ejpmr, 2018,5(6), 45-49

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH
www.ejpmr.com

Research Article
ISSN 2394-3211
EJPMR

EFFECT OF SHRIMP SHELL EXTRACT NANOPARTICLES ON ORAL SQUAMOUS CELL CARCINOMA CELL LINE: *IN VITRO* STUDY

[1]Dina Sabry Abd-El-Fattah, [2]Wael Sabry Mohammed and [3*]Marwa Mohammed Ellithy

[1]Professor of Biochemistry, Faculty of Medicine, Cairo University.
[2]Assisstant Professor at Polymers Department, National Research Centre.
[3]Researcher of Oral Pathology, Basic Dental Sciences Department, Nationl Research Centre.

***Corresponding Author: Marwa M. Ellithy**
Researcher of Oral Pathology, Basic Dental Sciences Department, Nationl Research Centre.

Article Received on 27/03/2018 **Article Revised on 17/04/2018** **Article Accepted on 07/05/2018**

ABSTRACT
To develop a shrimp shell extract based nanoparticles formulation and assess its effect on the viability and carcinogenic potential of human oral squamous cell carcinoma cell line Hep-2 in vitro. Nanoparticles of chitosan were prepared. Characterization was based on particle size, zeta potential, and morphology. Various concentrations of chitosan nanoparticles (0.3- 0.6- 1- 1.5 mg/ml) were used intreating cells for 48 hours then cell viability was assessed to calculate the IC50. VEGF and TNF-α were measured in plates of cells treated with Chitosan nanoparticles in its IC50 concentration. MTT assay results showed that chitosan nanoparticles exhibited concentration dependant cytotoxicity on the Hep-2 cell line. Chitosan nanoparticles are cytotoxic to Hep-2 cell line in-vitro and decreases the angiogenic marker VEGF and TNF-α in a dose dependant way. So, shrimp shell extract nanoparticles could be used as a natural agent in oral cancer therapy.

KEYWORDS: Shrimp shell, Chitosan, nanoparticles; oral cancer; angiogenesis, TNF-α.

INTRODUCTION
Shrimp shell waste is widely known as an abundant source for chitin biopolymers. After deproteinization in 10% NaOH and deacetylation in 40% NaOH, we get chitosan which is more stable and widely used in different industries as well as in medical purposes for its known biodegrability, non-toxicity and abundancy of health benefits (Qin et al, 2002). Chitosan helps in lowering levels of serum cholesterol. It has antioxidant properties, has also antibacterial and antiheminthic power (Zheng, 2003)In the latest years, the antitumoral effect of chitosan has been proved either by its direct effect on tumor cells or indirectly by acting as a biocarrier for many anticarcinogenic drugs.

Cancer therapy is still till now one of the fields where medicine has to be improved to achieve higher efficacy and lower side effects. In conjunction with surgery and radiotherapy, antineoplastic chemotherapy is a standard regimen used in the treatment of squamous cell carcinoma of the head & neck (HNSCC)(Janes, 2001).

Nanotechnology opens new era in targeting cancer cells with the least amount and concentration of drugs so decreasing the harmful effect of these chemotherapeutic agents on normal cells neighbouring the treated field (Pan et al, 2002-Xu and Du, 2003).

Researchers are now occupied by finding natural products that might have the same cytotoxicity and anticarcinogenicity as the usually used chemotherapeutic agents (Janes, 2001).

Chitosan has been used since years as a drug delivery for a long series of chemotheraprutic agents including herceptin, 5-fluorouracil, doxorubicin and methotrexate. But its own anticancer effect hasn't been elucidated clearly yet(Pan et al, 2002-Xu and Du, 2003).

Carcinogenesis is a multistep process and develops through many levels. Targeting any step may break the cycle of tumor formation and inhibit progrssion of this fatal disease. Researchers till now are seeking for a drug that can have an effect on several steps of cancer formation (Qi etal, 2004-2005).

