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Therapeutic Efficacy of Orally Delivered Doxorubicin Nanoparticles in Rat Tongue Cancer Induced by 4-Nitroquinoline 1-Oxide

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Abstract

Purpose: Oral cancer is one of the most significant cancers in the world, and squamous cell carcinoma makes up about 94% of oral malignancies. The aim of the present study was to compare the efficacy of doxorubicin plus methotrexate - loaded nanoparticles on tongue squamous cell carcinoma induced by 4NQO and compare it with the commercial doxorubicin and methotrexate delivered orally on seventy SD male rats.

Methods: 70 rats were divided into five groups. During the study, the animals were weighed by a digital scale once a week. Number of mortalities was recorded in the data collection forms. At the end of the treatment, biopsy samples were taken from rat tongues in order to evaluate the severity of dysplasia and the extent of cell proliferation. The results were analyzed using ANOVA, descriptive statistics and chi-square test.

Results: No statistically significant difference was found in the mean weight of five groups (p>0.05). No significant relationship was found between groups and mortality rate (P = 0.39). In addition, there was a significant relationship between groups and the degree of dysplasia (P <0.001). The statistical analysis showed a significant relationship between groups and the rate of cell proliferation (p <0.001).

Conclusion: The results of the present study showed that the use of doxorubicin plus methotrexate - loaded nanoparticles orally had more therapeutic effects than commercial doxorubicin plus methotrexate.

Introduction

Head and neck tumors include a major group of neoplasms in worldwide, and are on the rise in many parts of the world. The most common of this disease, is cancer of the mouth approximately 90% are squamous cell carcinoma (SCC).[1] Based on the reports of the World Health Organization, oral cavity carcinoma is the sixth most common cancer after lung, prostate, colorectal, stomach and bladder cancers in men and is the tenth most common, colorectal, cancer in women.[1] Most of the invasive oral carcinomas originate from altered oral pre-cancerous lesions and have a likelihood of progressing to SCC. Tumor progression in epithelial cells is classified into normal epithelium, hyperplasic (non-dysplastic), carcinoma in situ and invasive carcinomas.[1]

Squamous cell carcinoma is a multi-factorial disease. These factors are divided into two groups of internal and external factors. External factors, which include; smoking, alcoholism, syphilis and solar radiation (Vermilion lip cancer), and the internal factors, include systemic or the generalized conditions such as general malnutrition or iron deficiency anemia.[2,3] Developing

this type of cancer results in changes of the patient's appearance, decreased quality of life, and most importantly decreased eating ability.[4]

Various treatments have been carried out on squamous cell carcinoma. The standard treatments for this disease include surgery, chemotherapy and radiation, each of which has its own complications. The side effects of chemotherapy include soreness of the mouth, throat, weight loss, thrombocytopenia, vomiting, anorexia, diarrhea, and constipation. The surgical patients in the more advanced stages of this disease may face dysphagia and dysphonia, and most likely deformity of the face.[5] The side effects of radiation therapy include, dry mouth, sensitive teeth/ mucosa with extensive dental caries and dysphagia.[6]

Doxorubicin is one of the most effective anti-cancer drugs. It is prescribed often in combination with other anti-cancer drugs for the treatment of the various cancers. Doxorubicin is a subgroup of anthracycline, which is a branch of antibiotics produced by Streptomyces Peucetius and a variety Cassius.[7]

Anthracycline, doxorubicin, daunorubicin, adriamycin and epirubicin are antineoplastic antibiotics with a wide range of clinical applications, doxorubicin acts against a number of tumors, such as sarcomas and carcinomas. This drug is also used in hematological malignancies (leukemia, lymphoma and multiple myeloma).[7] However, the commercial form of this drug has severe side effects, one of which is cardiac toxicity. Hence, the use of doxorubicin for treatment of cancer patients has some limitations.[8,9] Much efforts has been taken to solve this problem and reduce the adverse effects, among which is its confinement within the nanoparticle systems. One of the goals of nanotechnology is (DNA-based nanobots) to target cancer cells, this is accomplished by the molecular delivery of drugs that trigger cell suicide in leukemia and lymphoma cells.[10] Nanoparticles include different types of colloidal systems with a submicron scale (smaller than one micrometer). They may be inorganic, liposomal or polymeric. One of the main advantages of nanoparticles is their dwarfed size, which makes it easy to bypass the biological membranes and deliver the drugs directly to the nuclei of cancer cells. Another advantage is the high density of the pharmaceutical agent that can be used to achieve different drug release characteristics.[11]

