anti-inflammatory action and reducing the adverse reactions associated with using higher doses of the unloaded drug. CNT carrier was loaded with prednisolone by nano-extraction, and the morphology and size were determined by a scanning electron microscope. X-ray diffraction patterns and FT-IR spectra were utilized for characterization. While the anti-inflammatory activity of the loaded drug was examined biochemically through the assessment of interleukin-1 levels as an inflammatory marker using an in vitro tissue culture model composed of HeLa cells. These cells were stimulated with TNF-α to initiate the activation of the nuclear factor-κB as an inflammatory response. Successful loading of drug was confirmed through microscopic examination results which showed that CNT formed a network with bundles of prednisolone molecules grafted onto the surface, and these results were confirmed by X-ray diffraction and FT-IR. Whereas, the loaded drug still has its anti-inflammatory action, by significant lowering in interleukin-1 levels which was detected in TNF-α-stimulated cells treated with prednisolone loaded on CNT compared to unloaded CNT. In conclusion, this study demonstrated, for the first time, a successful loading of prednisolone to a CNT with a potential anti-inflammatory action making it a promising complex in treating inflammatory diseases with reduced therapeutic doses.

KEYWORDS: Anti-inflammatory action; Interleukin-1; Carbon nanotube; Prednisolone; Drug delivery systems

1. INTRODUCTION

The enhanced and improved understanding of disease biology and aetiology is revealing new receptor targets for treatment. Nevertheless, due to insufficient tissue penetration and targeting efficiency, such treatments aiming at these receptors frequently fail [1]. A deeper comprehension is necessary to overcome obstacles with inadequate permeability across biological barriers and achieve targeted drug delivery to various tissues at the cellular level [1].

Drug delivery systems (DDS), either simple or sophisticated, are designed to enhance the therapeutic profile of biomolecules and protect them from deactivation while circulating in the body. Without DDS, effectiveness is completely reliant on drug's physico-chemical characteristics and capacity to enter a target region where the action is required. Safer (by decreasing the dose of administered drug) and more efficient drug delivery (by improving specific tissues targeting) may be achieved by hiring of targeted nanomedicines as DDS [2,3]. Thus, in the last two decades, the applications of nanomaterials (such as liposomes, dendrimers, carbon and titanium dioxide nanomaterials, iron oxide and polymers-based nanoparticles, and other types of nanoparticles) in the diagnostic and medical purposes have been escalated [4-6].

Carbon nanotubes (CNTs) have attracted tremendous interest in the drug delivery field because of their unique and promising characteristics including; high surface area to weight ratios, flexible interaction, reasonable strength, needle-like crystal structures and high loading capacities with cargo drugs, unique electrical and optical properties, high degrees of stability and biocompatibility, and reversible release of loaded bioactive cargos at targeted tissue) [7, 8].

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Furthermore, CNTs can be covalently or non-covalently conjugated to drug molecules to enhance its delivery. Because of their favorable characteristics, CNTs are readily taken up by a wide range of cell types. The cellular internalization of CNTs has been presumed by several mechanisms including; direct cell membrane penetration or passive uptake (independent energy pathway), or active uptake and endocytosis mechanisms (energy consuming pathway) [2, 9].

Numerous studies have investigated the potential of CNTs as carriers for anti-cancer drugs such as doxorubicin, docetaxel, methotrexate, paclitaxel, mangiferin and gemcitabine in the treatment of different tumors [10, 11]. In addition to anticancer drugs, CNTs have also been employed as DDS for the delivery of many anti-microbial and anti-inflammatory drugs such as dapsone, dexamethasone, ketoprofen, carvedilol (an antihypertensive drug), carbazochrome (a hemostatic agent) and amphotericin B (an antifungal drug) [12, 13]. CNTs can be conjugated with anti-inflammatory drugs, such as in transdermal drug delivery, which causes low concentrations and reduces systemic side effects associated with oral administration. Moreover, the conjugation may enhance the release of drug at the site of application with controlled release and decrease protein binding that happens with free drug, making its required therapeutic dose high.

Acute inflammation can turn into a serious illness that can even be life-threatening, while chronic inflammations can lead to systemic disorders. There are drawbacks to conventional therapies, including systemic side effects, the development of gastrointestinal ulcers, unstable continuous administration, insufficient local drug concentration, and uncontrolled drug concentration.