Angiogenesis is a physiological, normal mechanism that involves the formation of new blood vessels from already pre-existing vasculature. It is an important step not only as a physiological process but also in different diseases mainly in cancer development. VEGF is a key regulator of cancer angiogenesis and overexpression of VEGF helps tumors to grow better and to metastasize. This ensures declination of the clinical outcomes of cancer patients. So, targeting VEGF by inhibitor molecules is a promising era in cancer therapy (Qi, 2005).

Tumor necrosis factor-α (TNF-α) secreted by macrophages plays a crucial role in both the development and progression of cancer. Lowering TNF-α levels means that tumor cells are in their way to suicide losing their invasive properties (Qi etal, 2004-2005).

In the present study, we assess the antitumor effect of chitosan nanoparticles on human oral squamous cell carcinoma cell line in an attempt to develop a natural, cheap, safe agent that may be used alone or combined in ancer treatment plan. Cell viability, VEGF and TNF-α will be evaluated before and after CNP addition.

MATERIALS

- Primary culture of laryngeal squamous cell carcinoma (Cancer research institute, Cairo, Egypt).
- DMEM routine culture media.
- MTT assay kit, VEGF elisa kit, TNF-α kit, microRNA-21 kit (Sigma Aldrich Chemical co., St.Louis, U.S.A.)

METHODOLOGY

Nanoparticles preparation

This work was performed in the polymer and pigment department at the National Research Centre. Chitosan nanoparticles were prepared by using ionic gelation method as reported by Rafeeq et al. Different weight of chitosan was dissolved in 2% (v/v) of glacial acetic acid to form chitosan solution. Sodium tripolyphosphate (TPP) (1 %(w/v)) was used as an ionic cross linker. Chitosan nanoparticles were obtained upon the addition of 1mL of TPP into 10mL of chitosan solution under sonication at room temperature for 1 hour using 300 wt sonication doses.

Characterization of chitosan nanoparticles

The morphology and particle size of the prepared chitosan nanoparticles were examined using Transmission electron microscope (with JEOL Model JSM-T20 TEM).

Zeta potential measurement

The electrophoresis mobility (μe) of the prepared chitosan nanoparticles (0.6 mg/ml) dissolved in acetic acid in different pH values (pH 2:6) was using the Zetasizer from Malvern Instruments (3000-HS model) in 37oC. The zeta potential (ζ) was calculated from the electrophoresis mobility using the Smoluchowski's equation.

$\zeta = (\eta / \varepsilon) \times \mu e$

Where, η is the viscosity and ε the permittivity of the medium

For zeta potential measurement, sodium chloride solution of 10-3 mol/l was used.

Cell line culture and IC 50 calculation (minimum inhibitory concentration)

This work was done in the stem cells research unit at the National Research Centre. Human laryngeal squamous cell carcinoma primary culture was used in this work. Cells were plated in96 multiwell plate at a density of 10^4 cells/well. for 24 h.before treatment with chitosan to allow attachment of cells to the wall of the plate. Different concentrations of chitosan in nanoparticles formulation (0.3 - 0.6- 1-1.5 mg/ml) were added to the cell monolayer. Plates were incubated with chitosan for 48 h. at 37ºC, in 5% co_2 atmospheric pressure. After that, cells were fixed, washed and cell viability was assessed by the tetrazolium dye assay. Color intensity was measured with ELISA reader and the relation between surviving fraction and drug concentration was plotted to get a survival curve. Ic 50 was calculated and was 0.6 mg/ml.

Elisa for VEGF

Vascular endothelial growth factor (VEGF) was assessed to evaluate the ability of cancer cells for angiogenesis (pg/ml). Human VEGF ELISA kit (WUHAN HUAMEI BIOTECH CO., LTD, Donghu, China) was used according to manual instructions. The supernatant of cultured cell was collected in each group to measure VEGF presence using ELISA reader.

