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Intraperitoneal administration of PLGA nanoparticles could deliver the cargo to tumor-associated macrophages with less spreading peritoneal macrophages in the treatment of peritoneal carcinomatosis

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ABSTRACT

The survival time of patients with peritoneal metastasis is limited despite all treatments. Recent studies revealed that tumor-associated macrophages (TAMs) are educated in the tumor microenvironment for tumor growth and progression. The use of nanoparticles is considered an opportunity to deliver the therapeutics for depletion or re-education of these TAMs. In this study, it was planned to answer whether Poly(lactic-co-glycolic acid) (PLGA) nanoparticles applied to the peritoneum could be successful in delivering drugs to TAMs in peritoneal metastasis. For this purpose, Nile red loaded PLGA nanoparticles were prepared and characterized. Nil-PLGA NPs and nil red solution were injected intraperitoneally to mice model of CT-26 colon adenocarcinoma peritoneal metastasis, and nile red labeled macrophages were evaluated with the flow cytometer. The average particle size and zeta potential of nanoparticles were found as 238.4 ± 22.9 nm and −1.38 ± 1.35 mV, respectively. Labeled macrophages percent in the tumor was not found statistically different. On the other hand, after intraperitoneal administration of nil red solution, there was a very high nile red transfer to macrophages within the peritoneum. This widespread transfer was prevented by the application of nile red-loaded nanoparticles. This result shows that intraperitoneal application of PLGA nanoparticles could be an opportunity for drug delivery to TAMs with less damage to intraperitoneal macrophages.

1. Introduction

The peritoneal cavity is a frequent site for metastasis of many cancers such as ovarian, gastric, colorectal and pancreatic cancer, and it is generally seen that peritoneal carcinomatosis or widespread peritoneal metastasis spread throughout the peritoneal cavity in the last phase of cancer [1,2]. Although the use of cytoreductive surgery and intraperitoneal chemotherapy in addition to systemic chemotherapy brings promising results, the survival time of patients with colon cancer with peritoneal metastasis is limited despite all treatments [3–5].

Recent studies revealed that tumor-associated macrophages (TAMs) are educated in the tumor microenvironment for tumor growth and progression. The two main strategies for the prevention of these TAMs that cause tumor progression are; depletion of these TAMs with anticancer drugs or re-education of these macrophages [6–8]. The use of nanoparticles is considered an opportunity to deliver the necessary therapeutics to TAMs [9–11]. The use of nanoparticles administered directly into the peritoneum in the treatment of peritoneal metastasis caused by colon cancer provides the advantages of providing longer drug release in the peritoneal cavity and the application of especially water-insoluble drugs without using any organic solvent [12]. However, studies investigating the efficiency of intraperitoneally injected nanoparticles to transport drugs to TAMs are limited.

Poly(lactic-co-glycolic acid) (PLGA) is one of the most used polymers with its biodegradability and biocompatibility properties. The main reasons why PLGA is so preferred are that it is approved for parenteral use by the United States Food and Drug Administration and European Medicines Agency, it has well-known production processes and it can be successfully loaded with hydrophilic or hydrophilic drugs [13–15]. For this reason, PLGA was selected as a polymer to prepare nanoparticles in this study.

Considering the fact that actively targeted nanoparticles have not yet achieved the desired clinical success, it may be easier for nanoparticles to be applied to the target area and prepared in a simple way to achieve

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clinical success [16]. So this study was planned to answer whether PLGA nanoparticles applied to the peritoneum could be successful in delivering drugs to TAMs. For this purpose, Nile red (model drug) loaded PLGA nanoparticles (Nil-PLGA NPs) were prepared and characterized. Nil-PLGA NPs and nil red solution were injected intraperitoneally to the mice model of CT-26 colon adenocarcinoma peritoneal metastasis in Balb/c mice, and nile red labeled macrophages were evaluated with the flow cytometer.

2. Material and methods

2.1. Materials

PLGA RG 503 (Mw: 26,500 g/mol; Mn: 14,700 g/mol), nile red, Poly (vinyl alcohol) (PVA), dimethyl sulfoxide (DMSO), acetone were purchased from Sigma-Aldrich (Saint Louis, USA). Fetal bovine serum (FBS), RPMI 1640, L-glutamine, and penicillin-streptomycin solution were obtained from Lonza (Basel, Switzerland). Anti-mouse F4/80-FITC antibody Biolegend #123108. All other chemicals were at least reagent grade.