Because of the high level of nanoparticles, these particles are capable of carrying a payload of multiple drug particles directly to the nuclei of the cancerous cell. The nanoparticles are also capable of penetrating deep into the tumor tissue, thus delaying the detection by the immune cells, and reaching the targeted cells. The particles enter into cancerous tumors in large numbers, and form a homogenous fluid with low viscosity and remain in the tumor tissue for an extended period. In view of the fact that the cancerous cell mediums have an acidic pH of 4-5.8, the controlled delivery systems for the treatment of cancer should be designed in such a way that prevents drug release at a physiologic pH, and/or minimizes the release, with the maximum delivery of the drug released in the acidic environment. Since the ideal controlled delivery systems for the treatment of cancer contain a maximum release in acidic environments, the delivery system used is injection or implants depending on the type of medication prescribed; moreover, this delivery system is necessary to prevent the toxic effects of these drugs in an oral form.[12]

The aim of this study was to assess the efficacy of doxorubicin plus methotrexate - loaded nanoparticles (in a soluble form and loaded on polymeric nanoparticles) on the tongue squamous cell carcinoma. The SCC was induced by 4-Nitroquinoline 1-Oxide(4NQO) and compare with the commercial doxorubicin and methotrexate delivered orally on seventy Sprague Dawley (SD) male rats in the laboratory animals section of Drug Applied Research Center and School of Dentistry, Tabriz University of MedicalSciences.

Materials and Methods
Studied Animals
In this experimental study, 70 (5 groups of 14 each) SD male rats were selected with the following features: Age range of 2.5- 3 months, an approximate weight of 150 ± 50g. The rats were kept at 22 ± 2 °C and 12 hours light cycle, 60 ± 0.5 percent ambient humidity and available food conditions.

Chemicals
The powder 4-nitroquinoline1-oxide (4NQO) was purchased from Sigma (Poole, UK) to induce carcinoma of tongue.[13-15] The 500 ml of 4NQO solution 30 ppm was regularly prepared for each cage three times per-week (every other day for 14 weeks). The solution preparation was 15 mg of 4NQO weighed by a digital scale, and dissolved in 498.5ml of water, bottled and covered with foil to protect it from light exposure.[13-15]

Anticancer drug loaded nanoparticles
Nanoparticle synthesis was studied and discussed by Salehi et al, briefly, an appropriate amount of synthesized nanoparticles was stirred in a solution of methotrexate by ultrasonic device for 5 minutes. After stirring the mixture (methotrexate-loaded nanoparticles), it was placed in a dark condition, next doxorubicin hydrochloride was added to the mixture of methotrexate and loaded with nanoparticles by ultrasonic device for 3 minutes (Sonics Vibra cell, Model: VCX 130PB, Newton, CT). The final ratio of carrier/drug was 5 to 1 for both drugs. This mixture was kept in a magnetic stirrer at room temperature for 24 hours in a dark condition; until the Methotrexate-doxorubicin was loaded nanoparticles remained for 2 hours to allow the deposition. The methotrexate-doxorubicin loaded nanoparticles were centrifuged at 14,000 rpm for 15 minutes. Subsequently, it was dried for 24 hours at room temperature and stored in the container until further use by the study.[16]

The doxorubicin were loaded with anti-cancer drugs were diluted with a physiologic saline solution to the appropriate concentration before use on the animals.

Experimental design
The seventy rats were divided into 5 groups of 14. The Table 1 shows the studied groups briefly.
Group N: the control-group, were fed a normal diet and no intervention was performed.
Group K: the carcinoma control group, received 4-NQO at the concentration of 30 ppm in their drinking water for 14 weeks without any treatment.
Group F: the nanodrug control group, received 6mg the doxorubicin plus methotrexate-loaded nanoparticles once a day on days of 2, 5 and 8 of the study in order to evaluate the probable side effects of the drug.
Groups E and D were the treatment groups and received 4-NQO at the concentration of 30 ppm in their drinking water for 14 weeks and then they also orally received commercial doxorubicin (made by a pharmaceutical

company, Italy) and the DOX-MTX-loaded nanoparticles respectively once a day on days of 2, 5 and 8 of the study.

