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Investigation of the Exosome-Based Drug Delivery System Potential in the Treatment of Glioblastoma *in vitro* Experimental Models

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ABSTRACT

In our study, it was aimed to create a new drug delivery system by loading the chemotherapeutic drugs into exosome vesicles. This system act as a Trojan horse to targeting GBM cancer. Exosomes were isolated from the T-98G cell line, and characterized. Exosomes were encapsulated with Temozolomide. Then, the effectiveness of this new delivery system was evaluated by using MTT, LDH, TAC, TOS, GR. MTT results show a dose-dependent decrease in the TMZ, and Exolimer+TMZ groups. But this rate decreased significantly in the Exolimer+TMZ groups. The lowest viability was observed at the Exolimer+TMZ 800 ng/ml concentration. Our results in LDH, GR, TAS, and TOS analyzes shows correlation with MTT. Based on this study, we think that targeting the exosome to cancer by combining with special molecules, and nanotechnology will bring a new perspective to cancer treatment.

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Introduction

Glioblastoma Multiforme (GBM) is the most common primary malignant brain tumor, comprising 16% of all primary brain, and central nervous system neoplasms [1]. The incidence rate is 3.2 individuals per 100,000 population. In these individuals, cancer as location; is seen in all four lobes of the brain, frontal (25%), temporal (20%), parietal (13%), , and occipital (30%), , and sometimes in the brain stem, cerebellum , and spinal cord (12%) [2].

Exosomes are secreted by various cell types, including immune, and tumor cells. These vesicles are produced within multivesicular endosomes , and are released through the plasma membrane [3]. The diameter of exosomes released from cancer cells ranges from 30 to 100 nm. Released exosomes as content; proteins (including oncoproteins) [4], lipids [5], DNA [6] , and RNAs [7, 8], microRNAs [miRNAs (including resistance genes)] , and

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other non-coding RNAs It carries a variety of bioactive molecules such as [ncRNAs] [9]. In addition, tumor-derived exosomes promote tumor progression by regulating drug resistance. These drug resistance mechanisms; (1) tumor cells can use exosome secretion to metabolize anticancer drugs; (2) reducing therapeutic effects of anticancer drugs by competing with exosome (3) exosomes transmit resistance from drug-resistant cells to drug-sensitive cells [10].

Nanoscale drug delivery systems, which are used to increase the therapeutic effectiveness of chemical , and biomolecular drugs, are of great importance today [11]. However, these transport systems have some limitations. Besides of small capacity, the cancer exosome shows the cytotoxic effects, and rapidly was removed by the reticuloendothelial system (RES) or the mononuclear phagocyte system (MPS) [12]. In recent studies, it is emphasized that these vesicles can be used as a drug delivery system as a result of modification, and that the drug delivery system developed with this method will be more advantageous than other methods. However, studies on the use of exosomes as a drug delivery system are very few in the literature. These studies, on the other h, and, are mostly in the form of compilations [13, 14].

Based on this information, and this deficiency in the literature, we are planning to create a new drug delivery system by loading chemotherapeutic agents into exosome vesicles used as Trojan horses *in vitro*. However, with the help of the new drug-immuno-active transport system developed, it is thought to eliminate drug resistance, and increase treatment efficacy.

Material Method

Chemical and reagent

Temozolomide, High glucose Dulbecco eagle medium, Fetal Bovine Serum (FBS), Antibiotic, Hanks Balanced Salt Solution (HBSS) were obtained from Sigma, USA.

Exosome isolationand characterization

Exosome isolation

Exosomes isolated from T98G cells. For this aim T98G cells were seeded in a fresh medium (High glucose DMEM, FBS %10, Antibiotic %1). After reaching 70% confluency ratio. The cells were kept in FBS free medium for 48 hours. At the end of the 48^{th} hour, the liquid in the flask was obtained and various centrifugation processes were

done. The supernatant was taken and ultracentrifugation was performed. All centrifugation processes were carried out at 4 °C.