2.2. Methods

2.2.1. Preparation and characterization of nanoparticles

Nil-PLGA NPs were prepared by the nanoprecipitation method [17,18]. For the preparation of the organic phase, 1 mL of an acetone solution containing 100 μg of nil red was prepared. Then, weighed 30 mg of PLGA polymer was added to the acetone solution containing nile red. It was dissolved by mixing well. The aqueous phase was prepared by dissolving PVA in water with a concentration of 1%. Then, the prepared organic phase was added dropwise to 10 mL of 1% PVA solution, which was mixed in a magnetic stirrer at 1100 rpm. The prepared suspension was evaporated in an organic rotary evaporator. Nanoparticles from the suspension were collected by centrifugation at 13000 rpm for 15 min. After being redispersed in ultrapure water again, it was washed by centrifugation again and dispersed in ultrapure water. Average particle size, polydispersity index (PDI) and surface charge were measured with Malvern Nano ZS (Malvern Instruments, UK). Nile red loaded in nanoparticles was determined by direct method [19]. Briefly, a known amount of nanoparticles were weighed and 2:1 mixture of dimethyl sulfoxide (DMSO) and acetone was dissolved in the mixture and nile red absorbance was measured by UV spectrophotometry (Shimadzu UV-1800 spectrophotometer, Japan) at 550 nm. For preparing nile red solution, firstly 0.7 mg of nile red was dissolved in 1 ml DMSO, and then this solution was diluted via water to obtain the target concentration: 1.25 μg/200 μl.

2.2.2. In vivo analyses

For animal studies, CT-26 colon adenocarcinoma peritoneal metastasis model was formed in male 8-week-old 18–20 g BALB/c mice (Kobay Laboratory of Experimental Animals Ethics Committee No:539, 2021). Laboratory animal facility is maintained under a 12-h light/dark cycle at a temperature of 22 °C and a relative humidity of 40%.

The CT26 mouse colon cancer cell line was grown in RPMI cell medium (10% FBS, 1% penicillin/streptomycin antibiotic, and 1% L-glutamine) and in an incubator, at 37 °C and 5% CO_2. Cells were harvested after trypsinazination when it reached 70% cell density. Cells were washed with PBS and centrifuged (2000 rpm, 10 min) then 2×10^6 cells in 500 μl PBS were injected intraperitoneally. After waiting for 10 days in early period of colon cancer [20], Nil-PLGA NPs (n = 10) and nil red solution (n = 10) were injected intraperitoneally (Table 1). After 4 h, the mice were sacrificed with a high dose anesthetic and peritoneal lavage was performed with cold PBS. Tumor tissue and liver were collected. Half of the organs, tumor tissue and peritoneal lavage were used for immunofluorescence studies and the other half for flow cytometry studies. It was incubated with the collagenase II enzyme for 2 h at 37 °C for flow cytometry. Cells were centrifuged 2000 rpm 10 min and washed with PBS and then stained separately with F4/80 (with FITC) antibody and analyzed in flow cytometry (FACS Aria II, BD, USA) with FITC-PE quadrant for antibody and Nile Red dye respectively. For microscopy, after 5 μm sections were taken with the cryostat (Leica, Germany), sections were fixed with formalin, washed with PBS and analyzed (Fig. 1).

2.3. Statistical analysis

Data were presented as the mean ± standard deviation. Measurements were performed at least in triplicates. The comparison of more than two groups was investigated Student's t-test was used for parametric comparisons and Mann-Whitney *U* test was used for nonparametric comparisons. The comparison of more than two groups was investigated using ANOVA followed by Fisher LSD post hoc test. Tests were performed with Minitab®16 (Minitab Inc.; State College, PA, USA).

3. Results and Discussion

3.1. Preparation and characterization of nanoparticles

Many methods are used in the preparation of PLGA nanoparticles and these methods have their advantages and disadvantages. However, all preparation methods are based on mixing PLGA dissolved in the organic phase with antisolvent [21,22]. The nanoprecipitation method, which is one of these methods, is based on the principle of forming stable nano-emulsions by emulsifying spontaneously when the water-miscible organic solvent is mixed with water, polymer dissolved in the organic phase aggregates as nanoparticles. Compared to other methods, it does not require an additional energy application, there is no need for equipment, and its suitability for scale-up makes it a frequently used method [21,23]. So, PLGA NPs formulations were prepared with the nanoprecipitation method in this study. For the production of nanoparticles different organic phases, organic phase volume, polymer amount, water phase and surfactant could be used. In our previous studies, we investigated in detail the factors that can affect the properties of PLGA nanoparticles in the nanoprecipitation method [24,25]. Based on our previous experience, parameters were selected as described in the Methods section [18,25]. Acetone was used as an organic phase in this study, nile red and PLGA were solved in this phase. Nile red was selected as a fluorescent dye for labelling nanoparticles and using a model drug. Because nile red is frequently used dye as a model drug in previous studies and it has a wavelength that does not interfere with the cell auto fluorescence signal [18,19,26–28]. In this way, the model drug loaded nanoparticle easily determined in *in vitro* and *in vivo* studies. Although hydrophilic and hydrophobic drugs can be loaded into PLGA nanoparticles, high burst release is a disadvantage for hydrophilic drug loaded PLGA nanoparticles. On the other hand PLGA nanoparticles provide a great opportunity to prepare and delivery of hydrophobic drugs. When the potential drugs for the tumor associated macrophages targeted therapy are examined, it is seen that hydrophobic drugs, such as