Real Time PCR

TNF-α

For quantitative expression of TNF-α; the following procedure was assessed. 10 ng of the total RNA from each sample were used for cDNA synthesis by reverse transcription using High capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA). The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (Fermentas) in a 48-well plate using the Step One instrument (Applied Biosystem, USA) as follows: 10 minutes at 95 ºC for enzyme activation followed by 40 cycles of 15 seconds at 95ºC, 20 seconds at 55 ºC and 30 second at 72 ºC for the amplification step. Changes in the expression of target gene was normalized relative to the mean critical threshold (CT) values of β-actin as housekeeping gene by the ΔΔCt method. We used 1 μM of both primers specific for each target gene. Primer sequence specific for TNF-α was forward primer (5' ATCCGGGACGTGGAGCTG 3') and reverse primer 5' AAAGTAGACCTGCCCAGAC 3') (Gene Bank accession number NM_000594.3) and for β-actin was forward primer (5' GGC GGCACCACCATGTACCCT 3') and reverse primer (5' AGG GGCCGGACTCGTCATACT 3') (Gene Bank accession number NM_001101.3).

MiR-21

MiRNAs were extracted from cells with the miRNeasy extraction kit according to the manufacturer`s directions (Qiagen, Valencia, CA, USA). The miRNAs were converted into cDNA with the TaqMan micro-RNA kit (Applied Biosystems) according to the manufacturer's directions. The cDNA generated from the RT reaction was amplified using the TaqMan PCR Master Mix kit (Applied Biosystems), and a Step One System (version 2.1, Applied Biosystem) was used for the reactions. To determine the threshold cycle (CT) value from the amplification plot, RNU6B (Applied Biosystems) was

used as the endogenous reference control gene for normalization control. The cDNA was amplified using FAM/MGB probe (5' FAM-TCGGGCCTGGTTAGT MGB 3'), forward primer (5' CGTCTGATCTCGGAAGCTAAGC-3'), and reverse primer (5' GGCGGTCTCCCATCCAA 3') (Gene Bank accession number NG043905.1). The qRT-PCR reaction mix per well consisted of 5 µL nuclease-free water, 0.5 µL TaqMan microRNA Assay, 6.5 µL TaqMan Universal Master Mix, and 3 µL cDNA. The RT-PCR instrument was programmed as follows: denaturation (95°C, 10 min) followed by 40 cycles of denaturation (95°C for 15 sec) and annealing/ extension (60°C for 60 sec).

Statistical assessment

Data were coded and entered using the statistical package SPSS version 21. Data was summarized using mean ± standard deviation for quantitative variables. Comparisons between groups were done using analysis of variance (ANOVA). P-values less than 0.05 were considered as statistically significant.

RESULTS

Size and Morphology of chitosan nanoparticles

The average particle size of chitosan nanoparticles was 46 nm. From the figure, it is clear that, there is a good dispersion of chitosan nanoparticles in the acetic acid solution and the particle size of the prepared nanoparticles ranged from 31 to 49 nm. A solid dense structure and a round shape of chitosan nanoparticles were shown under TEM (fig.1-2).

Zeta potential result

Zeta potential (ζ) for the prepared chitosan nanoparticle with concentration 0.6 mg/ml in different acidic pH values was measured and data illustrated in figure-3. From the figure, it is clear that all the zeta potential were found in positive charge values where the positive charged of chitosan is due to the presence of amino group which protonated in acidic medium and give a net positive charge. The maximum zeta potential value was 42 mV in pH 3.

MTT assay result

Chitosan nanoparticles showed concentration dependant cytotoxicity on the Hep-2 cell line with an IC 50 of 0.6 mg/ml (600µ/ml) (fig.4).

ELISA for VEGF, TNF-α evaluation and micRNA-21 assessment results

In all tests, there is a significant difference between the chitosan group used in its IC50 and the control group using routine culture medium ($P < 0.05$). The chitosan, when used, decreases the expression of VEGF, TNF-α and micRNA-21 which are all biological markers for squamous cell carcinoma cell line (fig.5) (table-1).