Exosome characterization

Transmission electron microscope (TEM)

TEM results were obtained from Ataturk University DAYTAM center. Briefly, exosomes were mounted on grids, and after adding 1% glutaraldehyde it washed in sterile distilled water for 5 minutes at pH 7. More cellulose was removed, and the samples were dried for permanent protection. TEM was used to image exosome samples at a voltage of 80 kV.

Temozolamide Exolimer loading

For the bulk synthesis of nanocapsules (NCs), a solution of PDMS-PEG in THF was prepared under appropriate conditions. Nanoprecipitation, and formation of NCs were accomplished by adding this solution dropwise to water under vigorous stirring.

For drug-loaded NCs, Temozolomide was dissolved in CDCL3 (Deuterated chloroform) , and mixed with polymeric (PDMS-PEG , and Pluronic® F-127) solution.

For the microfluidic synthesis of nanocapsules (NCs), a solution of PDMS-PEG/Pluronic in THF was prepared under stirred conditions, and then fed to the microchip as the mainstream. The water flows act as a non-solvent, and the lateral flow ensures self-assembly during mixing. The flow rate between polymer, and water streams can be easily adjusted using Syringe Pumps to change the degree of focus, and thus mixing time.

For drug-loaded NCs, TMZ was dissolved in THF at four different initial loadings of 5, 10, 15 , and 20% by weight mixed with the polymeric solution. A mixture of Pluronic F127, , and TMZ (10 wt% initial loading weight) without polymer at a constant flow rate of 0.076 was used as control samples.

Cell culture

T98 cell line for our study was obtained from Ataturk University (Erzurum, Turkey) Medical Pharmacology Department. Briefly, the cell suspension was centrifuged at 1200 rpm for 5 minutes. Cells were resuspended in a fresh medium (DMEM, FBS 15%, and antibiotic 1%), and seeded in flask (stored in an incubator 5% CO_2; 37 °C). When 80% of the flask was covered with cells, it was removed with Trypsin- EDTA (0.25% trypsin-0.02% EDTA) to 96 well plate.

Cytotoxicity analysis

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) analysis

To determine the cytotoxicity of the materials, the 'direct contact test method' was applied, and an evaluation was made with the MTT substance (Sigma Aldrich inc, St. Louis, USA) containing 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltatrazilium bromide. To determine cytotoxicity with the MTT test, a mixture of 5 mg MTT powder in 1 ml of PBS was prepared, passed through a sterile 0.20 μm filter (Corning, Wiesbaden, Germany), , and kept at +4 °C until the time of use after its outer surface was covered with aluminum foil. After the medium liquids of the incubated cells were withdrawn, the previously prepared samples were placed in each well and incubated again for 24 , and 72 hours at 37 °C in an environment containing 5% CO_2. Thus, the cytotoxic effects of the materials used at the end of 24, and 72 hours were evaluated.

To solubilize the formazan crystals formed as a result of the application of MTT, 99.4 ml dimethylsulfoxide (DMSO), 0.6 ml (HCl), and 10 g sodium lauryl sulfate (SDS) were added to the mixture as 100 μl/well and incubated again for 4 hours. After this process, absorbance (optical density) was measured in a spectrophotometer (μQuant, Bad Friedrichshall, Biotek) at a wavelength of 570 nm. The data are presented as graphs after SPSS analysis [15].

Measurements of lactate dehydrogenase (LDH) activities

Ready-made elisa was taken as a kit from Elabscience (Houston, Texas, USA). Cells lysed with the help of the kits were prepared in accordance with the protocol, and the results were determined by spectrophotometric method

Measurements of glutathione reductase (GR) activities

Ready-made elisa was taken as a kit from Elabscience (Houston, Texas, USA). Cells lysed with the help of the kits were prepared in accordance with the protocol, and the results were determined by spectrophotometric method.