Inflammatory diseases, such as inflammatory bowel diseases and rheumatoid arthritis have been mostly influenced by the pathogenesis of inflammation. Therefore, proper delivery of anti-inflammatory drugs could reduce therapeutic dosage and enhance the drug's effect [14, 15]. Regardless of the cause of inflammation, glucocorticoids, including prednisolone (PDN) can act as potent anti-inflammatory agents. In addition to suppressing immune responses, these drugs may also inhibit the production of prostaglandins and leukotrienes, which are usually elevated in inflammatory conditions [16, 17].

Prednisolone is a corticosteroid drug with molecular weight equal to 360.4 g/mole and it has poor water-solubility. The drug exhibits a wide range of actions and is mainly used for its anti-inflammatory and immunosuppressive activities. It interferes with glucose levels in the blood and potassium and sodium secretions. The main route of prednisolone elimination is via renal excretion where over 98% of the administered dose is eliminated by this route. The plasma half-life of the drug is about 2-3.5 hours. The drug clearance is about 0.09 L/kg/h [18].

However, untargeted delivery of PDN, may result in loss of drugs activity because of reduced concentrations at the site of action which may necessitate the utilize of increased doses of these drugs that will be accompanied by higher incidence of adverse effects [19]. To overcome this problem, this study focuses on developing and applying safe and efficient delivery of PDN using CNT as a carrier to promote its transport to the targeted sites and thereby improving its anti-inflammatory action through the assessment of interleukin-1 levels as an inflammatory marker, in turns this could avoid using higher doses of the drug which eventually lead to reduction in the side-effects.

2. RESULT AND DISCUSSION

2.1. Examination of morphology of drug-loaded carrier molecule

The morphology of the carrier by TEM is shown in Fig.1, which appeared as single filaments that made possible by the reduction of the Van der Waals forces between the CNT bundles caused by the development of C-O, C=O, and C-OH groups on the surface of the CNTs. The morphology of the PDN-CNTs and free PDN by SEM are shown in Fig. 2, where Fig. (2a and b) show the SEM images for PDN and PDN-CNTs, respectively. According to the SEM results, the CNTs formed a network with bundles that were approximately 58.61 nm in diameter and attached to one another to produce a porous structure. PDN appears to have a smooth surface in Fig. 2a; however, the carbon nanotube surfaces of PDN-CNTs are rough and contain some connected clusters (Fig. 2b). PDN macromolecular has apparently been grafted onto the surface of CNTs, according to the phenomenon. While Fig. (2c and 2d) show the histogram of the

distribution of PDN alone, and the distribution of PDN-CNTs, respectively, that is indicating the crafting of the drug molecules into the nanocarrier based on changes in particle size distribution of the free drug and loaded molecule.