Accordingly, groups D and E were divided into two groups of 7 rats:

Group D1 and D2: Received 1.5 and 6 mg doxorubicin plus methotrexate-loaded nanoparticles, respectively.

Group E1 and E2: Received 1.5 and 6 mg commercial doxorubicin plus methotrexate, respectively.

During the study, the animals were weighed by digital scale within an accuracy of 1g once a week, and recorded in grams on the data collection form.

The rats in each group were counted and the number of mortalities was recorded in the data collection forms daily, and evaluated with consideration of their relationship to their groups and the other groups.

Table 1. The studied groups

Groups	4NQO	Drug
N	-	-
K	×	-
F	-	×(Nanodrug)
D	×	×(Nanodrug)
E	×	×(commercial drug)

-: The groups that didn't receive 4NQO or any drugs
×: The groups that received 4NQO or any drugs

Histopathologiocal evaluation

Following treatment for three month with the doxorubicin plus methotrexate, the rats were anesthetized with ether by use of the anesthetic chamber and tongue biopsies were taken from the groups-N, K, D and E.[17] The samples were fixed in 10% formalin for 24 hours, and paraffin blocks were prepared from the samples. Next, sections of 5-micron thickness were prepared for staining with hematoxylin and eosin, the prepared samples were studied by both pathologists using light microscope, Olympus BX50F4 with a magnification of ×400. Every morphological changes of the epithelium including enlarged nuclei and cells, increased nuclear to cytoplasmic ratio, pleomorphic nuclei and cells, dyskeratosis and increased mitotic activity were scaled.

For evaluation of the malignant changes in each sample, the changes of the epithelium were classified as follows: no lesion, mild dysplasia, moderate dysplasia, severe dysplasia and squamous cell carcinoma. Ki-67 was used as an immunohistochemistry marker to study the cellular proliferation. The 4-micron sections were prepared from paraffin blocks using the specific assay kit for staining, Ki67 (monoclonal antibody Ki-67 protein; DO-7 Novocastra, Anti-mouse) in groups N, K, D and E. Immunostaining for Ki-67 was performed according to the manufacturer's instructions (Dako, Denmark). Ki-67 precipitate on the cellular nuclei renders them a brown color. Therefore, the sections were studied by both pathologists using a light microscope, OlymposBX50 F4, made in Japan, with a magnification of ×400. It should be noted that assessing and evaluating Ki-67 marker

begin with counting the total number of cells in the microscopic field with a magnification of × 400 and the number of cells, which had stained nuclei, were counted and was expressed as a percentage of the total cells in the field. Cells that were associated with the expression of Ki-67 create brown insoluble sediments; Ki-67 transforms the nuclei brown. Therefore, they were separately counted in10 fields, thus, the end the mean percentage was expressed as a total of10 fields. Therefore, the percentage data obtained was placed in one of the subgroups. Based on these studies, these sections were divided into four categories (negative, weak, moderate, severe). If less than 10 percent of the section was stained, it was considered negative, 10-25%, weak(+), 26-50%, moderate(++), more than 50%, severe(+++).[18,19]

Statistical analysis

The obtained data was analyzed using analysis of variance (ANOVA) statistical methods, SPSS.15 and descriptive statistic methods (frequency and percentage) and Chi square test were used to study the mortality rate. The data was also analyzed by the nonparametric ANOVA test (Kruskal-Wallis) to compare the mean weight loss in the different groups, the significance level of tests was at 0.05.

Ethical considerations

All ethical considerations were met based on Helsinki Protocol and the University Research Ethics Committee approvals. Since this article is the result of a research project approved by the Research Committee, Tabriz University of Medical Sciences, the ethical considerations have been approved by the its ethics committee.

Results

Weight changes

In total, no weight loss was observed in any of the study groups at their first and last weight measurement. The maximum amount of weight gain was observed in-group D (Figure 1). One-way ANOVA test with repeated measurements were used to investigate the statistical significant differences between the mean weights. The results are shown in Table 2, No statistically significant difference was shown in mean weight in the five groups (p>0.05).The mean weight in Groups D1 and E1 were greater than D2 and E2 respectively, the difference was significant (p<0.05), the results are illustrated in Table 3.

