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ORIGINAL ARTICLE / ÖZGÜN MAKALE

CELL INCORPORATION STUDY OF [^{99m}Tc]Tc-NAB-PACLITAXEL ON HUMAN COLORECTAL ADENOCARCINOMA CELL LINE

[^{99m}Tc]Tc-NAB-PAKLİTAKSEL'İN İNSAN KOLOREKTAL ADENOKARSİNOMA HÜCRE HATTI ÜZERİNDE HÜCRE BAĞLANMA ÇALIŞMASI

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ABSTRACT

Objective: *The development of new radiopharmaceuticals capable of specifically imaging cancer is an important research area. For this reason, the aim of this study is to investigate cell binding of novel developed nanoradiopharmaceutical ([^{99m}Tc]Tc-nab-PTX) on HT-29 cell line (human colorectal adenocarcinoma cells).*
Material and Method: *In this study, nab-PTX was labeled with [^{99m}Tc]Tc, and labeling efficiency and in vitro stability were investigated by radioactive thin layer chromatography (RTLC). Then, cell incorporation of [^{99m}Tc]Tc-nab-PTX was performed using HT-29 cell line.*
Result and Discussion: *According to obtained results, nab-PTX was labeled with high radiochemical purity (>99%). The [^{99m}Tc]Tc-nab-PTX was found to be stable in saline for 6-h, and in cell medium up to 2-h. It was determined that [^{99m}Tc]Tc-nab-PTX had a greater cell binding activity on HT-29 cells than R/H-[^{99m}Tc]$NaTcO_4$ at 30 min. The results were found to be promising for future in vivo studies with [^{99m}Tc]Tc-nab-PTX.*
Keywords: *HT29 cell line, nab-paclitaxel, radiolabeling, technetium-99m*

ÖZ

Amaç: *Spesifik olarak kanseri görüntüleyebilen yeni radyofarmasötiklerin geliştirilmesi önemli bir araştırma alanıdır. Bu nedenle bu çalışmanın amacı, yeni geliştirilen nanoradyofarmasötiğin ([^{99m}Tc]Tc-nab-PTX) HT-29 hücre hattına (insan kolorektal adenokarsinom hücreleri) hücre bağlanmasını araştırmaktır.*
Gereç ve Yöntem: *Bu çalışmada, nab-PTX [^{99m}Tc]Tc ile işaretlenmiş ve işaretleme etkinliği ve in vitro stabilitesi radyoaktif ince tabaka kromatografisi (RTLC) ile araştırılmıştır. Daha sonra, [^{99m}Tc]Tc-nab-PTX'in hücre bağlanması, HT-29 hücre hattı kullanılarak gerçekleştirilmiştir.*

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Sonuç ve Tartışma: *Elde edilen sonuçlara göre, nab-PTX yüksek radyokimyasal saflıkla (>%99) radyoişaretlenmiştir. [^{99m}Tc]Tc-nab-PTX'in oda sıcaklığında 6 saate kadar ve hücre ortamında 2 saate kadar stabil olduğu bulunmuştur. [^{99m}Tc]Tc-nab-PTX'in HT-29 hücreleri üzerinde 30. dakikada R/H-[^{99m}Tc]NaTcO$_4$'ten daha yüksek bir hücre bağlama aktivitesine sahip olduğu belirlenmiştir. Sonuçların [^{99m}Tc]Tc-nab-PTX ile gelecek in vivo çalışmalar için umut verici olduğu bulunmuştur.*

Anahtar Kelimeler: *HT29 hücre hattı, nab-paklitaksel, radyoişaretleme, teknesyum-99m*

INTRODUCTION

Cancer has long been one of the world's top causes of death [1,2]. According to 2020 data, a total of 19.3 million new cancer cases developed in the world, and 10.0 million people died due to cancer [3]. Cancer can be defined simply as uncontrolled cell proliferation. Although uncontrolled proliferation is the main feature, the cancer cell also has other biological characteristics. These include avoiding contact inhibition in cell cultures, not requiring external stimuli to divide, insensitivity to proliferation suppressive signals, avoiding apoptosis, stimulating angiogenesis and metastasis. Although there are many different types of cancer, they all start with the out-of-control proliferation of abnormal cells. If left untreated, it can cause serious illness and even death [4].