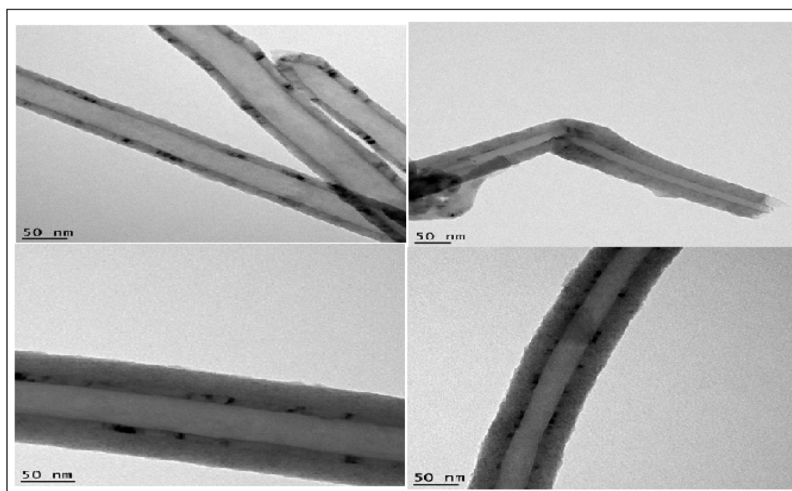

Figure 1. TEM images of the synthesized carrier

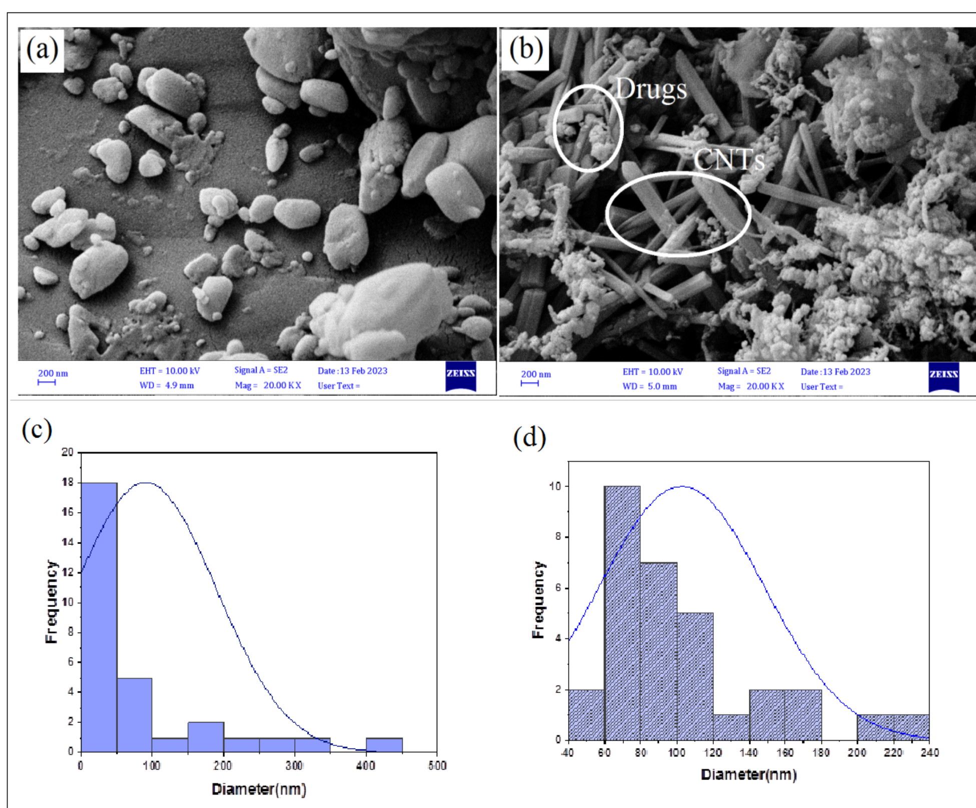

Figure 2. SEM of (a) Prednisolone drug, (b) Loading of Prednisolone into CNTs, (c) Histogram shows the distribution of Prednisolone drug, and (d) Histogram shows the distribution of PND-CNTs

2.2. Analysis by XRD

Fig.3 depicts the XRD pattern of PDN, CNT carreir and PDN-CNT surfaces. The XRD patterns present many broad bands peaking at 26.0°, and 43.2°. The sharp and tight peak at 26.0° with interplanar spacing corresponds to graphite, as it is due to the presence of carbon atoms. The peaks near 43.2° are attributed to the nanotube structure, as previously stated by other studies [20]. Fig. 3 depicts the drug molecules as well as those of the created formulations. Drug's crystallinity was demonstrated by the compound's multiple reflections and strong peaks at 15.758, 17.648, 21.338, 22.778, 25.278, and 26.318. The amorphous nature was most apparent, as indicated in the diffractograms obtained for the produced formulations. The characteristic PDN peak at 2 of 15.78 was discovered to be undetectable in all formulations. The XDR results of PDN-CNT molecule showed further distinctive peaks at 2θ = 27.38° and 47.98° (which are marked with the sign (*)) in Fig. 3, indicating the successful loading of PDN molecules within the CNT matrix as confirmed by early findings regarding PDN's XRD analysis [21].