Mortality

Statistical analysis indicated a lack of relationship between the groups and the rate of mortality (p= 0. 39). The results of mortality rates of study the groups are illustrated in Table 2.
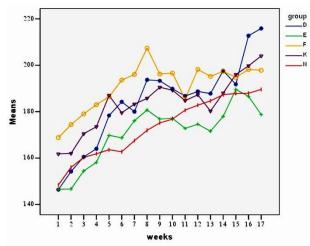
Figure 1. Mean weight of the studied groups in the seventeen –week length

Table 2. Weight gain and death rate of treated and nontreated rats in groups

Groups	N	K	F	D	E
Weight gain Mean (grams) (SD)	173.2 (±32.23)	170.9 (±20.4)	178.1 (±29.52)	170.98 (±36.67)	183.87 (±43.99)
Death rate n (%)	0	7%	0	0	0

Table 3. Mean weight in two doses

Variables	D		E	
	D1	D2	E1	E2
Weight(SD)	206.6(±9)	161.1(±9)	199.4(±4.1)	142.6(±4.1)

Dysplasia

In Group-D, the most common lesion was mild dysplasia (Figure 2), in Group-E moderate dysplasia and in Group-K, SCC (Figure 3). The results of the evaluation of the lesions in the study groups are given in Table 4.The statistical results demonstrated a relationship between the study groups and the dysplasia rate (p<0.001). There was no relationship between drug dose and dysplasia rate within each group (p>0.05). The results of the evaluation of the lesions in the study groups and the drug doses are shown in Table 5.
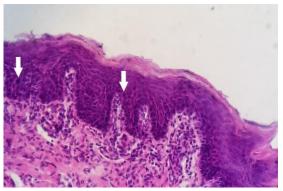

Figure 2. Mild epithelial dysplasia (H&E ×200), shows nuclear hyperchromatism and plomorphism (arrows) in the basal and parabasal layers.
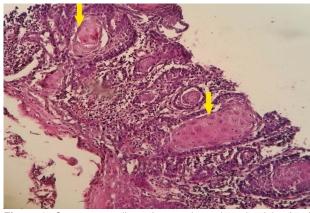

Figure 3. Squamous cell carcinoma shows invasive islands of dysplastic epithelial cells (arrows) (H&E, ×400)

Table 4. Frequency and percentage of the generated lesions in the studied group

groups	Without Dysplasia	Mild epithelial dysplasia	Moderate epithelial dysplasia	Severe epithelial dysplasia	SCC
D	2(14.3)	10(71.4)	2(14.3)	0	0
E	0	0	8(517)	6(42.9)	0
K	0	0	0	2(14.3)	12(85.7)

Table 5. Frequency and percentage of lesions in the study group based on drug dose

groups	Without Dysplasia	Mild epithelial dysplasia	Moderate epithelial dysplasia	Severe epithelial dysplasia	SCC
D1	0	5(53.7)	2(14.3)	0	0
D2	2(14.3)	5(53.7)	0	0	0
E1	0	0	3(21.4)	4(28.6)	0
E2	0	0	5(53.7)	2(14.3)	0

Ki- 67 expression

The results revealed that the level of Ki-67 expression in Group-N, which was the control-group, was entirely negative(-), and Group-D was a typically weak expression (+),while the expression of this marker in group-E was moderate (+ +), and in Group K, severe (+++) (Figure 4). Figure 5 displayed a mild Ki-67 expression (+) in group-D (which received the nano drug).

Results of the evaluation of Ki - 67 expressions in the study groups are illustrated in Table 6. The Chi-square test was used to evaluate the relationship between the study groups and rate of Ki-67 expression. In each group, the results demonstrated a significant relationship between the study groups and the rate of Ki-67 expression (P <0.001). In addition, there was no relationship between the drug dose and Ki-67 expression within each group (p>0.05). Ki-67expression results based on the drug doses in the study groups are given in Table 7.
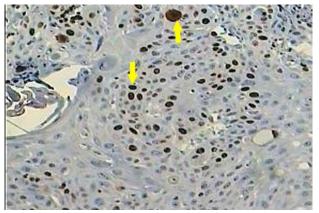
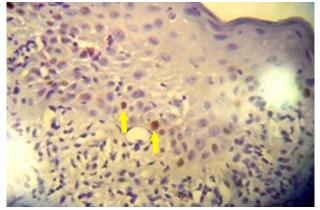**Figure 4.** Severe expression of ki-67 in epithelial cells with nuclear staining (arrows) (IHC, ×400)