The most common types of cancer are lung, breast, and colorectal cancers. Among them, colorectal cancers are the 4th most common cancer in women and 6th in men globally [3], while their incidence in both genders is 3rd in our country [5]. It usually gives symptoms as the disease progresses. Therefore, early diagnosis is very important for colorectal cancer. Detection and effective treatment of cancer at early levels significantly reduces the cost and duration of treatment, and the risk of mortality and morbidity. Anatomical imaging techniques are not sufficient for imaging in the initial stage of this disease, as they rely on morphological changes. Since scintigraphic imaging is a non-invasive imaging technique based on the detection of physiological changes, it allows diagnosis at an early stage [6-9]. Therefore, it is critical to develop radiopharmaceuticals that can detect physiological changes before anatomical changes occur [10]. With nuclear medicine imaging techniques, both the diagnosis of the disease and its prevalence, or in other words, at what stage is determined [7,11,12].

Radiopharmaceuticals are defined as radioactive drugs used in nuclear medicine, consisting of two parts, a radionuclide, and a pharmaceutical part, that do not cause a pharmacological response in the patient, and are used for diagnosis and treatment. As the target/non-target tissue uptake of radiopharmaceuticals increases, so does the diagnosis and treatment efficiency. Therefore, by targeting the radiopharmaceutical, it is aimed to reduce the radiation damage outside the target tissue and to increase the image quality obtained [13].

Ideal radionuclide to be used in diagnosis should emit pure gamma rays, have an energy between 100-250 KeV, be easily available, reasonably priced, suitable for use with effective half-life, high target/non-target ratio, sterile, nonpyrogenic, isotonic, and isohydric. Considering all these features, [^{99m}Tc]Tc is the most ideal of the radioisotopes used for diagnostic purposes in nuclear medicine. The physical half-life of [^{99m}Tc]Tc is 6-h, and [^{99m}Tc]Tc only emits gamma radiation with an energy of 140 KeV, does not emit beta particles and has a short half-life, so the radiation dose to which the patient is exposed is very low [14-16].

Nanoparticle albumin bound paclitaxel, also known nab-PTX, is an antineoplastic agent effective against many cancer types including breast, ovarian, colon, pancreas, and non-small cell lung cancers [17-19]. nab-PTX has been shown to be effective, even in individuals with poor prognostic factors and aggressive disease characteristics [20], thus nab-technology a focus of interest made for anti-cancer drug delivery systems [21]. In preclinical research, nab-PTX demonstrated anticancer effect as a single agent and synergistic activity when combined with other chemotherapeutic agents [22,23].

Albumin is a drug carrier in the anticancer drug delivery system, and also has the capacity to actively target tumors, and nab-PTX, which has 130 nm of particle size, is a new form of solvent-free albumin-bound PTX [24,25]. It is prepared in a nanoparticle colloidal suspension by high pressure homogenization of PTX in the presence of serum albumin [25]. The difference of nab-PTX from PTX is that PTX requires the use of solvents such as Cremophor EL and Colliphor EL in the treatment. These

solvents used in the solvent-based (sb) PTX formulation are associated with extremely serious hypersensitivity reactions. To prevent this situation with sb-PTX, patients are generally treated with antihistamines and corticosteroids. Also, some studies have shown that Colliphor EL can retain PTX in solvent micelles, so the drug is less available to enter tumors, and clinical efficacy of PTX reduces [26-28].

Furthermore, compared to sb-PTX, nab-PTX has several practical advantages such as delivering significantly higher doses of PTX over a shorter infusion time (about 30-min), and eliminating the require for premedication to prevent hypersensitivity reactions. Also, nab-PTX can better transport of PTX between endothelial cells than sb-PTX [24]. Since nab-PTX contains albumin in its formulation, it is presumed that the drug utilizes endogenous albumin transport routes, including receptor-mediated transcytosis, to enter tumors by crossing endothelial cell monolayers, eliminating the effect of Cremophor-EL on PTX pharmacokinetics [24,29]. In a preclinical study, four times more nab-PTX transported between endothelial cells than sb-PTX was found [24]. Due to leaky vascularity around tumors, albumin-bound molecules like albumin or nab-PTX are also able to escape from the bloodstream through spaces between endothelial cells, finding a pathway in the tumor microenvironment *via* increased permeability and retention effect [30].