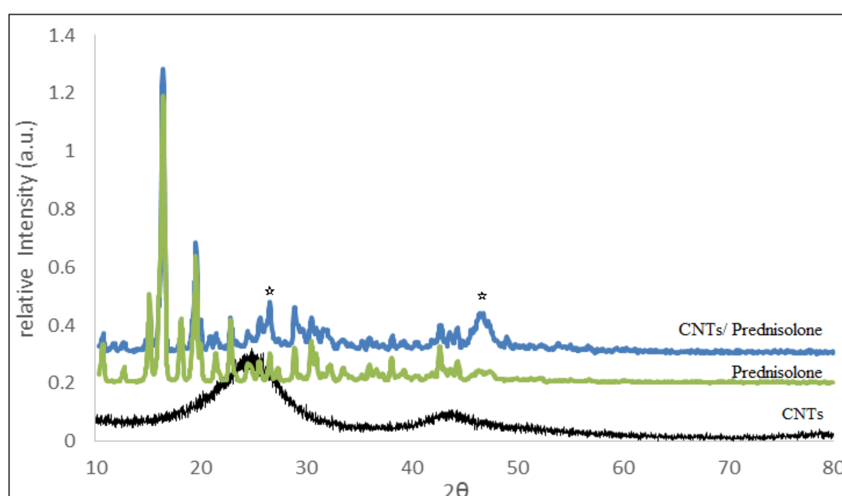

Figure 3. XRD of free Prednisolone drug, CNT carrier, and loaded drug on CNTs

2.3. Fourier Transform Infrared (FT-IR) Spectroscopy

To produce the hydrophilic surface structure of oxygen inside a certain surface group, CNTs are chemically oxidized using H_2O_2 and UV. On the surface of the CNTs, this oxidation with H_2O_2 introduces a number of functional groups, including -OH (hydroxl), -COOH (carboxyl). The strong properties of functionalized CNTs are shown in Fig. 4, along with a broad band between 3173 and 3600 cm^{-1} that is due to the O-H stretching vibrations in the C-OH groups. On the other hand, the C=O stretching vibrations in the carboxyl, aldehyde, and acid anhydride groups are what create the broad band between 1766 and 2017 cm^{-1} [22]. To analyze potential interactions between the PDN and the CNTs, FT-IR spectroscopy was employed. Fig. 4 shows the FT-IR spectra of the loaded and unloaded formulations. Free PDN's spectra displayed OH group-specific bands between 3200 and 3500 cm^{-1} (OH-group is involved in intermolecular association). The bands related to the PDN drug molecule appeared in the PDN-CNT molecule spectrum where strong bands of C=C at 1660 cm^{-1} and C=O at 1700 cm^{-1} are detected. Furthermore, strong band due to carbonyl groups at 1720 cm^{-1} (C=O stretch), as well as the two bands related to ester bonds at 1267 cm^{-1} and 1165 cm^{-1} related to PDN molecule also appeared in the spectrum of the loaded molecule. These findings indicate the successful crafting of the drug molecule on the CNT carrier.

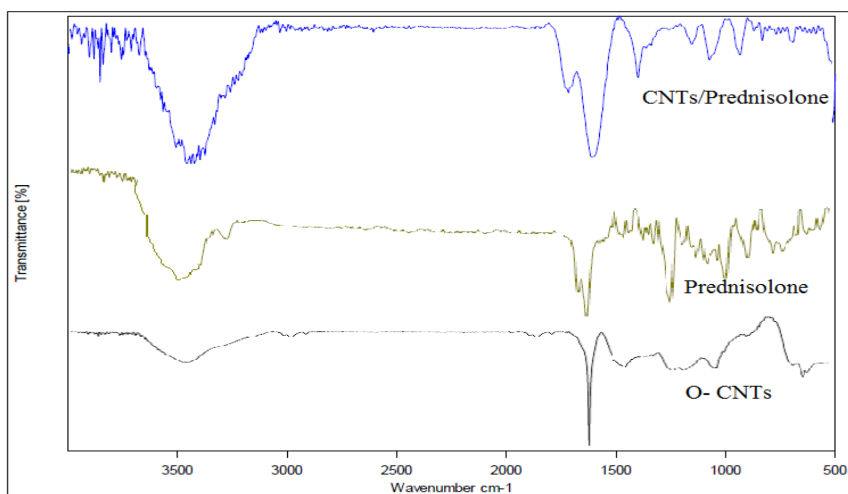

Figure 4. FTIR spectra of unloaded O- CNTs, free Prednisolone drug molecule, and PDN-CNTs

2.4. Cells spiking by TNF-α

To initiate inflammatory responses in the cultured cells, to be later used to assess the anti-inflammatory activity of the loaded drug, these cells were treated with TNF-α. HeLa cells stimulation with TNF-α is highly recommended for investigating programmed cell death (apoptosis) and for the analysis of immune response activation 's role on inflammation levels, cell growth, and cell survival rates [23]. The spiking was achieved as indicated by the elevated levels of interleukin-1 in cells treated with the inflammatory initiator compared to untreated cells (Fig. 5). The levels of linterleukin-1 increased to 30 pg/mL after 4 hours of treatment and above 55 pg/mL after 12 hours, with significant difference from untreated cells (P <0.001).