Figure 5. Mild Ki-67 expression in group D which received nanoparticle-based doxorubicin. A few nucleus have been stained in this figure (arrows)(IHC,× 400)

Table 6. Frequency and percentage of Ki-67 expression in the studied group

groups	Negetive(-)	Weak(+)	Moderate(++)	Severe(+++)
N	14(100)	0	0	0
D	3(21.4)	9(64.3)	2(14.3)	0
E	0	6(42.9)	6(42.9)	2(14.3)
K	0	0	0	14(100)

Table 7. Frequency and percentage of proliferation in the studied groups based on the drug dose.

groups	Negative(-)	Weak(+)	Moderate(++)	Severe(+++)
D1	1(7.1)	4(28.6)	2(14.3)	0
D2	2(14.3)	5(35.7)	0	0
E1	0	2(14.3)	3.(21.4)	2(14.3)
E2	0	4(28.6)	3.(21.4)	0

Discussion

In this study, 4NQO was used to induce cancer, 4-Nitroquinoline 1-oxide (4NQO) is a potent chemical carcinogens and its carcinogenic activity is due to its enzymatic activity and reduction of nitro groups. The carcinogenic metabolite of 4-Nitroquinoline 1-oxide (4NQO) is created by the loss of four electrons resulting in the production 4-hydroxy amino quinoline 1-oxide (4HAQO).[20] This material has been used in previous studies to induce oral cancer in rat tongues.[13,14] Clinical and histopathologic studies have shown that squamous cell carcinoma due to 4NQO is considered the equivalent to human squamous cell carcinoma.[20,21,22] Squamous cell carcinoma(SCC)makes-up approximately 95% of all the oral malignancies. It is associated with various factors such as smoking, alcohol, nutritional deficiencies, and radiation exposure, also to a number of viruses and suppression of immune system.[2]

The conventional treatments for this cancer are radical surgery, radiation and chemotherapy. Despite the recent advances in cancer treatments today, the survival rate in patients with squamous cell carcinoma remains at a 50 to 59%.[2] Chemotherapy is a systemic treatment which controls the metastatic spread of tumors by destroying the cancerous cells. Using the appropriate dose of anti-cancer agents, and chemotherapy can destroy a large percentage of the cancerous cells. There are a number of various chemotherapy drugs available in the market today, some of which are used separately, however, in most cases, a combination of medications are used adjunctly.[23]

A range of drugs have been used for the purpose of chemotherapy such as cisplatin and doxorubicin, of which each can have severe side effects.[10,23]

The creation of nanotechnology has given medicine a new option and tool for the delivery and accurate transfer of the medications necessary for treating cancer.[24]

The main purpose of nanotechnology in the field of pharmacology is providing an efficient and accurate transfer of the drug to the desired target with minimal side effects and toxicity. Nanocarrier may have the following functions:
- protect a drug from destruction;
- increase drug absorption through facilitating its distribution across the intestinal membrane;
-result in the balanced effect of the drug on the body and alter the tissue distribution.[7,8]

Thus it can lead to the protection of large amounts of prescribed medication, which could be destroyed by the liver and spleen.Without a doubt, the use of colloidal metals is one of the most important parts of nanotechnology in the field of pharmacology. The first direct application is in the treatment of cancer.[7,8] To date, numerous efforts have been made and are being carried out for the active controlled transfer of drugs to the cancerous cells, most of which have focused on loading active molecules, such as antibodies that bind to cancer cells on the liposome's containing the anti-cancer drug.[9] Because of the ability of the chemotherapy nanodrug with its different coatings to penetrate the tumor tissues, Peer and Valetti revealed that the nanodrugs made with a liposomal coating were one of the most reliable carrier systems designed to receive moreover, deliver the chemotherapy nanodrugs.[25,26]

In this study, doxorubicin plus methotrexate-loaded stimuli responsive silica based nanoparticles was used. The results established that the above-mentioned system had a greater efficacy. Doxorubicin is a drug that is used to treat a variety of tumors such as sarcomas and carcinomas. Additionally, these drugs are used in hematologic malignancies (leukemia, lymphoma and multiple myeloma).[10]

Multiple studies have been carried out on the efficacy of doxorubicin in animal models.