The aim of this study is to determine cell incorporation of novel developed radiopharmaceutical ([^{99m}Tc]Tc-nab-PTX) on HT-29 cell line (human colorectal adenocarcinoma cell line). For this aim, nab-PTX was labeled with [^{99m}Tc]Tc, and radiochemical purity (RP) and *in vitro* stability (in cell medium) were investigated *via* radio-thin layer chromatography (R-TLC). Then, cell incorporation of [^{99m}Tc]Tc-nab-PTX was studied.

MATERIAL AND METHOD

Material

Panacea Biotec (India) provided nab-PTX. Sigma-Aldrich (USA) provided stannous chloride. [^{99m}Tc]$NaTcO_4$ was provided from the Nuclear Medicine Department of Ege University (Turkey). Cell culture supplements were obtained from Gibco Invitrogen (Grand Island, NY). The HT-29 cells were purchased from the American Type Culture Collection.

Preparation of [^{99m}Tc]Tc-nab-PTX

nab-PTX was previously radiolabeled with [^{99m}Tc]Tc by our research team [31]. Briefly, 0.5 mg nab-PTX was dispersed in 1 ml of saline (SF). To this solution, 100 $\mu g.ml^{-1}$ reducing agent (stannous chloride solution in distilled water) was added. Then, to radiolabel nab-PTX, 37 MBq (0.1 ml) [^{99m}Tc]$NaTcO_4$ was added to the system. The resulting radiolabeled complex was shaken for 45 s, and incubated for 15-min. The RP of [^{99m}Tc]Tc-nab-PTX was analyzed by R-TLC.

Quality Control Study of [^{99m}Tc]Tc-nab-PTX

In order to determine the RP of nab-PTX, R-TLC was done with ITLC-SG chromatography paper using 3-5 µl of [^{99m}Tc]Tc-nab-PTX and acetone as mobile phase. The radioactivity of the papers was scanned *via* a R-TLC device (Bioscan AR 2000) up to 6-h.

In vitro Stability of [^{99m}Tc]Tc-nab-PTX

For *in vitro* stability of [^{99m}Tc]Tc-nab-PTX in McCoy's 5A containing 10% fetal bovine serum (FBS) (cell media), 0.1 ml of [^{99m}Tc]Tc-nab-PTX was added to cell medium (0.4 ml). The complex was incubated in an incubator at 37°C and RP of [^{99m}Tc]Tc-nab-PTX were performed up to 2-h.

Cell Culture Study

The HT-29 cells were used for cell incorporation studies. McCoy's 5A containing 10% FBS was used as cell media. Cells were seeded 1×10^6 cells on plates under 90% humidity and 5% CO_2 at 37°C.

Cell Incorporation Study of [^{99m}Tc]Tc-nab-PTX

18.5 MBq of [^{99m}Tc]Tc-nab-PTX and R/H-[^{99m}Tc]$NaTcO_4$ (as control) were treated with HT-29

cells in six well plates for 2-h at 37°C. The radioactive cell culture media was collected at determined times. The HT-29 cells were trypsinized with 0.5 ml of trypsin to remove the cells. 0.75 ml of cell medium and 0.75 ml of phosphate-buffered were also added to the cells, and the system was centrifuged at 1.200 rpm for 3-min. The activities both cells and cell medium were counted using a gamma counter (Sesa Uniscaller), separately. The radioactivity of cells (%) was determined from the following equation (Eq. 1):

Radioactivity of HT-29 cells (%) = (Radioactivity of HT-29 cells/Total counted radioactivity) × 100 (Eq. 1)

Statistical Analysis

The *in vitro* results were calculated using Microsoft Excel, reported as the mean ± standard error, and studies were performed three times. The statistical significance was assessed using the t test. Differences that were significant at the 95% level of confidence (p>0.05) were noted.

RESULT AND DISCUSSION

In this study, nab-PTX was successfully radiolabeled with [^{99m}Tc]Tc. The RP of [^{99m}Tc]Tc-nab-PTX was evaluated by R-TLC studies for 6-h. ITLC-SG chromatography paper and acetone were used to determine the amounts of radioactive contaminant (free [^{99m}Tc]Tc). Using these systems, the R-TLC chromatogram of [^{99m}Tc]Tc-nab-PTX was presented in Figure 1.