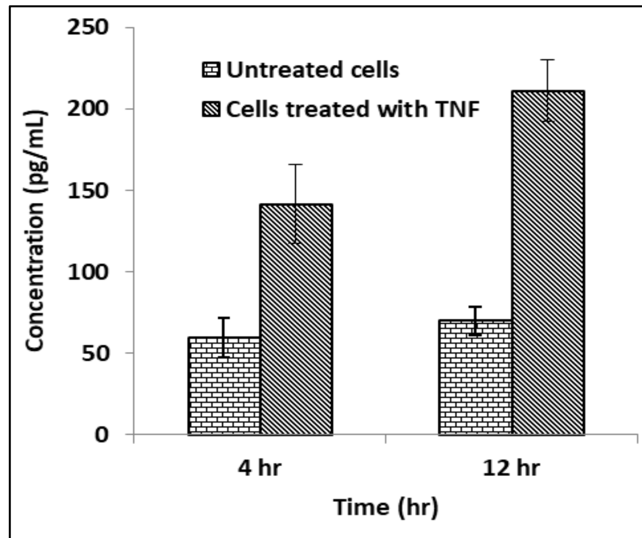

Figure 5. Interleukin-1 levels after cells treatment with TNF-α for 4 and 12 hours compared to treatment of free cells

2.5. Biochemical assessment of anti-inflammatory activity

The levels of interleukin-1 were measured following treatment of cells with TNF-α alone, TNF-α with free PDN, TNF-α with unloaded CNTs and TNF-α with CNTs loaded with PDN (Fig. 6). Treatment with TNF-α caused detected elevation in the inflammatory markers' levels at both time intervals of treatment (4 and 12 hours). However, combining free PDN with the spiking mediator result in lowering the inflammatory marker levels to almost half and one third concentrations after 4 and 12 hr treatment respectively, in comparison with the levels seen in cells treated with TNF-α (P<0.01). The results are expected due to the strong anti-inflammatory activity of the glucocorticoid drug, PDN. Many of the earliest steps in an

inflammatory response are inhibited by glucocorticoids which eventually promote the resolution of inflammatory status [24]. Nevertheless, comparing the inflammatory status represented by interleukin-1 in cells treated with TNF-α-drug free CNTs with cells treated with TNF-α alone did not reveal any significant difference after 4 and 12 hours of exposure (Fig. 6) (P=0.082 and 0.11 respectively). CNTs don't possess any anti-inflammatory actions however the activity is related to the loaded drug [25].

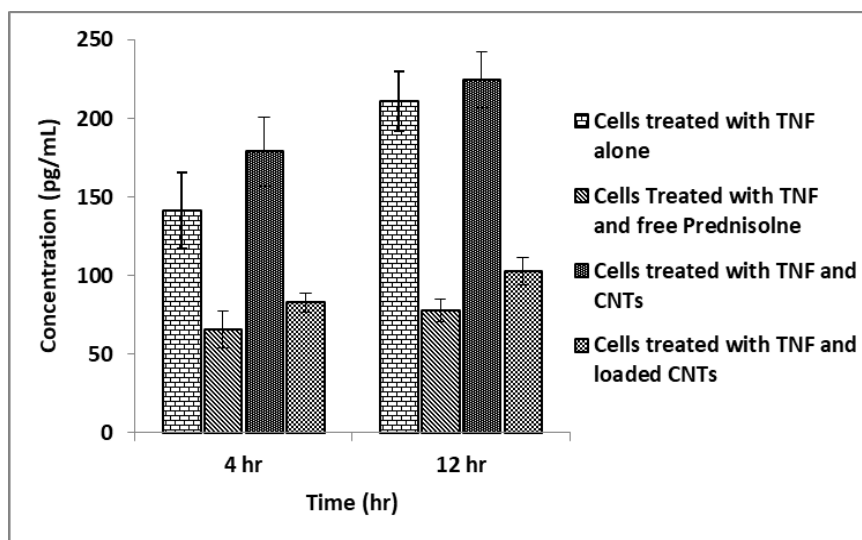

Figure 6. Interleukin-1 levels in cells treated with TNF-α alone, TNF-α with free Prednisolone, TNF-α with unloaded CNTs and TNF-α with CNTs loaded with Prednisolone after 4 and 12 hours. Values are expressed as mean ± standard deviation

An essential part of this test is to measure the ability of loaded PDN on CNTs in alleviating inflammatory status in the tested cells. Significant lowering in interleukin-1 levels ($P<0.01$) were detected in cells treated with PDN loaded on CNTs compared to cells treated with TNF-α alone or unloaded CNTs (Fig. 6). However, no such difference was detected in comparison with the levels of cells treated with free drug.

