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New Approach to Inducing Rat Tcell Activation Against Colon Cancer by Using Cancer Mediate Exosome: An *in vitro* Study

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ABSTRACT

Although exosomes were first described as cellular waste in the late 1980s, their role in cellular communication has been revealed by recent studies. In addition to components such as DNA, RNA, and protein, it is thought that it may also be associated with the immune system, as it contains tetraspanins such as CD9, CD81, and major histocompatibility complex (MHC) molecules. Therefore, it has been seen as a new source for immunotherapy. Immunotherapy is one of the methods used for colon cancer, which is one of the most common and deadly cancers, where traditional treatments are insufficient. In our study, we first performed exosome isolation from the CaCo-2 cell line, then lymphocyte (T lymphocyte) activation by the exosome. Then, we counted the activated lymphocytes (10,000, 20,000, 40,000, and 80,000 cells) and applied them to the CaCo-2 cell line in vitro. After 48 hours, we performed viability (MTT), antioxidant (TAC), oxidant (TOS) and lactate dehydrogenase (LDH) analyzes. Exosome characterization was demonstrated with TEM, SEM, and AFM images. According to our results, it was seen that the lymphocytes activated by exosomes act at similar rates with the lymphocytes activated by IL-2. In the groups given 80,000 cells, a significant decrease was observed in the viability and antioxidant level of the cancer line, while an increase in oxidant and lactate levels was observed. The tumor-suppressive properties of exosomes obtained from immune cells have been demonstrated in the literature. We have successfully produced this study with our own experience and knowledge of the literature. We have successfully produced this study with our own experience and knowledge of the literature.

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Introduction

Exosomes are type of microvesicles with endosomal origin. The earliest identification of exosomes in the extracellular space was in the late 1980s. Until this date, exosomes secreted from cells were thought to be cellular wastes resulting from cell damage or by-products of

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cell homeostasis. However, recently it has been shown that these vesicles have structures such as proteins, lipids, nucleic acids and have important effects on intercellular communication [1]. Exosomes exert very important effects on many cellular processes such as immune response, signal transduction, and antigen presentation. In addition, exosomes contain structures such as tetraspanins (CD9, CD63, CD81, etc.), heat shock proteins (such as HSP70 and HSP90), and major histocompatibility complex (MHC) molecules and play a role in antigen presentation [2]. It is thought that due to increased growth rates or stressful conditions, cancer cells tend to release more exosomes than healthy cells. The use of exosomes as an immune activator together with the above feature and ability to present antigen makes our study very valuable in terms of specificity [3-5]. It is now clear that exosomes play important roles in various diseases, including cancer, infection [6], cardiovascular and autoimmune diseases [7]. Due to the MHC molecules in the structure of exosomes, their effects on the immune system including maturation, antigen presentation, activation and differentiation of immune cells are becoming increasingly interesting [8]. It has also been shown that exosomes secreted by cancer cells can promote cancer cell growth [9], as well as trigger anti-tumor responses dependent on CD8⁺ T cells by antigen presentation and stimulation of T cells [10].

The main purpose of cancer immunotherapy is to increase the activity of intracellular cytotoxic T cells (CTL) and to create effective and permanent anti-cancer immunity. Therefore, CD8⁺ T cells play a key role in controlling cancer [11]. The fact that exosomes released from cancer cells can also respond to CD8⁺ T cells shows that they can be used in immunotherapy.

Surgical resection has traditionally been the first choice in the treatment of colorectal cancer. It is effectively preferred with chemotherapy, radiation therapy or both to increase the patient survival rate. However, 25% of patients in the metastatic stage have a five-year survival rate of only 10% [12]. For all these reasons, more effective therapeutic strategies in the treatment of the disease are still being investigated. Various difficulties have been observed in the application of immunotherapy, which is one of these therapeutic approaches. Disadvantages are seen, such as insufficient immune effector response, limited recognition of tumor targets, or introduction of foreign components into the body.

In our study, we aim to activate T lymphocytes by using exosomes obtained from colon cancer, and to reveal the effects of activated T cells on colon cancer in vitro as cell viability, oxidant and antioxidant.