Oxidant and antioxidant activity analysis

Detection of total antioxidant capacity (TAC), total oxidant number (TOS) and presence of oxidative stress in the cell

The determination of total antioxidant capacity (TAC) level , and Total Oxidant Status (TOS), were determined by commercial kits produced by Rel Assay Diagnostics® (Gaziantep, Turkey)[16].

By adding Reagent 1 solution TAC, and TOS first absorbance were read at 660 nm and 530 nm respectively. Then the second read was done after adding the Reagent 2 solution. Absorbance value was read at the same wavelength as the first reading. TAC levels in were determined as Trolox Equiv./L^{-1} , and mmol TOS levels were determined as H_2O_2 Equiv./L^{-1}[17].

Results

Exosome, and Exolymer+TMZ characterization

TEM

TEM images showed that the size of the exosomes, and Exolimer. Using TEM imaging, a well-accepted technique for nanoparticle validation, we observed that the diameters of our particles were in the expected range for exosomes (40-150 nm). In addition, in the direction of the data obtained from the image, it was determined that the exosomes were spherical, and circular structures. (Fig 1).

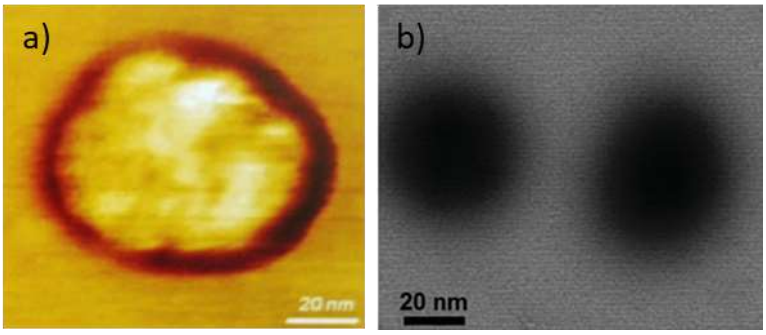

Fig 1 TEM image a) Exosome b) Exolimer

Cytotoxicity analysis

MTT results of T98-G cells

At the end of T 98-G 24, and 72 hours, the cytotoxicity results of the application groups (Exosome, Polymer, Polymer-TMZ, Exolimer, TMZ, and Exolimer-TMZ) are given in Fig. 2-4. Cell viability was increased by %21 in the Exosome 200 ng/ml group compared to the control group. According to our results, we observed that T98-D cancer cells increase the proliferation of cancer exosomes. Studies are showing that exosomes belonging to the cell in which they have secreted increase cell proliferation, and cell migration.[18]. Our results show a correlation with the literature.