Conjugating drug with a specific carrier system can improve its efficacy, permeability and targeting at the desired site of action and for various medical applications [26, 27]. One approach to drug delivery is to use nanoparticles, including liposomes, polymeric nanoparticles, and dendrimers, to encapsulate and deliver the drug to specific target cells or tissues [28, 29]. For instance, PDN-loaded liposomes have been shown to improve drug delivery, potentially reducing the side effects associated with systemic administration [30].

Likewise, CNTs have mostly been used in applications for the treatment of cancer and other diseases. CNTs can be used for the delivery of various molecules since their surfaces are easily functionalized by noncovalent and covalent attachment. Conjugation of Cisplatin, an anticancer drug, with CNT improved its antitumor effect and decreased the side effects associated with applying the drug in its free form [31]. CNTs have been extensively used for delivering Doxorubicin for treatment of different tumors to target its delivery and to potentiate its antitumor activity [32].

CNTs-based drug delivery systems have also been utilized for the delivery of different non antitumor drugs. A previous study has demonstrated that loading Dexamethasone, an anti-inflammatory and immunosuppressant drug, on CNTs stimulated its delivery with a complete drug release and accelerated cellular uptake [33]. Moreover, the anti-microbial and anti-inflammatory drug, Dapsone, has been loaded onto CNTs that enhanced the drug's efficiency [33]. Further study has shown that Ketoprofen, a non-steroidal anti-inflammatory drug, when delivered by CNT via electrospinning could enhance the release of the drug with applied potentials [34].

Due to their unique properties, CNTs may be used as excellent carriers with a high loading capacity for many drugs, including PDN. PDN drug is an ideal candidate drug to be delivered by CNTs due to its low molecular weight and poor water solubility. The drug can be easily dispersed on and inside the carrier molecules. This may increase the drug's therapeutic efficiency by reducing its excretion through urine and improving its loading capacity and solubility [35].

3. CONCLUSION

This study demonstrated, for the first time, a successful loading of PDN on CNT which was confirmed by X-ray diffraction and FT-IR results. The loaded drug showed a potential anti-inflammatory action as indicated by the lowering of inflammatory marker levels, interleukin-1, similar to the action of unloaded drug. Therefore, the encapsulation of drug on the carrier system retained its pharmaceutical activity. The designed DDS could be used for treatment of inflammatory disease at the site of target with a prospect of decreased therapeutic dose and in turn lowering systemic side effects that are associated with unloaded drug.

Overall, utilizing CNTs for drug delivery has the potential to revolutionize the field of medicine by enhancing drug effectiveness, decreasing its toxicity, and addressing delivery to specific tissues or cells. Once loaded onto CNTs, PDN can be delivered to specific cells or tissues in the body, offering targeted drug delivery and potentially reducing side effects associated with systemic administration of the drug. In addition, the drug can be delivered using different dosage form rather than conventional dosage form since its physic-chemical properties will be quite different in loaded form than the parent drug. However, it is important to note that the use of CNT in PDN drug delivery is still an active area of research, with further studies are required for a comprehensive understanding of the safety and efficacy of this approach as well as its pharmacokinetics profile for the new molecule.

4. MATERIALS AND METHODS

4.1. Carrier system preparation

The synthesis of CNT carrier was performed by flame-fragments deposition method using a specialized designed chamber device, as described in details in previous published studies [36-38]. To enhance the surface and functionalization properties for loading drug molecules, 100 mg of the synthesised CNTs were dissolved in 50 ml of acetone using an ultrasonic waterbath. This process can introduce more functional groups on the inner walls of these nanotubes, thereby diversifying the chemical nature and reactivity enabling more efficient loading of various drug molecules [39]. The chemicals used in this process were purchased from Dubi Chem In. UAE.

