In *Vitro* Evaluation of Nanochitosan Derivatives and Streptomycin on RD Cell Line and *Leishmania Tropica*

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Abstract

The study aimed to evaluate *in vitro* the efficiency of the nano-encapsulated streptomycin within carboxymethylnanochitosan synthesized by an acid-base method on Rhabdomyosarcoma RD cell line and *Leishmania tropica*. Results showed that the concentrations 100 and 50 µg/ml of encapsulated streptomycin were more effective than 50 and 100 µg/ml of streptomycin alone and inhibited the growth of RD cells to 56 and 35 vs. 19.45 and 23% respectively after 24 hrs of incubation. Carboxymethylnanochitosan at a concentration of 50 µg/ml has the anticancer ability since it inhibited the growth of RD cells up to 20% in comparison with 19% for streptomycin at the same concentration. The growth inhibition of *L. tropica* in the presence of streptomycin, carboxymethylnanochitosan and streptomycin encapsulated within carboxymethylnanochitosan at 100 µg/ml for each was 34.4, 36 and 29.5% respectively. Carboxymethylnanochitosan is then considered the most effective inhibitor of *L. tropica* growth. [DOI: 10.22401/JNUS.20.4.12]

Keywords: Carboxymethylnanochitosan, Encapsulation, RD cell line, *Leishmania tropica*, Growth Inhibition%.

Introduction

The efficiency of many drugs is limited by their prospect to reach the site of action because of different problems like poor bioavailability, solubility, *in vivo* stability, intestinal absorption, sustained and targeted delivery to the site of action, side effects, therapeutic competence, and fluctuations of drug concentration in plasma which either, drop below the minimum efficacious concentrations or surpass the safe therapeutic concentrations. Mostly a little amount of drug reaches the target site, on the other hand, most of the administered dose distributes throughout the body in accordance with its biological and physicochemical properties [1]. Streptomycin (S) is a water-soluble aminoglycoside derived from *Streptomyces griseus* and it is commonly referred as sulfate salt of streptomycin. This antibiotic was first isolated in 1943 by Albert Schatz. S binds to the 30S ribosome and blocks protein synthesis. Streptomycin also slows down protein synthesis that has already initiated and induces misreading of the mRNA. Streptomycin is used in treating acute infections caused by different types of bacterial strains, such as infections of the respiratory tract (Pneumonia, Bronchitis and Laryngopharyngitis) and infections of the urinary tract (UTI) [2]. Many of water-soluble derivatives were prepared from chitosan. One of these derivatives is carboxymethyl chitosan (CMC) which has been widely studied because it is simple to synthesize and it is potential of wide of applications [3]. The properties and applications of CMC are relied on its structural characteristics and synthesis, mainly the percentage of substitution and amine location or OH-groups of the carboxymethylation [4]. The active agents can be carried on the surfaces of nanocapsules or embedded in the polymeric membrane [5]. Therapy-loaded polymeric nanocapsules have extended possible applications in the drug delivery systems recently [6]. Polymeric nanocapsules that are a specific kind of polymeric nanoparticles are used to improve biological effects [7]. RD cell line: Rhabdomyosarcoma (RMS) is a malignancy, which arises from skeletal muscle precursors [8]. In children and adolescents less than 20 years old, it is considered the most common kind of soft tissue sarcoma [9]. One of the most common cell lines used in RMS research are RD cells, that can be obtained from ATCC and are grown in Eagle's medium with (10%) FBS [9].

The MTT assay is a colorimetric assay to assess the cell metabolic activity. NAD (P) H-dependent cellular oxidoreductase enzymes may under specific conditions, reflect a number of viable cells that are present. [10]. Therefore, this study was carried out to investigate the efficacy and effect of nanocarboxymethyl nanochitosan as an anti-cancer and antileishmania when carried with streptomycin, compared to antibiotic alone**.** Different concentrations 50 and 100 µg/ml of CMNC-S, streptomycin alone and CMNC were prepared and the inhibitory effect was tested on RD cell line and *L. tropica*.

Materials and Methods

Streptomycin: powder was purchased from a local pharmacy. Two concentrations (50 and 100 µg/ml) were prepared in sterile deionized water.

Preparation of CMNC [11]: Nanochitosan (3g) was dissolved in 65 ml of isopropanol. After stirring for 20 minutes at room temperature, 20.4 g of 40% aqueous NaOH and 14.4 g of monochloroacetic acid/ isopropanol solution (1:1 v/v) were melted together and added to the suspension. The reaction continued at room temperature for 24 hrs. The product was centrifuged at 14000 rpm for 10 minutes using a high-speed cooling centrifuge and suspended in 150 ml of methanol then, neutralized by glacial acetic acid. Finally, the product was washed with 80% ethanol and dried at room temperature.

Preparation of CMNC-S: CMNC-S was prepared by mixing 14.4 g of monochloroacetic acid/ isopropanol solution (1:1 v/v), 20.4 g of 40% aqueous NaOH with Nanochitosan-Isopropanol solution. Then streptomycin at a ratio 1:5 was added and mixed well for 24 hrs at room temperature. The solution was centrifuged at 14000 rpm for 10 minutes using a high-speed cooling centrifuge. The supernatant was discarded and the deposit was suspended with methanol and then neutralized with 10% glacial acetic acid. Finally, the deposit was washed with ethanol (80%) and dried at room temperature.