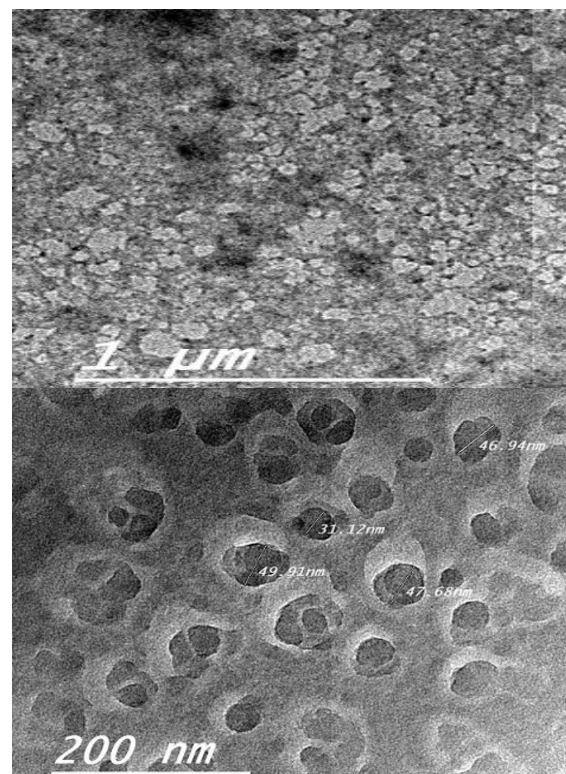

Fig 1-2: Showing characterization of chitosan nanoparticles.

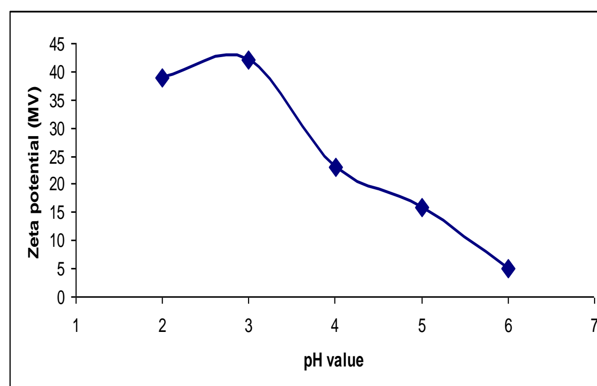

Fig. 3: Showing zeta potential measurement of chitosan nanoparticles.

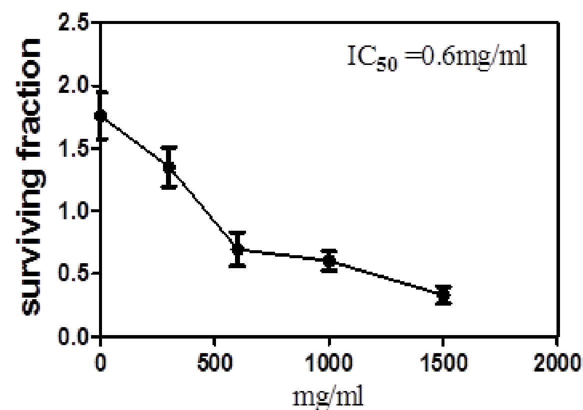

Fig. 4: Showing Ic 50 calculation of chitosan nanoparticles.

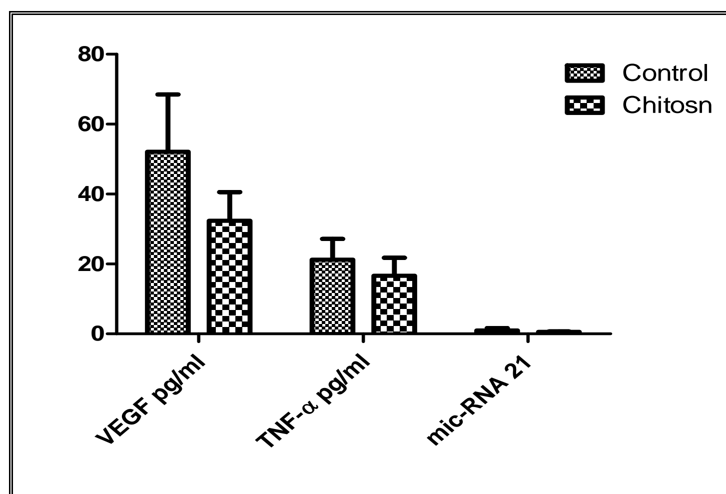

Fig. 5: Showing ELISA for VEGF, TNF-α evaluation and micRNA-21 assessment results.