Material and Methods

Cell culture and doses

The Caucasian colon adenocarcinoma (CaCo-2) cell line was obtained from Ataturk University, Department of Medical Pharmacology. It was allowed to grow to sufficient density (80%) in 25 cm³ flasks (incubation medium at 37°C and 5% CO₂). After the CaCo cells were grown, their medium was removed and exosomes were isolated according to the procedure. Blood was cultured from animals, and white blood cell isolation was performed in vitro. The exosome was applied at a dose of 10 µg/ml to each blood culture medium and the isolated T cells were activated.

Exosome-activated T lymphocytes were applied to the CaCo-2 cell line as 10,000, 20,000, 40,000 and 80,000 cells.

Lymphocyte (T lymphocyte) isolation

Whole blood from the donor is collected in heparinized blood tubes, then diluted with double volume of sterile PBS. Pipette the density gradient solution into a separate centrifuge tube. An equal amount of diluted blood is added onto the density gradient solution for phase separation. Centrifuge for 20 minutes at 1200 x g. Then, the Peripheral Blood Mononuclear Cell (PBMC) layer containing lymphocytes is carefully removed with a sterile pipette. Add 3-4 times the volume of PBMCs in PBS and mix gently. Centrifugation at 200 x g for 10 minutes is repeated 3 times and the cells at the bottom are lymphocyte cells. Isolated lymphocyte cells are allowed to grow in a medium. After the cells reach a sufficient density, activation is performed with the exosome 10 µg/ml dose and the doses are adjusted as described above.

MTT tetrazolium assay concept

MTT analysis was performed with a purchased kit (Sigma Aldrich, USA). MTT reagent was added to each well of the 96-well plate and the plate was incubated for 4 hours (5% CO₂; 37°C). The medium was removed after 4 hours and 100 µL of dimethylsulfoxide (DMSO)

(Sigma, USA) was added to dissolve the formazan crystals. Cell viability (%) was measured using a spectrophotometer reader based on optical density at 570 nm (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Canada, USA). The control group was accepted as 100, other groups were calculated according to the formula below [13].

$$\text{Viability Rate (\%)} = (\text{O.D of groups/Control O.D}) \times 100$$

Total antioxidant capacity (TAC) assay

Total antioxidant assay testing was performed with commercially purchased kit (Rel Assay Diagnostics® Company (Gaziantep, Turkey)). Briefly, to determine the antioxidant level, 30 µL samples followed by 500 µL Reagent 1 solution were added to the wells and the first absorbance value was measured at 660nm. For the second absorbance measurement, 75 µL Reagent 2 solution was added to the same wells, and measurement was made at 660nm after 10 minutes. Absorbance values were applied according to the formula below and antioxidant values were calculated as Trolox Equiv mmol/L⁻¹ [14].

$$A2-A1 = \Delta \text{ Absorbance (Standard, sample or H}_2\text{O)}$$

$$\text{Result} = \frac{(\text{H}_2\text{O } \Delta \text{ Abs} - \text{Sample } \Delta \text{ Abs})}{(\text{H}_2\text{O } \Delta \text{ Abs} - \text{Standard } \Delta \text{ Abs})}$$

Total oxidant status (TOS) assay

Total oxidant status (TOS) is the evaluation of color intensity in the spectrophotometer based on the number of oxidants in the sample (from Rel Assay Diagnostics® Company (Gaziantep, Turkey)). To determine the TOS level, 500 µL of reagent 1 was added to the wells containing 75 µL of sample and the first absorbance value was measured at 530 nm. Then, 25 µL of Reagent2 solution was added to the same wells, incubated for 10 minutes at room temperature, and the second absorbance value was measured. Absorbance values were changed according to the procedure as stated below, and TOS values were calculated as H₂O₂ Equiv/mmol L⁻¹.

$$A2-A1 = \Delta \text{ Absorbance (Standard or sample)}$$

$$\text{Result} = \frac{\text{Sample } \Delta \text{ Abs}}{\text{Standard } \Delta \text{ Abs}} \times 10$$

Lactate dehydrogenase (LDH) analyze

Lactate dehydrogenase level was determined according to the LDH detection kit (Cayman Chemicals, USA) and manufacturer's instructions. OD values were determined after the measurement made at 440 nm with the spectrophotometric method.