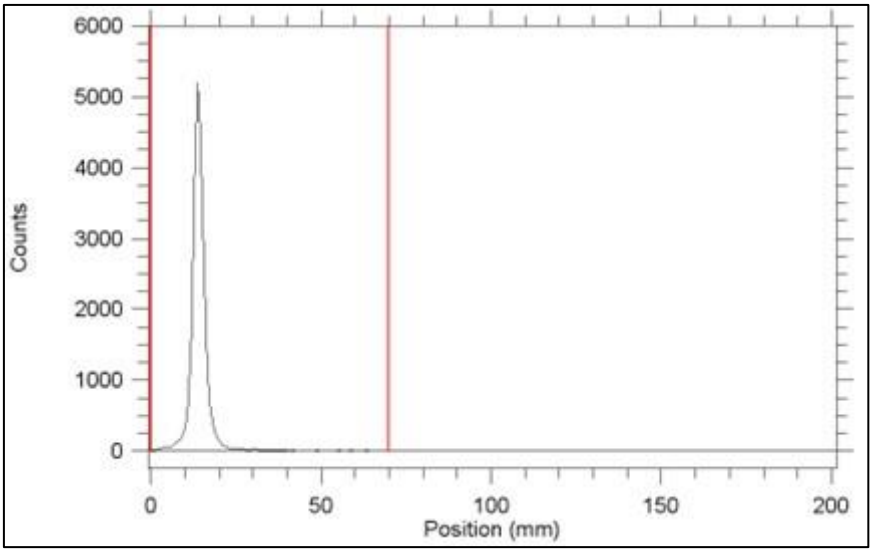

Figure 1. R-TLC chromatogram of [^{99m}Tc]Tc-nab-PTX in acetone as mobile phase.

[^{99m}Tc]Tc-labeled compounds consist of a targeting vector linked to a [^{99m}Tc]Tc-ligand complex which is carried [^{99m}Tc]Tc to a specific site. [^{99m}Tc]Tc-labeled compounds include nanoparticles, blood cellular elements, and receptor-specific transporters, such as peptides and antibodies [32]. In general, labeling of the prepared nanoparticles might be performed (a) by surface sorption of the radionuclide to the surface of the nanoparticle directly, (b) intrinsic encapsulation of the radionuclide into the core of the nanoparticle during the synthesis, (c) chelation of radionuclide by ligands (mostly polydentate, e.g., DTPA (Diethylenetriamine pentaacetate), DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), and NOTA (1,4,7-triazacyclononane-N,N′,N″-trisacetic acid) derived analogs) directly attached or linker spaced on the surface of the nanoparticle. Several factors influence the labeling yield and the stability of the complex, such as the amount of reducing agent and ligand, pH, and temperature. The chemical groups suitable for direct radiolabeling by chelating [^{99m}Tc]Tc radionuclide are –OH, –COOH, –C=O, –PO_4, –P_2O_7-, –NH_2, –SOOH, –SOONH, –$SOONH_2$, –OCH_3. By using these chemical groups,

radiolabeling can be done directly with [^{99m}Tc]Tc and through different chelate groups [33].

According to the results, the RP of [^{99m}Tc]Tc-nab-PTX was found greater than 99%. Over the experimental period, [^{99m}Tc]Tc-nab-PTX was quite stable and, RP was found >99 % up to 6-h without any significant change (p>0.05) (Table 1). At the same time, [^{99m}Tc]Tc-nab-PTX was incubated with 37 MBq of [^{99m}Tc]Tc during 6-h for radiolabeling. The loaded amount of [^{99m}Tc]Tc in nab-PTX was also presented in Table 1. Our results showed that over 99% of added [^{99m}Tc]Tc were loaded into the nab-PTX.

Table 1. The radiochemical purity of [^{99m}Tc]Tc-nab-PTX and loaded amount of [^{99m}Tc]Tc up to 6-h.