Table 1
Summarize of intraperitoneally injected groups.

Group	Dose	Injection Route	Injection Volume	Frequency of Administration
Nil-PLGA NPs	1 mg NPs/200 μl (Containing 1.25 μg of nile red)	Intraperitoneal	200 μl	Once
Nile red solution	1.25 μg/200 μl (Nile red concentration)	Intraperitoneal	200 μl	Once

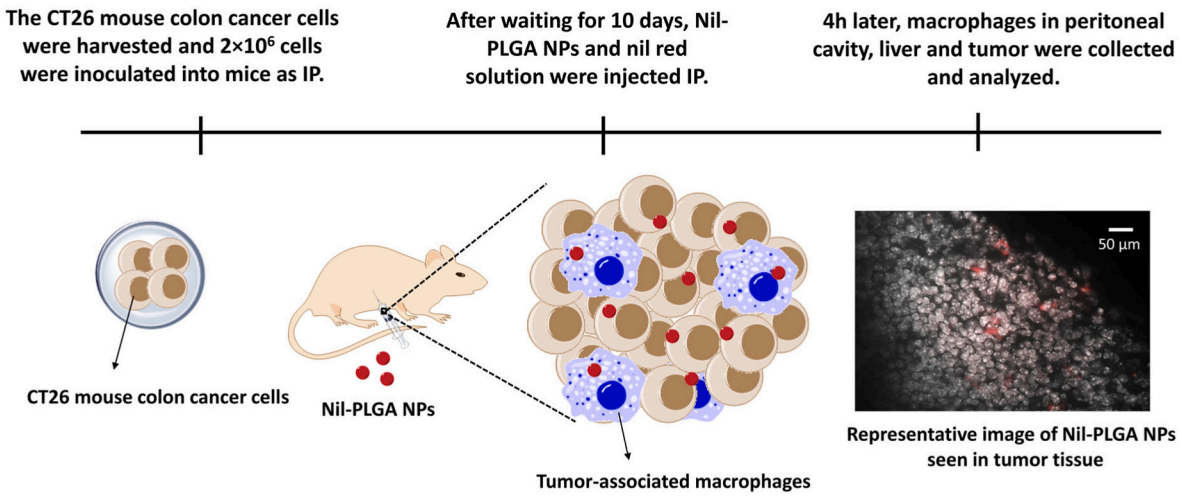

Fig. 1. Schematic presentation of *in vivo* evaluation method.

GDC-0941, Sorafenib, JQ1, Ruxolitinib, Cyclopamine, Lapatinib, provide important treatment options. In the light of all this information, nile red was used as a model drug in this study. 1% PVA was used as a water phase to stabilize obtaining nanoparticles. After organic phase evaporation and washing steps, the average particle size and polydispersity index of nanoparticles were found as 238.4 ± 22.9 and 0.388 ± 0.088 nm. Zeta potential of nanoparticles was measured as −1.38 ± 1.35 mV (Fig. 2).

These results are similar to the results obtained previously with PLGA nanoparticles obtained by the nanoprecipitation method. This particle size is in the range defined as the ouzo range [29,30], which is an expected particle size when acetone is used as the organic phase. The zeta potential value was found to be slightly negative, while the polydispersity value indicates a wide particle size distribution. In the work to be done, it will be critical to compare the distribution of nile red labeled nanoparticles to macrophages with the difference from the dye given in the solution. Since it can be thought that the dye used imitates a model drug, its difference becomes important with application in solution form. For this purpose, the most critical factor is to determine the amount of dye contained in the nanoparticles and to use the solution containing this dye amount as a control group. In this study, the amount of nile in the nanoparticles was determined by the method described by Xu et al. [19], and the amount of dye in one mg nanoparticle was calculated as 1.25 µg. To prepare the solution to be used in the control group, nile red was first dissolved in DMSO and then diluted with water to prepare the control group. Confirmation that the same amount of dye was given also overcome one of the biggest problems in the comparison of solution and nanoparticle groups.