Exosome isolation and characterization

When the cell line (CaCo-2) grown in flasks reached the desired density (80%), the cell medium was removed from the medium. Ultracentrifugation method was applied to the cell medium collected for exosome isolation. First, the cell medium is centrifuged for 10 minutes at 300 x g at 4°C. The resulting supernatant was filtered through a 0.22 µm filter to remove microparticles and cell debris. Centrifugation was then repeated at 100,000 x g at 4°C for 90 minutes. The supernatant was carefully removed and the pellet portion containing the exosomes was resuspended in cold PBS. Finally, it was centrifuged a second time at 100,000 x g for 90 minutes at 4°C and pure exosomes were suspended in 500 µL of PBS.

Scanning electron microscopy (SEM)

SEM, Exosome were seeded on glass slides. Three glass slides were selected. For inspection, the specimens were commissioned to the Eastern Anatolia High Technology Application and Research Center (DAYTAM).

Transmission electron microscopy (TEM)

TEM was used to observe the Exosome. The Exosomes were fixed on clean glassed then kept at 4°C until further analysis. The specimens were subsequently commissioned to the Eastern Anatolia High Technology Application and Research Center (DAYTAM) for post-processing.

Statistical analysis

Results were calculated as mean ± standard error. Statistical comparison between groups was calculated using One-way ANOVA and Tukey LSD method. For statistical analyzes, all calculations were performed using SPSS 20 software and statistically P<0.05 was considered to be a significant difference in all tests.

Results

MTT tetrazolium assay concept results

In our study on the CaCo-2 cell line, after the cells reached the appropriate density (80%), we applied the lymphocytes activated with exosome and IL-2 to our Caco-2 cells in vitro and showed their effects on cell viability after 48 hours in figure 1. According to our results, we revealed that cell viability decreased as the number of cells increased in lymphocyte groups activated with exosome and IL-2. The most significant results were seen in the exosome/40,000 and exosome/80,000 groups, while the exosome/80,000 group, which reduced viability by 35%, was more effective. Another important result of the exosome/80,000 group is that the interleukin-2 (IL-2) cytokine, which is known to play a role in the stimulation of lymphocytes (Ross & Cantrell, 2018), has similar results with the IL-2/80,000 group. Thus, we have shown that lymphocytes activated by exosome, which is the main purpose of our study, are effective.

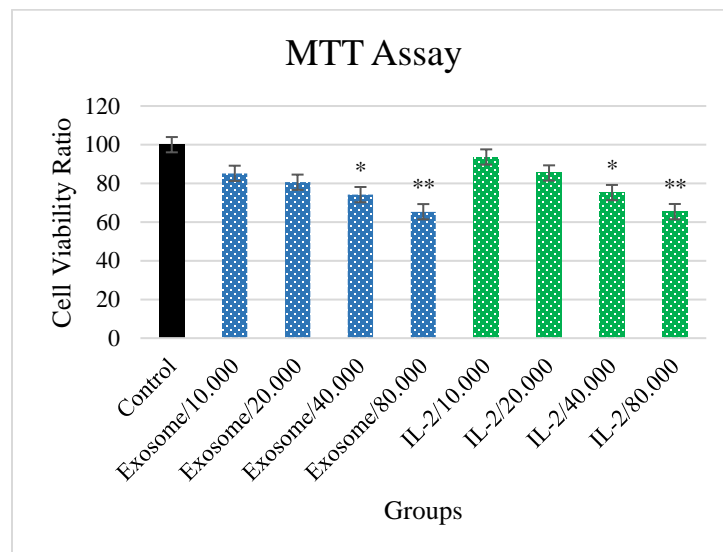


Fig 1 MTT assay results for CaCo-2 cell line after 48h exosome and IL-2 activated lymphocytes (T lymphocyte) treatment. (*P < 0.05 compared to control group; **P < 0.001 compared to control group)

Total antioxidants capacity (TAC) and total oxidant status (TOS) results

When our results in figures 2 and 3 were examined, it was found that there was no significant difference between exosome/10,000 and exosome/20,000 groups and IL-2/10,000 and IL-

2/20,000 groups in antioxidant and oxidant levels. The exosome/40,000 and exosome/80,000 groups, which reduced the antioxidant level to 4.1 and 3.8 Trolox equiv mmol/L⁻¹, respectively, showed a significant difference. In the effects of the same groups on the oxidant level, it was observed that the oxidant level increased in parallel with the antioxidant results (exosome/40.000, 1.6-time increase; exosome/80.000, 1.8-time increase).