Time (h)	[^{99m}Tc]Tc-nab-PTX (%)	Loaded amount of [^{99m}Tc]Tc (MBq)
0.25	99.28 ± 0.11	36.73 ± 0.03
1	99.46 ± 0.26	36.80 ± 0.02
2	99.36 ± 0.15	36.76 ± 0.03
3	99.18 ± 0.06	36.70 ± 0.03
4	99.34 ± 0.16	36.76 ± 0.02
5	99.27 ± 0.21	36.73 ± 0.04
6	99.45 ± 0.19	36.80 ± 0.01

In cell culture studies, [^{99m}Tc]Tc-nab-PTX was treated with cell media for 2-h. So, the stability of [^{99m}Tc]Tc-nab-PTX in cell media was assessed, and the results were presented in Figure 2.

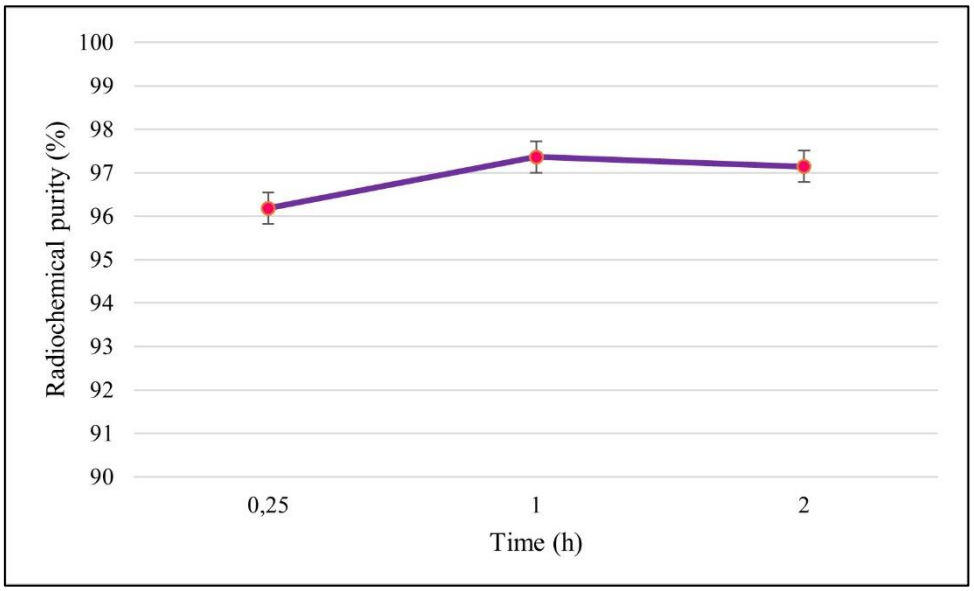

Figure 2. The radiochemical purity of [^{99m}Tc]Tc-nab-PTX in cell medium up to 2-h.
*Cell medium: McCoy's 5A containing 10% fetal bovine serum

The RP of [^{99m}Tc]Tc-nab-PTX in cell medium was found to be quite stable with >95% labeling efficiency ($p < 0.05$) (Figure 2). So, [^{99m}Tc]Tc-nab-PTX was found suitable for cell binding study.

Cell culture studies have an important place in recent research, since it is a very costly process to develop a cancer model in experimental animals due to the cost, technical infrastructure, and the need for specialized personnel. For this purpose, evaluating the efficacy of radionuclide-labeled drug formulations by cell culture studies is a very innovative research area [34-36]. In this study, the capacity of [^{99m}Tc]Tc-nab-PTX to bind to HT-29 cells was investigated. The tests were evaluated for 2-h due to the available half-life of [^{99m}Tc]Tc. The cell binding percentage to HT-29 cell lines of [^{99m}Tc]Tc-nab-

PTX and R/H-[^{99m}Tc]$NaTcO_4$ (as a control group) were shown in Figure 3.