***In Vitro* Anticancer Activity:** The anticancer efficacy of S, CMNC and CMNC-S against RD cell line was evaluated. The colorimetric cell viability MTT assay was used as described by [12, 13]. Aliquot of 100µl of RD cells (10^6 cell/ml) was dispensed using 96-well tissue culture plates. Two concentrations of S, CMNC, CMNC-S (50 and 100 µg/ml) were prepared by dissolving in sterile distilled Water (DW). Aliquots of 100 µl of various concentrations were added to each well and incubated for 24 hrs at 37◦C. After that, 10 µl of MTT solution (5mg/ml) was added to the wells and incubated for 4 hrs at 37◦C. Then, 50 µl of dimethyl sulfoxide (DMSO) was added to the wells and incubated for 10 minutes. RD cells were cultured in the medium without S, CMNC and CMNC-S as a positive control (AC), and the medium without cells and test solutions as a blank. The absorbance of each well was read at 620 nm by using an ELISA reader. The inhibition percentage was calculated according to the following equation:

Inhibition (%) = (AC – AS / AC) x 100

Where (AC) and (AS) are the optical density for positive control and tested samples respectively.

***In Vitro* antileishmanial activity:** The antileishmanial efficiency of S, CMNC and CMNCS against promastigote forms of *L. tropica* was assayed. The colorimetric cell viability MTT assay was used as follows [14]:

L. tropica promastigotes were cultured in 96-well tissue culture plate100 µL well (10^6 parasite ml^{-1}), then a 100 µL of various concentrations of S, CMNC and CMNC-S test solutions were prepared (50 and 100 µg/ml) by dissolving in sterile distilled water and added to each well then incubated for 4 hrs at 26◦C. After that, 50 µL of (DMSO) was added to the wells and incubated for 10 minutes. Promastigotes were cultured in the medium without S, CMNC and CMNC-S as a positive control, and the medium without promastigotes and test solutions as a blank. Finally, the absorbance was read at 620 nm for each well by using an (ELISA) reader. Finally, the living promastigotes, the viability and inhibition percentage were calculated using the same equation that used for RD cell line.

Results and Discussion

Caroxymehtylnanochitosan (CMNC)

CMNC was prepared according to [11]. The steps included except that filtration were substituted with centrifugation at high speed under cooling to obtain a good yield. Because of the low solubility of chitosan or nanochitosan when pH is more than 6.5 and this represent a serious barrier in many of its possible applications and the CMNC is soluble in a wide range of pH mentioned by Fernanda and Campana-Filho (2005). The yield was 420 mg dry weight of CMNC from 3g of NC The supernatant of centrifugation was maintained for subsequent experiments.

Caroxymehtylnanochitosan loaded with Streptomycin

The ratio1:5 w/w of (CMN:S) was added to one portion of CMNC during the preparation of CMNC based on the dry weight of the CMNC produced by the method of preparation and after the addition of solvents ensuring the loading of the S within the structure of CMNC to obtain formula of encapsulation and surface attachment. The subsequent steps of preparing CMNC were followed. The supernatant of centrifugation was maintained for subsequent experiments of loading efficiency%.

In vitro inhibitory effect on RD cell line

Results showed that the inhibitory effect of CMNC-S at 50 µg/ml was significantly high (35.5±4.40)% followed by CMNC (20.55±2.65) and S (19.45±1.55) respectively. In addition, the inhibitory effect of CMNC-S at 100 µg/ml was 56.0 ± 1% followed by CMNC (16.02±5.06) and S (22.98±12.02) respectively. The Table (1) show that the best treatment was CMNC-S at concentration 100 µg/ml where the growth inhibition was 56%, compared with 23% of S at the same concentration. In addition to 50µg/ml of CMNC-S. Note that the loading efficiency was 90% means the real concentration 45 mg/ml. The growth inhibition for CMNC-S (100 µg/ml) approximated 50 µg/ml and this may due to the mechanism of the S release, which almost the same rate within only 24 hours of culture incubation. Therefore, prolonging the incubation time increases the percentage of inhibition based on the sentiments [14]. It was found that carboxymethylnanochitosan at the concentration 50 µg/ml has anticancer ability since it inhibited the growth of RD cells to 20% in comparison with 19% for streptomycin at the same concentration.

Table (1)
In vitro inhibitory effect of CMNC and CMNC-S on RD cell line.

Treatment (µg/ml)	% growth Inhibition (PGI % Mean ± SE)
S (50)	19.45 ± 1.55
S (100)	22.98 ± 12.02
CMNC (50)	20.55 ± 2.65
CMNC (100)	16.02 ±5.06
CMNC-S (50)	35.5± 4.40
CMNC-S (100)	**56 .0 ± 1**

****S:Streptomycin; CMNC: Carboxymethyl nanochitosan.***

In vitro inhibitory effect on *Leishmania tropica.*

The inhibitory effect CMC at 50 µg/ml was a significant (23.3±0.007)% followed by CMNC-S (24.8±0.012) and S (35.5 ±0.014) respectively. In addition, the inhibitory effect of CMNC at 100 µg/ml was significantly higher (36)% than others. Streptomycin and encapsulated streptomycin were (34.4 and 29.5)% respectively. Nanochitosan derivatives and streptomycin effect on the growth of *L. tropica* was exhibited in the Table (1). The growth inhibition of *L. tropica* in presence of S, CMNC and CMNC-S at 100 µg/ml for each was 34.4, 36 and 29.5% respectively. CMNC is then considered the most effective inhibitor on the growth of *L. tropica*.

Table (2)
In vitro inhibitory effect of nanochitosan derivatives and antibiotic on Leishmania tropica.

Treatment* (µg/ml)	% growth Inhibition (PGI % Mean ± SE)
S (50)	35.5 ±0.014
S (100)	34.4 ±0.009
CMNC (50)	23.3±0.007
CMNC (100)	36±0.004
CMNC-S (50)	24.8±0.012
CMNC-S (100)	29.5±0.002

****S:Streptomycin; CMNC: Carboxymethyl nanochitosan.***

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