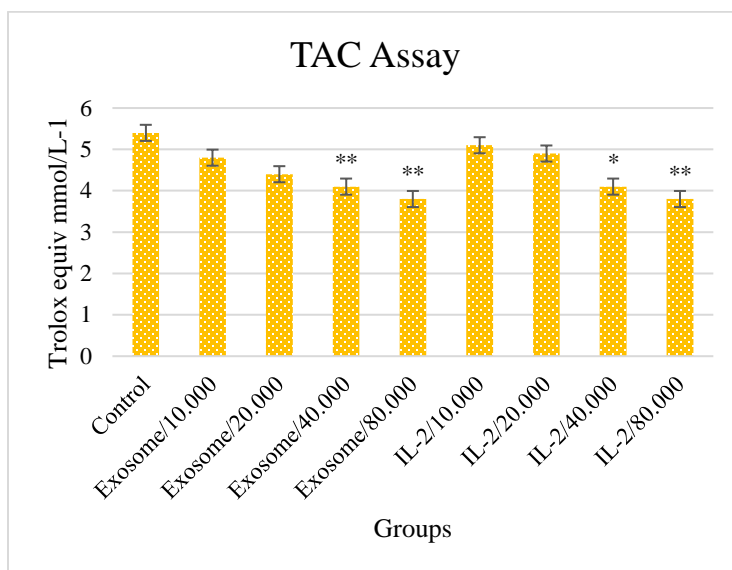


Fig 2 TAC Assay for CaCo-2 cell line after 48h exosome and IL-2 activated lymphocytes (T lymphocyte) treatment. (*P < 0.05 compared to control group; **P < 0.001 compared to control group)

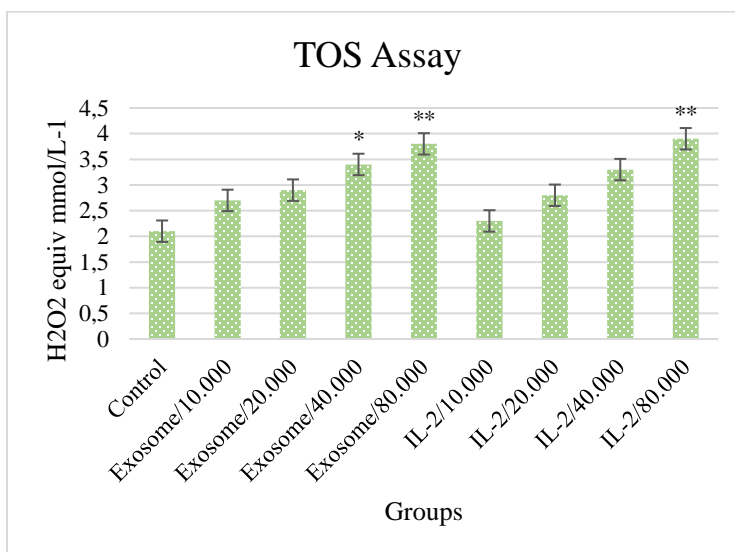


Fig 3 TOS Assay for CaCo-2 cell line after 48h, exosome and IL-2 activated lymphocytes (T lymphocyte) treatment. (*P < 0.05 compared to control group; **P < 0.001 compared to control group)

Lactate dehydrogenase (LDH) result

Lactate dehydrogenase (LDH) can be used as a marker for cell death both in vitro and in vivo. We can say that cellular viability decreases due to the increased LDH value in vitro. When we examine our results in Figure 3 based on all this information, we see that lymphocytes activated by exosome and IL-2 increase the LDH level due to the increasing number of cells. The most significant result was the exosome/80.000 group, which increased the LDH level 2.2 times and produced results close to the IL-2/80.000 group.