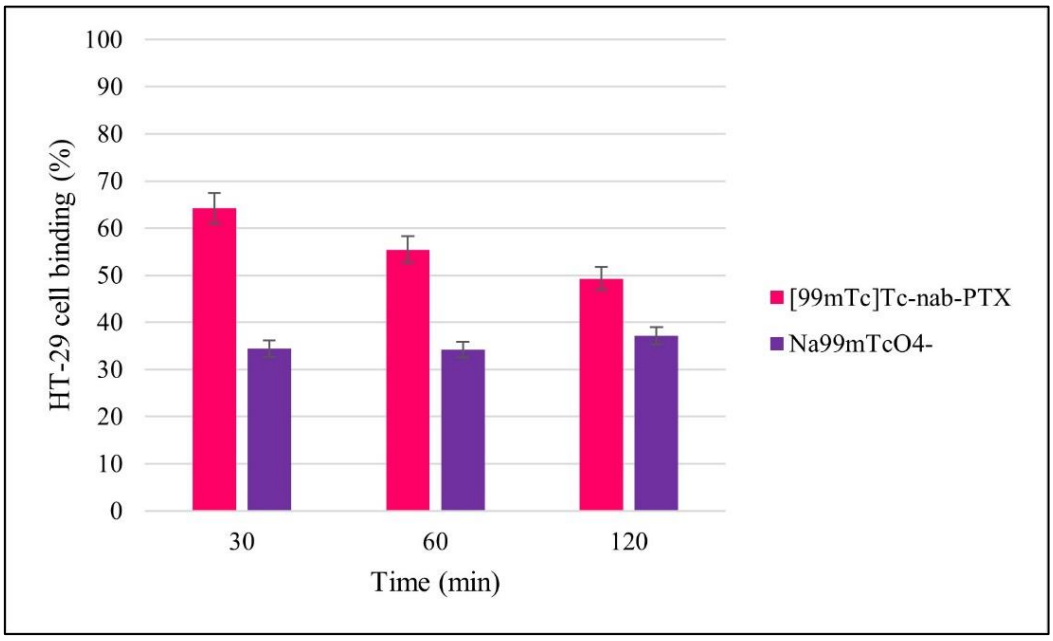

Figure 3. Cell incorporation of [^{99m}Tc]Tc-nab-PTX on HT-29 cell line.

In addition to avoiding radiation damage to non-target tissue, the high binding ratio of radiolabeled formulations in the target tissue enables us to collect high-quality images. Low target/non-target ratio can localize in nontargeting organs and harm these tissues, while also affecting the quality of target organ images [37]. As seen in Figure 3, [^{99m}Tc]Tc-nab-PTX had a greater cell binding activity on HT-29 cells than R/H-[^{99m}Tc]$NaTcO_4$ during experimental time *via* passive targeting. The cell binding percentage of [^{99m}Tc]Tc-nab-PTX was ranged from 64.24±3.51% at 30-min to 49.29±2.53% at 120-min. [^{99m}Tc]Tc-nab-PTX had a rate of almost 2 times higher to cancer cells then R/H-[^{99m}Tc]$NaTcO_4$ [9,38].

Also, to control the study, the cell binding percentage of R/H-[^{99m}Tc]$NaTcO_4$ was ranged from 34.43±1.56% at 30-min to 37.14±1.78% at 120-min. This finding demonstrates that our radiolabeled formulation reacted differently in cell medium than R/H-[^{99m}Tc]$NaTcO_4$ and verified the high labeling efficiency and *in vitro* stability.

In conclusion, nab-PTX was successfully radiolabeled with high radiochemical purity (>99%). The [^{99m}Tc]Tc-nab-PTX was found to be stable in saline up to 6-h, and in cell media up to 2-h. According to the *in vitro* cell culture studies, [^{99m}Tc]Tc-nab-PTX had a greater cell binding activity on HT-29 cells than R/H-[^{99m}Tc]$NaTcO_4$ at 30 min. In the light of the data obtained, it was found that this nanoparticular radiopharmaceutical prepared for colorectal cancer imaging is a step for *in vivo* studies. Further studies with [^{99m}Tc]Tc-nab-PTX are in progress in order to evaluate biodistribution and imaging of the complex in experimental animals.

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AUTHOR CONTRIBUTIONS

Concept: M.E., D.İ.Ö.; Design: M.E., D.İ.Ö.; Control: M.E., D.İ.Ö.; Sources: M.E., D.İ.Ö.; Materials: M.E., D.İ.Ö.; Data Collection and/or Processing: M.E.; Analysis and/or Interpretation: M.E.; Literature Review: M.E.; Manuscript Writing: M.E.; Critical Review: M.E., D.İ.Ö.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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