Wang et al carried out a study targeting the therapy of hepatocellular carcinoma using pro-drugs (an inactive form) of doxorubicin. In the above-mentioned study, an increased anti-metastatic activity and decreased toxicity was observed.[27]

In a previous study by Siegal et al, they compared the efficacy of free doxorubicin with its liposomal form in animal models that had brain tumors as a secondary cancer and revealed that the use of liposome's as a carrier of cytotoxic drugs improved the treatment efficacy of the drug in brain tumors.[28]

Lingha evaluated the amount of drug released after treatment with free doxorubicin as compared to the injectable nanocarrier doxorubicin in mice implanted with breast cancers, and concluded that the application of liposomal nanodrugs increased the bioavailability of the drug and consequently, its effectiveness.[29]

Steiniger studied the effects of the nanodrugs in vivo in the treatment of glioblastoma and concluded that doxorubicin bound to nanoparticles provided greater therapeutic potential in the treatment of human glioblastoma.[30] However, to the knowledge of this study, no research has been published on the effects of nanodrugs on squamous cellcarcinoma, which was the investigative work of this study.

This investigative research on the histopathologic evaluation of the study groups revealed a significant decrease in the malignancy grade of the groups receiving doxorubicin plus methotrexate-loaded stimuli responsive silica based nanoparticles compared with its commercial form (P <0.001),which demonstrated the efficacy of the nanodrug as compared to that of commercial formulary. The results of previous studies regarding various tumors are consistent with those of the present study.[27-30]

In this study, Ki-67 marker were used to evaluate the efficacy of doxorubicin plus methotrexate-loaded nanoparticles in the treatment of squamous cell carcinoma. Ki-67 is one of the most well known cell cycle-related proteins that are used in the diagnostic histopathology. This marker is a protein that is expressed on proliferated,malignant cells, and used as a marker of cell proliferation.[18,19] The efficacies of nanodrugs were evaluated using other immunohistochemical markers in various studies.[18,19]

The results of Stefani's study on glioblastoma tumor demonstrated a decrease in the expression of GFAP and VEGF markers in the groups treated by doxorubicin plus methotrexate-loaded stimuli responsive silica based nanoparticles. Since the decrease in the expression of these markers is effective in the improvement of patient's prognosis, the results revealed that the combination doxorubicin and methotrexate-loaded stimuli responsive silica based nanoparticles played a more effective role in the healing of the tumor than a free drug.[31]

Milane et al evaluated the effect of nanodrug chemotherapy on breast cancer. The study used EGFR marker to study the effects of the nanodrug. The results validated that EGFR markers and tumor sizes in the groups receiving nanodrug reduced sharply in comparison with the groups receiving the simple form drug. Since the EGFR is considered the prognostic factor, it validated that the combination doxorubicin and methotrexate-loaded stimuli responsive silica based nanoparticles is more effective in the treatment of tumors,[32] which is consistent with the results of this study. In this study, Ki -67 expression in the groups receiving oral doxorubicin plus methotrexate loaded stimuli responsive silica based nanoparticles were significantly lower than the groups receiving the commercial drug (p <0.001).

Conclusion

Based on the investigative results of this study, the combination of the orally prescribed medication, doxorubicin plus methotrexate-loaded stimuli responsive silica based nanoparticles, were used for the treatment of oral squamous cell carcinoma. This combination medication demonstrated a significant reduction in the cell proliferation and severity of the malignancy, when compared with the commercial formulary form presently available for treatment of oral squamous cell carcinoma. However, it is the recommendation of this study that further research and investigative work be continued on the side effects and efficacy of the combination drug doxorubicin plus methotrexate-loaded stimuli responsive silica based nanoparticles, as compared to other pharmaceutical forms presently available for treatment of squamous cell carcinoma.

Acknowledgments

Ethical Issues

Not applicable.

Conflict of Interest

Authors declare no conflict of interest.

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