Table-1: Showing ELISA for VEGF, TNF-α evaluation and micRNA-21 assessment results.

	VEGF pg/ml		TNF-α pg/ml		mic-RNA 21	
	Control	**Chitosn**	**Control**	**Chitosn**	**Control**	**chitosn**
Mean	52.11	32.37	21.23	16.66	0.9525	0.5821
SD	16.42	8.217	5.957	5.110	0.7025	0.1414
T	5.728		2.671		2.368	
P	< 0.0001		0.0109		0.0228	

*$p<0.05$, **$p<0.01$ compared with the control.

DISCUSSION

Chitosan has been used in drug delivery systems for preparing micro or nano spheres used to encapsulate enzymes, drugs, proteins and DNA because of its well known biocompatibility and biodegradability(Kim etal, 2004)(Mao etal, 2001).

It has been demonstrated that nanoparticles of chitosan have an effective anticancer activity either in vitro or in vivo through many researches(Qi etal , 2005). However, the proper mechanisms that may be responsible for their potential anticancer activity haven't been revealed yet . In this research, the effects of chitosan nanoparticles on oral squamous cell carcinoma cell line has been studied. CNP were able to hit several steps of carcinogenesis through inhibition of angiogenesis, TNF-α and micRNA-21. All the previous parameters are well known to direct cancer formation and to increase cancer agresiveness.

Due to its biological activities, we can use chitosan alone or combined with other drugs.. Carreno-Gomez showed that soluble chitosan and chitosan microspheres are cytotoxic in some way towards B16F10, the murine melanoma cell line and that amng many members of its family, chitosan hydrochloride has the highest cytotoxicity with an IC50 of 0.21±0.04 mg/mL(Gomez,1997). QI et al proved in their work that nanoparticles of chitosan are highly cytotoxic against MGC803, human gastric carcinoma cell line. MGC803 cells were sensitive towards CNP with an IC50 value of 5.3 µg/mL after 48-h treatment which means that CNP can be a good candidate as anticancer drugs (Qi etal, 2005).

Lee et al demonstrated that amino-derivatized cationic chitosan derivatives show an inhibitory effect on the proliferation of many tumor cell lines in a dose dependant way and that the lowest IC50 was 22±4 µg/mL towards liver cancer(Lee etal, 2002).

Polymers that have a high cationic charge densities proved to be highly cytotoxic than those that have a lower charge densities (Fischer etal, 2003). Chitosan was used in this work as an example of cationic polymers. Its surface charge is the major factor affecting the cytotoxic activity due to the electrostatic ionic interaction between the negatively charged groups of tumor cells and the positively charged amino groups of chitosans. So, the high surface charge about 42 mV of chitosan nanoparticles is responsible for its higher cytotoxic potential.

Harish and Tharanathan showed that chitosan inhibited angiogenesis in vitro(Harish etal, 2005) In the same way YINGLEI et al proved the antiangiogenic effect of chitosan in vivo in a xenograft of nude mice by BEL-7402, human hepatocellular carcinoma (HCC)(Yinglei etal, 2009)

CONCLUSION

CNP are cytotoxic to Hep-2 cell line *in vitro* and also inhibit the angiogenic marker VEGF and TNFα which is a proinflammatory cytokine favouring carcinogenesis in a dose dependant manner. So, Further preclinical studies are needed to enable shrimp shell extract nanoparticles to be used as a natural anticarcinogenic agent sparing the

deleterious and hazardous side effects of chemotherapeutic agents as well as radiotherapy used till our day in treating oral cancer.

ACKNOWLEDMENTS
This work was supported by the National Research Centre, Egypt.