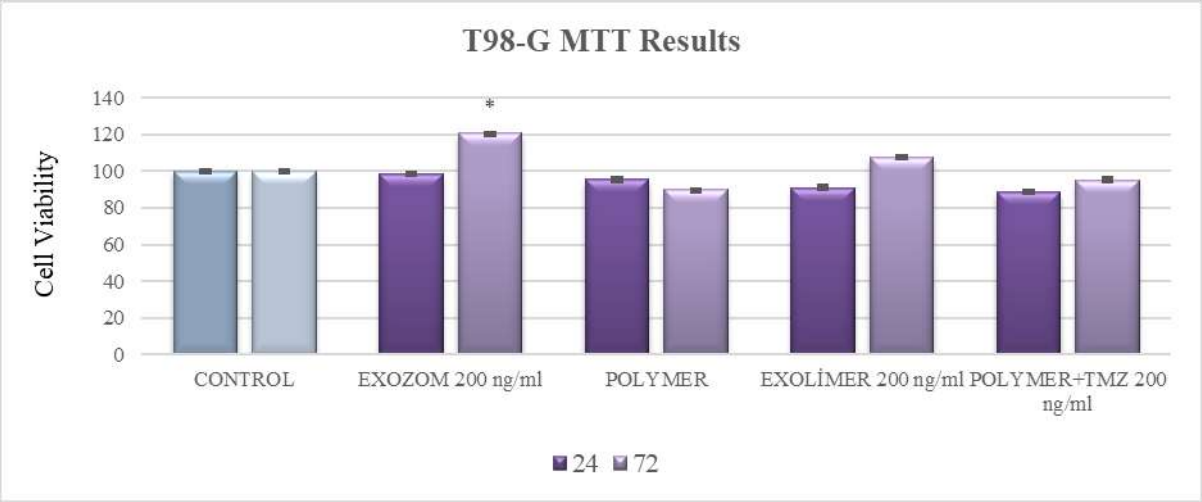

Fig 2 MTT results of control, and positive controls (exosome, polymer, exosome, and polymer exosome (200 ng/ml)). (The data in Fig. are expressed as percent (%) values compared to the control group. Only the Exosome group was found to be statistically significant in the data obtained. (P<0.05)

According to the MTT results of TMZ applied at different doses, the viability rate decreased in both hours depending on the dose (Fig. 5). After 24, and 72 hours of application, the viability rate decreased the most (%29) at TMZ 800 ng/ml, and was determined as 72.99±1.78, and 71.06±0.6438, respectively. The results of the TMZ 800 ng/ml group were found to be significant when compared to the control (P<0.05).

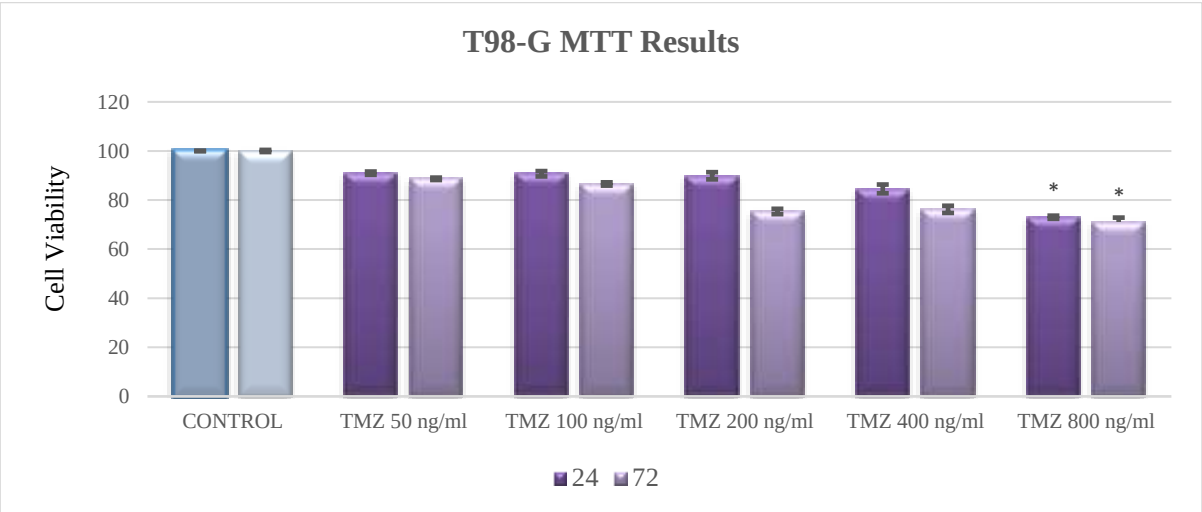

Fig 3 MTT results of control, and different doses of TMZ (50,100,200,400, and 800 ng/ml) are expressed as percent (%). (* P<0.05 values are marked as significant, ** P<0.001 values are marked as very important).

The Exolimer+TMZ group significantly decreased cell viability in both hours compared to the control group. Cell viability at Exolimer+TMZ 50 ng/ml was found to be 88.11±2.1776, and 89.34±6.5328 at 24, and 72 hours, respectively. Cell viability was found to be 84.83±2.925, and 86.27±5.85 at Exolimer+TMZ 100 ng/ml, to be 78.46± 3.56, and 86.19±7.13 at Exolimer+TMZ 200 ng/ml. It was found to be 73.70±1.28, and 72.29±3.86 at 400 ng/ml. Exolimer+TMZ 800 ng/ml group caused the most cytotoxicity in both hours. Cell viability decreased by %39, and %45, respectively. The values obtained were 69.66±2.33, and 55.77±6.99. The results were statistically significant at P<0.05, and P<0.001.