3.2. *In vivo* analyses

The peritoneal cavity is a region where metastasis is seen in colon cancer and TAMs are cells of great importance in tumor development and progression in peritoneal metastasis [5,31]. Studies have shown that macrophages in the tumor region can be trained to advance the tumor. After these studies, treatment approaches targeting TAMs have increased rapidly [17,32]. The main axis of these studies is the killing or re-education of TAMs. On the other hand, the view that more effective treatment can be achieved with the use of nanoparticle drug delivery systems for this purpose is gaining importance day by day [11]. In this study, nil red fluorescently labeled nanoparticles obtained with PLGA were evaluated in the macrophages and peritoneal fluid in the tumor tissue after intraperitoneal nanoparticle injection in the peritoneal metastasis model.

When the results were examined, when we looked at the percentage rate of macrophages marked with nile red in the nanoparticle injected group, it was found that 10.9 ± 1.8% of macrophages in the peritoneal lavage, 3.8 ± 1.6% of macrophages in the liver and 13.9 ± 8.2% of macrophages in the tumor. In the solution injected group, 27.8 ± 14.5% of the macrophages in the peritoneal lavage, 4.7 ± 4% of the macrophages in the liver, and 12.4% ± 7.7% of the macrophages in the tumor were found (Fig. 3).

The percentage of macrophages marked with nile red was the highest in peritoneal macrophages after injection of nile red solution ($p < 0.01$).

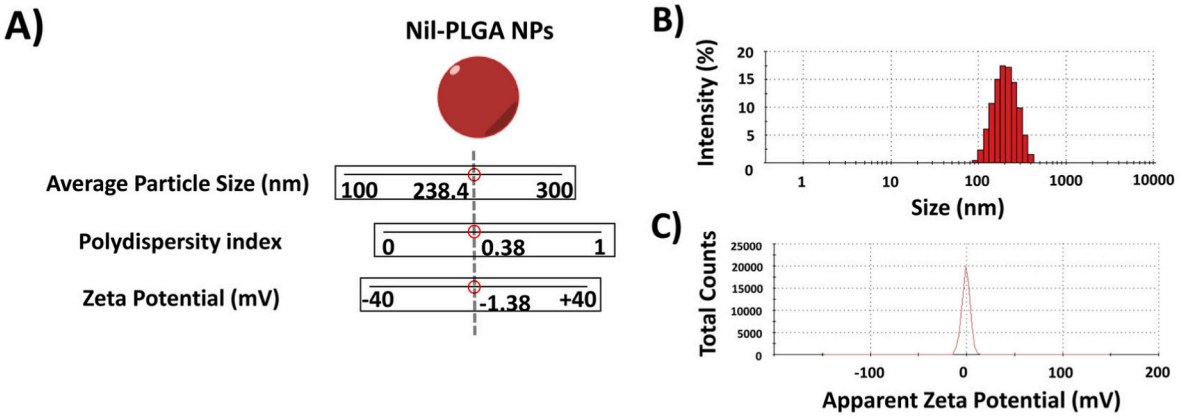

Fig. 2. A) Characterization results of Nil-PLGA NPs, B) Particle size distribution graph of nanoparticles, C) Zeta potential distribution graph of nanoparticles.

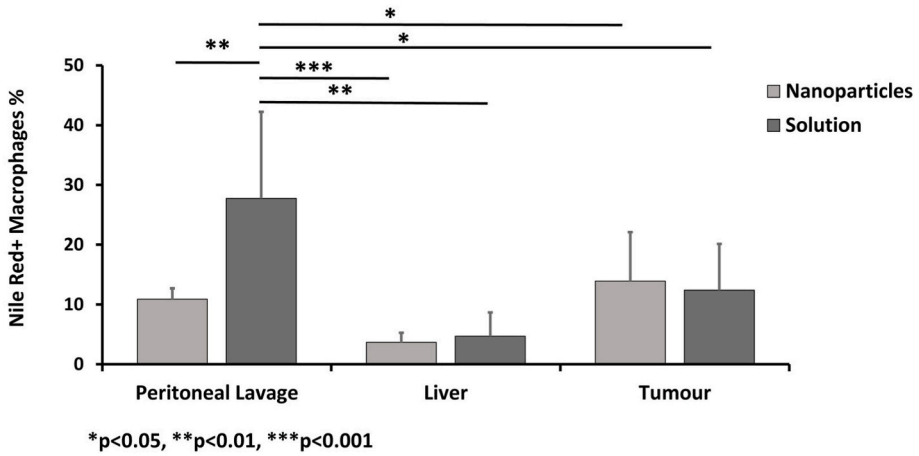

Fig. 3. Percentage of macrophages marked with nile red in nanoparticles and solution injected group.