4.2. Loading prednisolone on the carrier

A nano-extraction method was used to load PDN into CNTs [37]. A solvent of ethanol/water (1:2) mixture was used to dissolve PDN at a concentration of 2 mg/ml and then mixed at room temperature for an overnight period with oxidized CNTs (10 mg /ml). PND-CNTs were obtained by filtering the mixture and drying the collected black powder, at room temperature, on filter paper in flowing nitrogen gas.

4.3. Characterization of the drug-carrier molecule

Scanning electron microscope (SEM) (XL30 FEG; Philips) and transmission electron microscopic (TEM) (Carl Zeiss EM900, Germany) pictures were used to determine the morphology. For each developed formulation, Image-J software was used to determine the average particle diameter, particle size distribution, and standard deviation of the population. The crystalline structure, placement, and structural characteristics in the range of 0°-100° and scan rate 10 deg /min were discovered using the X-ray diffraction pattern (XRD) (PW1730: Philips) with Cu K of 0.541oA as the X-ray radiation's source. Using a Fourier-transform infrared spectroscopy (FT- IR) (SHIMADZU 8400S) in transmission mode with regions having a resolution of 4 cm-1, the drug-loaded and unloaded carrier system were characterized, in which the infrared spectra at ambient conditions were diluted with KBr.

4.4. In vitro anti-inflammatory test

Following loading of PDN on CNTs, the anti-inflammatory activity of the loaded drug was examined utilizing an in vitro tissue culture model consists of HeLa cells. The test aimed to examine whether the drug loaded on the drug carrier system (CNTs) retained its therapeutic efficacy compared to the free drug.

Hela cells spiking with TNF-α (which is a member of cytokines inflammatory markers that cause stimulation of the acute phase reaction of the immune response), which in turn leads to apoptosis and initiates the activation of the nuclear factor-κB (NFκB) inflammatory transduction pathway [40, 41]. The

NFκB transcription factor is activated in all inflammatory diseases, and it has an essential role in amplifying and perpetuating the inflammatory responses, which are suppressed by prednisolone [42, 43].

4.4.1. Tissue culture preparation

HeLa cells (CLS catalog number 300194) and culturing media with other supplements were purchased from CLS Cell Lines Service GmbH, Deutschland. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) composing of 0.011% sodium pyruvate, 2 mM glutamine, 0.045% glucose, 100 Unit/ml penicillin and streptomycin and 10% foetal bovine serum in a 37◦C humidified incubator at 5% CO_2. Cells were passaged every 3-4 days. Prior to experiment, 80-90% confluence cell seeded in 24-well plates were washed with phosphate buffer saline and treated with TNF-α (25 ng/mL) to produce the highest possible inflammatory responses in the HeLa cells with minimal cell death at the same time [16]. The cell culture was allowed to incubate and treated later (after 4 and 12 h of cells spiking by TNF-α) with PDN loaded on CNTs in a concentration of 10 µg/mL suspended in complete culture medium. Other wells were treated with free drug or unloaded CNTs at the same time intervals for comparison. The culture supernatants were collected and then stored at -20°C and tested later for inflammation levels using the inflammatory marker, interleukin-1.

4.5. Biochemical examination of Interleukin 1 (IL-1)

The inflammatory status was estimated biochemically by measuring interleukin-1 levels using interleukin-1 ELISA kit, employing the double-sandwich ELISA technique. The kit was purchased from MyBioSource Co. / San Diego, USA. The test was performed based on the manufacturer instruction supplied with the kit. Monoclonal antibody acts as the pre-coated antibody, whereas the detecting antibody is represented by polyclonal antibody with biotin labeling. ELISA plates were washed with phosphate buffer saline and standard solutions and samples are added to wells for biotin labeling. The TMB substrate is added to color the ELISA wells, after an orderly addition of avidin-peroxidase conjugates, and the reactants were fully removed by washing. TMB turns blue when catalyzed by peroxidase, and then it turns yellow under the influence of acid. Color depth and sample testing variables have a positive correlation which can be measured at 450 nm wavelength. The supplied standard solution was used to produce a series of standard dilutions (1000, 500, 250, 125, 62.5, 31.2 and 15.6 pg/ml), which were measured and used to generate a standard curve (absorbance versus concentration) to be used later for sample measurements.

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Author contributions: S.A., A.H., and D.M. have designedthe study, collected, processed and analyzed data, and reviewed the manuscript. S.A. and D.M. have writtin and edited the manuscript. D.M. has supervised the study.

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