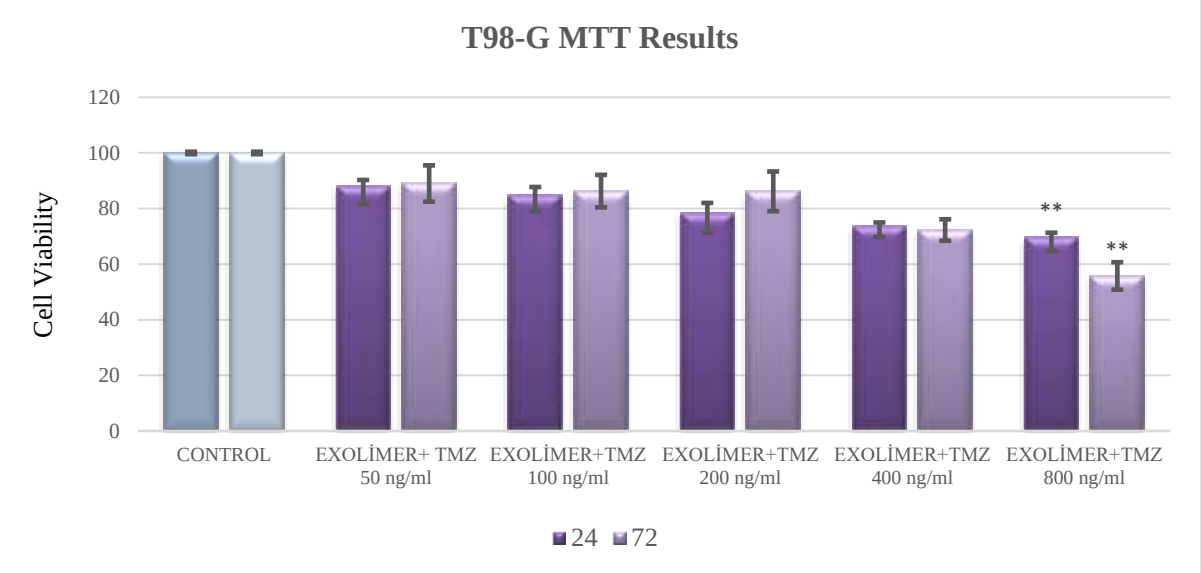

Fig 4 MTT results of control, and different doses of Exolimer+TMZ (50,100,200,400, and 800 ng/ml) are expressed as percent (%) values. (* P<0.05 values marked as significant, (* P<0.05 values marked as significant, ** P<0.001 values marked as very important)

Our MTT analysis results of T98-G cells show that the Exolimer carrier system has a cytotoxic effect on cancer cells.

LDH results of T98-G cell

In the T 98-G cell line, it was found that the Exosome 200 ng/ml, Polymer, Exolimer 200 ng/ml, and Polymer + TMZ 200 ng/ml groups increased lactate dehydrogenase activity compared to the control group, but no significant difference emerged. Despite this, a slight difference was observed in the Polymer + TMZ 200 ng/ml (72h) group compared to the control. However, this observed difference was statistically significant (Fig 5).