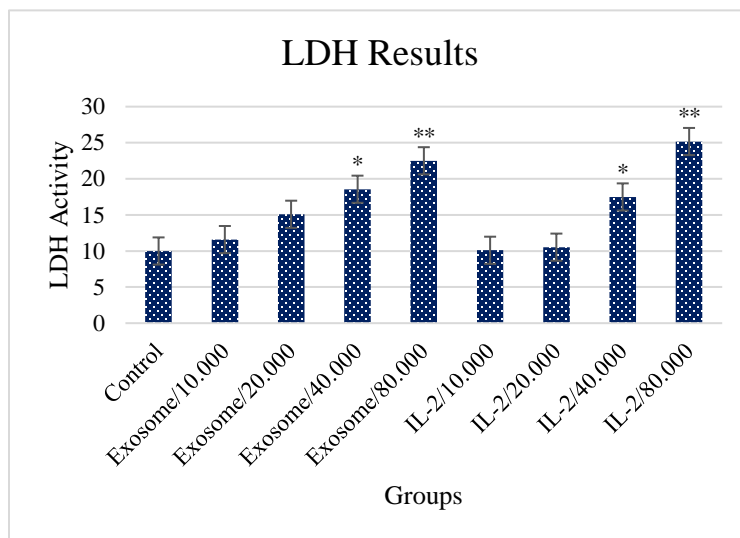


Fig 4 LDH Results for CaCo-2 cell line after 48h exosome and IL-2 activated lymphocytes (T lymphocyte) treatment. (*P < 0.05 compared to control group; **P < 0.001 compared to control group)

Atomic force microscopy (AFM) results

The microstructure of exosomes was visualized in figure 4 by atomic force microscopy (AFM). The vesicle sizes in the indicated images confirm that they consist of typical exosome-sized populations of vesicles, consistent with exosome AFM images reported in the literature (Di Noto et al., 2014; Raposo and Stoorvogel 2013; Sharma et al., 2010).

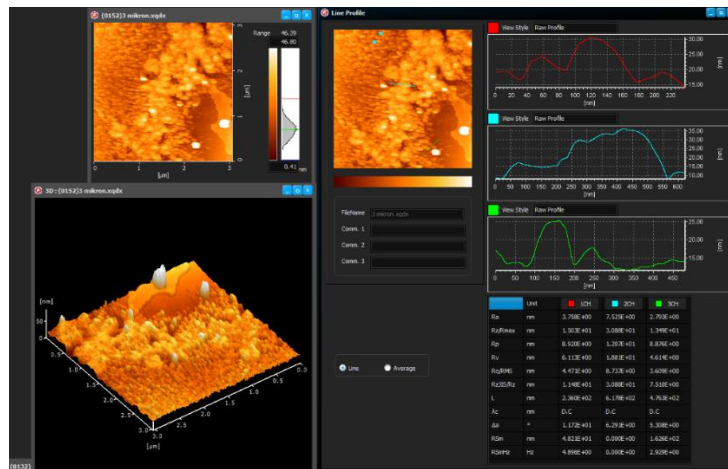


Fig 5 Atomic force microscopy (AFM) images of isolated exosomes

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)

Electron microscopy (EM) is a widely used technique to characterize and visualize a variety of samples. In biological sample studies, two types of EM are commonly used, namely transmission electron microscopy (TEM) and cryo-electron microscope (cryo-EM). In our study, we obtain TEM images with the hitachi TEM system to visualize exosomes and determine their diameter and show them in figure 5A. We also support the exosome characterization with the results we obtained with the ZEISS device at 200 nm magnification. With the scanning electron microscope (SEM) image in Figure 5B, we show vesicles with a size of 115 nm. In summary, the structures of colon cancer-derived exosomes were revealed by both TEM and SEM results. It is shown in figure 5 that they are round or oval in shape and their size ranges from 40-150 nm.

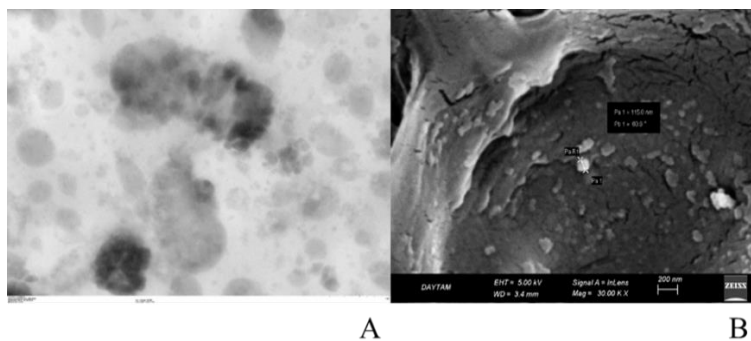


Fig 6 Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images of isolated exosomes

Discussion

In studies on exosomes and their biological functions, it has been revealed that exosomes obtained from different cell types provide intercellular communication [1]. The main cell types are fibroblasts, endothelial cells and immune cells that interact with the signals of exosomes in the tumor microenvironment (TME). As a result of these interactions, different pathways are triggered [15]. For example, exosomes in the TME can act as a cargo in carrying information, and may enable tumor cells to proliferate and cancer to progress. These exosomes are completely tumor-focused and can induce metastasis by silencing and altering antitumor immune responses Peinado et al., (2012) demonstrated in their study that exosomes derived from metastatic melanoma cells can program the bone marrow into an environment that supports the development of invasive melanoma cells.