REFERENCES

1. Bivas-Benita M, Laloup M, Versteyhe S, Dewit J, Braekeleer JD, Jongert E, Borchard G. Generation of Toxoplasma gondiiGRA1 protein and DNA vaccine loaded chitosan particles:
2. preparation, characterization, and preliminary in vivo studies.Int J Pharm, 2003; 266: 17-27.
3. Carreno-Gomez B, Duncan R. Evaluation of the biologica properties of soluble chitosan and chitosan microspheres. IntJ Pharm, 1997; 148: 231-240.
4. Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T. In vitro cytotoxicity testing of polycations: influence of polymer structureon cell viability and hemolysis. Biomaterials, 2003; 24: 1121-1131.
5. Harish Prashanth KV and Tharanathan RN: Depolymerized products of chitosan as potent inhibitors of tumor-induced angiogenesis. Biochim Biophys Acta, 1722: 22-29: 2005.
6. Janes KA, Fresneau MP, Marazuela A, Fabra A, Alonso MJ. Chitosan nanoparticles as delivery systems for doxorubicin. J Control Release, 2001; 73: 255-267.
7. Kim TH, Park IK, Nah JW, Choi YJ, Cho CS. Galactosylatedchitosan/DNA nanoparticles prepared using water-soluble chitosan as a gene carrier. Biomaterials., 2004; 25: 3783-3792.
8. Lee JK, Lim HS, Kim JH. Cytotoxic activity of amino derivatized cationic chitosan derivatives. Bioorg Med Chem Lett. 2002; 12: 2949-2951.
9. Mi FL, Tan YC, Liang HF, Sung HW. In vivo biocompatibilityand degradability of a novel injectable-chitosan-based implant. Biomaterials, 2002; 23: 181-191.
10. Mao HQ, Roy K, Troung-Le VL, Janes KA, Lin KY, Wang Y, August JT, Leong KW. Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J Control Release, 2001; 70: 399-421.
11. Pan Y, Li YJ, Zhao HY, Zheng JM, Xu H, Wei G, Hao JS, Cui FD. Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo. Int J Pharm, 2002; 249: 139-147.
12. Qin C, Du Y, Xiao L, Li Z, Gao X. Enzymic preparation of water-soluble chitosan and their antitumor activity. Int J BiolMacromol, 2002; 31: 111-117.
13. Qi L, Xu Z, Jiang X, Hu C, Zou X. Preparation and antibacterial activity of chitosan nanoparticles. Carbohydr Res, 2004; 339: 2693-2700.
14. Qi LF, Xu ZR. Lead sorption from aqueous solutions on chitosan nanoparticles. Colloid Surface A, 2004; 251: 183-190.
15. Qi L, Xu Z, Jiang X, Li Y, Wang M. Cytotoxic activities of chitosan nanoparticles and copper-loaded nanoparticles.Bioorg Med Chem Lett, 2005; 15: 1397-1399.
16. Qi L., Xu Z., Li Y., Jiang X. and Han X. In vitro effects of chitosan nanoparticles on proliferation of human gastric carcinoma cell line MGC803 cells. World J Gastroenterol, 2005;11(33): 5136-5141.
17. Roller S, Covill N. The antifungal properties of chitosan in laboratory media and apple juice. Int J Food Microbiol, 1999; 47: 67-77.
18. Truter EJ, Santos AS, Els WJ. Assessment of the antitumor activity of targeted immunospecific albumin microspheres loaded with cisplatin and 5-fluorouracil: toxicity against a rodent ovarian carcinoma in vitro. Cell Biol Int, 2001; 25:51-59.
19. Xu Y, Du Y. Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. Int J Pharm, 2003; 250: 215-226.
20. Yinglei X., Zhengshun W. and Zirong X.: Chitosan Nanoparticles Inhibit the Growth of Human Hepatocellular Carcinoma Xenografts through an Antiangiogenic Mechanism. Anticancer Research, 2009; 5103-5109.
21. Zheng LY, Zhu JF. Study on antimicrobial activity of chitosan withdifferent molecular weights. Carbohyd Polym, 2003; 54: 527-530.