The fact that this group contains a much higher percentage of labeled macrophages than peritoneal macrophages labeled with Nile-PLGA NPs has led to the conclusion that the nanoparticles can carry the drug without spreading to the macrophages in the peritoneum. This seems particularly successful in transporting the cargo directly to the target without contacting the macrophages in the peritoneum. On the other hand, accumulation in the tumor tissue did not reach a significant difference in the solution and nanoparticle groups constitute the limiting aspect of the treatment. These findings also suggest that active targeting may provide an improved option for drug delivery to TAMs in peritoneal metastases. Also, the dye density in that cells is evaluated as the main parameter of how much the drug is delivered. For this purpose, when dye intensity in macrophages was measured, the results presented in Fig. 4 were obtained.

When median fluorescence intensity (MFI) of macrophages marked with nile red in the nanoparticle injected group was evaluated, it was found that the macrophages in the peritoneal lavage were 122.8 ± 7.6, the macrophages in the liver were 145.5 ± 15.4 and the macrophages in the tumor were 128 ± 11.2. In the solution injected group, 201.8 ± 17.4 of the macrophages in the peritoneal lavage, 138.5 ± 17.9 of the macrophages in the liver and 116.8 ± 10.1 of the macrophages in the tumor were found (Fig. 4A). In the peritoneal lavage, the intensities of macrophages labeled with nile red appear to be much higher in the solution-administered group compared to the nanoparticle-applied group ($p < 0.001$). It was observed that the intensity of the macrophages in the liver in the solution-administered group was higher than that of the tumor tissue ($p < 0.05$), and in the nanoparticle-administered group, the intensity of the liver macrophages did not differ significantly compared to the macrophages in the tumor tissue. Although there was a slight increase in the intensities of the macrophages in the tumor in the nanoparticle applied group, it did not reach a significant level. In the sections obtained from the tumor tissue, the nanoparticle group can be displayed, but no signal can be obtained in the analysis of the solution group (Fig. 4B). This situation can be explained by the fact that nanoparticles may accumulate more intensely in other cells of the tumor. Similarly, the study by Haber et al. showed that relatively large anionic nanoparticles (>100-nm) applied intraperitoneally can accumulate in tumor tissue and TAMs [33].

4. Conclusion

Consequently, after intraperitoneal administration of nil red solution, there is a very high dye transfer to macrophages within the peritoneum, both in percentage and intensity. It has been determined that this widespread transfer is prevented by the application of Nil-PLGA NPs and the target molecule can be transported to macrophages in the tumor tissue as well as in a solution. This result shows that in addition to all the other advantages of intraperitoneal application of PLGA nanoparticles, there may be an opportunity for drug delivery to TAMs with less damage to intraperitoneal macrophages.

Declaration of competing interest

The authors declare that they have no known competing financial

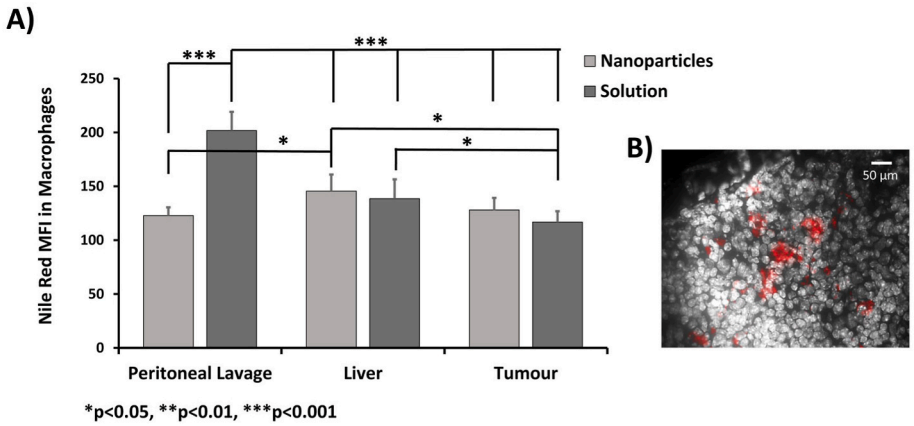

Fig. 4. A) MFI of macrophages marked with nile red in the nanoparticle and solution injected group, B) Microscopy image of Nil-PLGE NPs in tumor section. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Adem Sahin: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Suleyman Can Ozturk:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft.

Data availability

Data will be made available on request.

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