Exosomes in the TME can also stimulate immune cell functions or suppress antitumor activity. Factors such as the origin, capacity and load of exosomes are important in these stimulating and inhibitory functions. For example, exosomes in the TME may be the main source of activated immune cells in early-stage tumor progression, but not in advanced cancers. On the contrary, exosomes in TME are secreted to suppress the immune system [16]. Liu et al., (2006) showed that in vivo breast cancer-derived exosomes exert immunosuppressive effects by inhibiting NK cell proliferation by tumor-mediated exosomes. It has been shown that tumor-mediated exosomes can also target myeloid cells to modulate their function. Valenti et al., (2006) demonstrated that exosomes originating from human colon cancer cells exert a suppressive effect on T cells in vitro through TGF- β 1.

Immunotherapies, which use immune cells for treatment, aim to increase the activity of the immune system to destroy cancerous cells [17]. In colon cancer, T cell infiltration into the tumor site suggested that immune-regulation may play a role in tumor progression [18]. In the 1900s, Paul Ehrlich suggested that the human body constantly produces neoplastic cells that are destroyed by the immune system, and today, cancer immunotherapy has re-emerged and made significant progress [19]. While Halliday et al., (1995) showed the ability of T cells to fight cancerous tissue in their studies, Teng et al., (2015) proved that natural killer (NK) and active cytotoxic T cells (CD8+T) recognize and eliminate cancer cells. Again, in different

studies, it has been revealed how CD8 + T cells prevent the development of many cancers [20-22].

T lymphocytes effectively control almost all parts of the body to capture foreign substances. Eventually, naive and active T cells become migratory cells, which are highly skilled in enhancing immunity against undesirable conditions such as infection and cancer. In particular, CD8+ T cells are very important in immunotherapy as they are the main killers of pathogens and neoplastic cells. CD4+ T cells, on the other hand, are effective in maintaining the effect of CD8+ T cells and preventing depletion [23].

The main purpose of our study, it is revealed by studies that exosomes increase their function by adding a new perspective to immunotherapy and their importance is increasingly understood. Seo et al., (2018) showed that tumor mesenchymal cells were depleted and tumor progression stopped by applying exosomes obtained from activated CD8+T cells to the cancer site in vivo. When we examined the in vitro viability results in our study, we revealed that lymphocytes activated by exosomes obtained from the CaCo-2 cancer line also affected the viability of the CaCo-2 cell line. Similarly, Klibi et al., (2009) showed that tumor-mediated exosomes affect T cell proliferation, activation and apoptosis. They showed that exosomes obtained from human nasopharyngeal carcinoma (NPC) stimulate regulatory T cell (Treg) formation via miRNAs and inhibit T cell proliferation. On the other hand, we demonstrate that in addition to vitality, activated lymphocytes increase the oxidant level by decreasing the antioxidant level in the CaCo-2 cell line. Tokuda et al., (2021) also investigated the development of cancer by administering cancer-mediated exosomes in vivo intraperitoneally. As a result, they observed that cancer development was inhibited and NK cell stimulation increased. Various studies have shown that IL-2, which we used as a control point in our study, is effective in T cell proliferation and activation [24-26].

Conclusion

In our study, when we examined the effects on the CaCo-2 line of lymphocytes activated by exosomes against lymphocytes activated by IL-2, we found that both groups were effective, especially when 80,000 cells were applied. We demonstrated that exosomes can be as effective in activating as IL-2. The effective results found have been a source of hope for the

development of these studies, especially in the field of immunotherapy and in the treatment of a very important cancer type such as colon cancer. The idea of combining immunotherapy with exosomes, which is increasingly important, has been studied recently, but still no effective results have been obtained. We think that our work will be an important start in this sense.

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