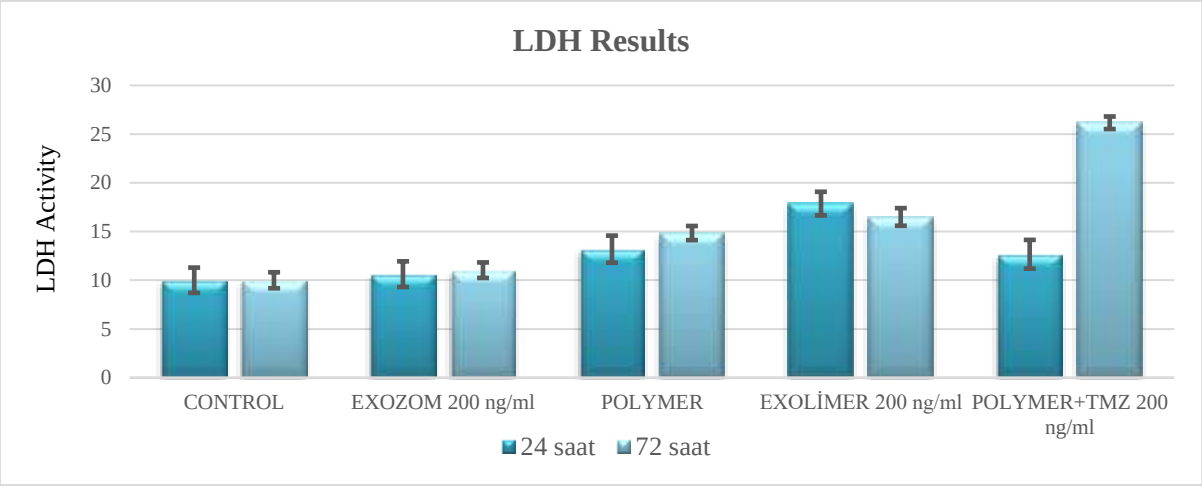

Fig 5 LDH results of Control (Negative Control), Positive Control (Exosome 200 ng/ml, Polymer, Exolimer 200 ng/ml, Polymer + TMZ 200 ng/ml) groups at 24, and 72 hours

Similar to MTT results, significant groups were observed at TMZ 400, and 800 ng/ml doses . It was observed to be three times more cytotoxic than the control at both 24, and 72 hours (P<0.05, P<0.001).

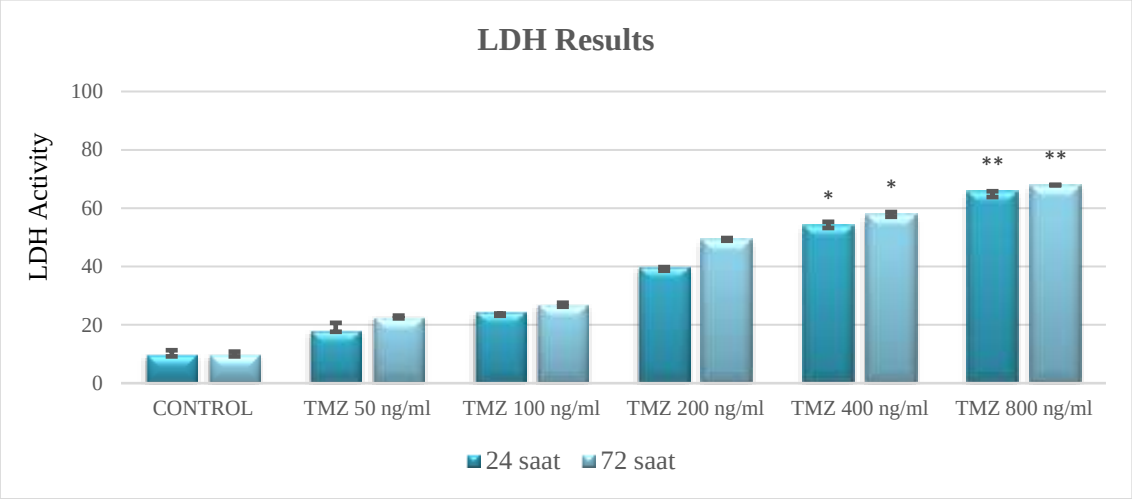

Fig 6 LDH results of the control, TMZ 50 ng/ml, TMZ 100 ng/ml, TMZ 200 ng/ml, TMZ 400 ng/ml, TMZ 800 ng/ml groups at 24, and 72 hours. (* P<0.05 values are marked as significant, ** P<0.001 values are marked as very important)

Although significant results were found in TMZ 800 ng/ml, the results were 52.6±3.8, and 60.7±1.74 hours at 24, and 72 hours, respectively, in the TMZ group encapsulated with Exolimer (Exolimer+TMZ 400-800 ng/ml), and 70.0±8.59, and 92.5±7.23, and the result was observed to increase almost 10 times compared to the control (Fig. 7). The data obtained were found to be statistically significant (P<0.05, P<0.001)

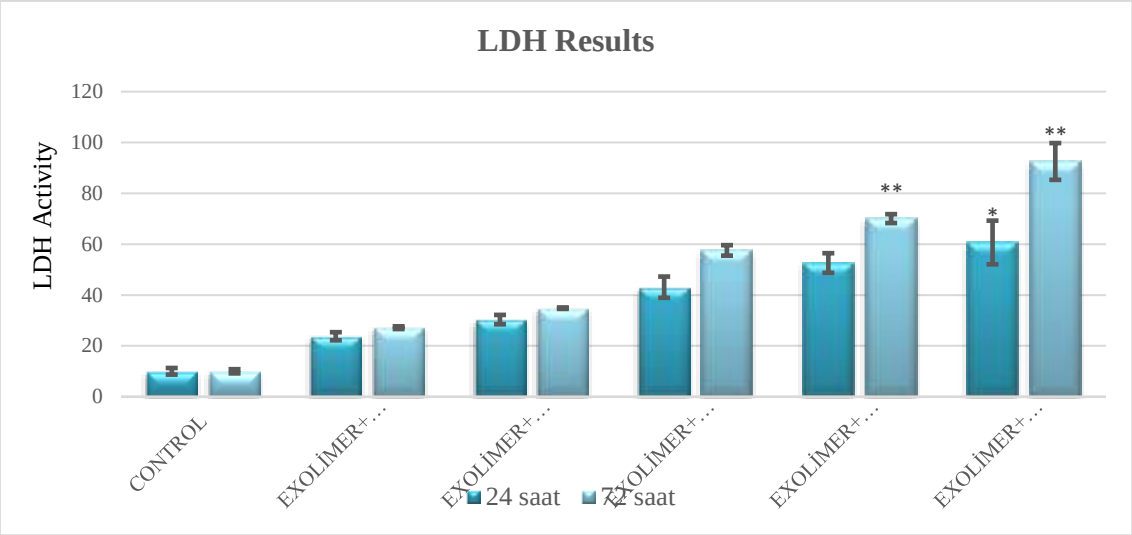

Fig 7 LDH results at 24, and 72 hours for Control, Exolimer+TMZ (50 ng/ml, 100 ng/ml, 200 ng/ml, 400 ng/ml, 800 ng/ml groups. ((* P<0.05 values) significant), ** P<0.001 values marked as very important)

GR results of T98-G cells

There was no significant change in GR level in positive controls compared to control in T98-G cells, with almost similar results with control (Fig 8).

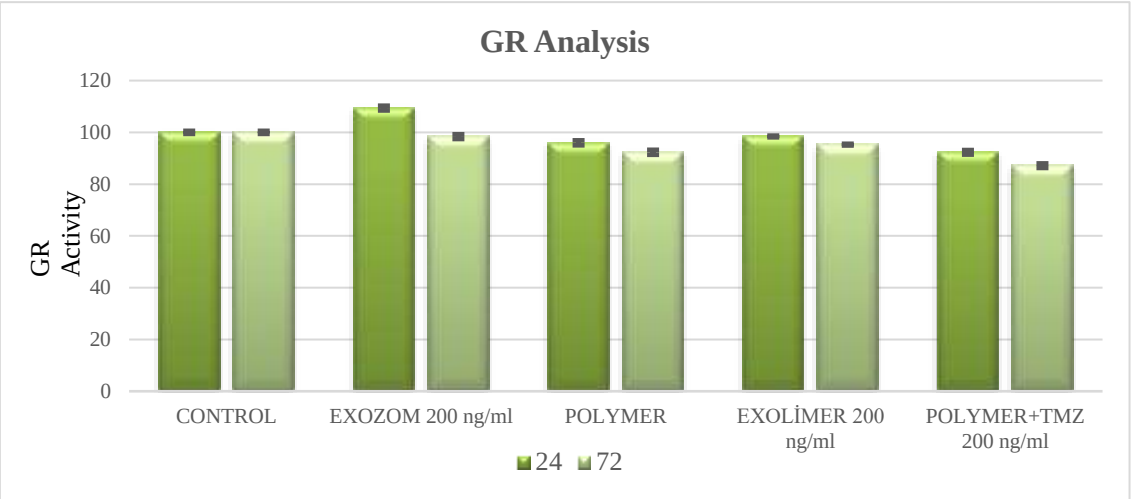

Fig 8 GR results of Control (Negative Control), Positive Control (Exosome 200 ng/ml, Polymer, Exolimer 200 ng/ml, Polymer + TMZ 200 ng/ml) groups at 24, and 72 hours

Contrary to positive controls, both hours of the TMZ high dose decreased the GR level to 61.43±0.921, and 61.69±1.868, and statistical significance was found only in this group (P<0.05).

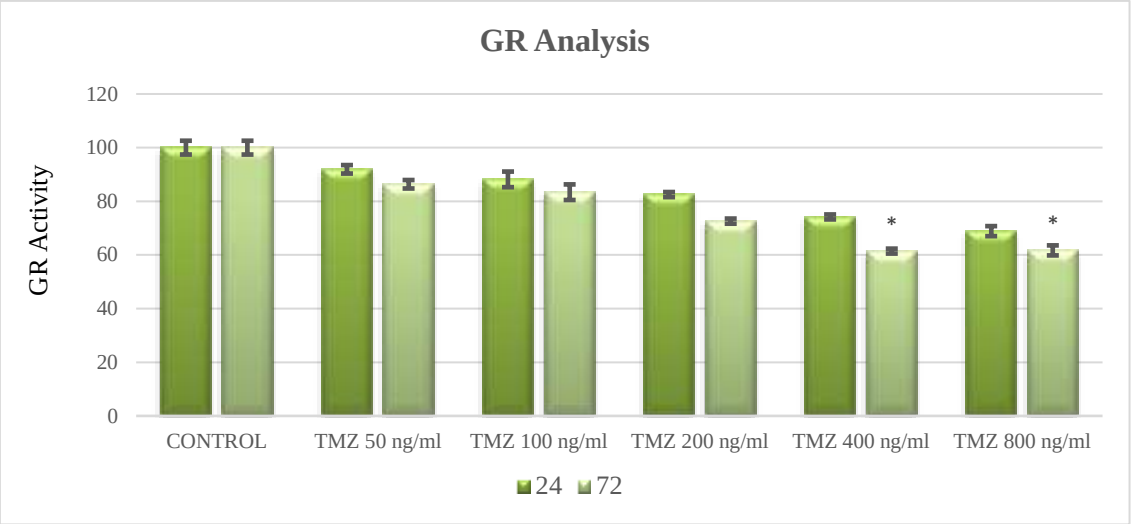

Fig 9 GR results at 24 , and 72 hours for the control, TMZ (50 ng/ml, 100 ng/ml, 200 ng/ml, 400 ng/ml, 800 ng/ml groups. (* P<0.05 values significant, ** P<0.001 values marked as very important)

As a result of the GR of the T98-G cell line, MTT, and LDH levels decreased considerably. Especially at the 72nd hour, the viability level at Exolimer+TMZ 400, and 800 ng/ml concentrations was determined as 58.125±6.66, and 50.95±5.79, respectively, and it was found to be statistically significant (P<0.05, P<0.001).

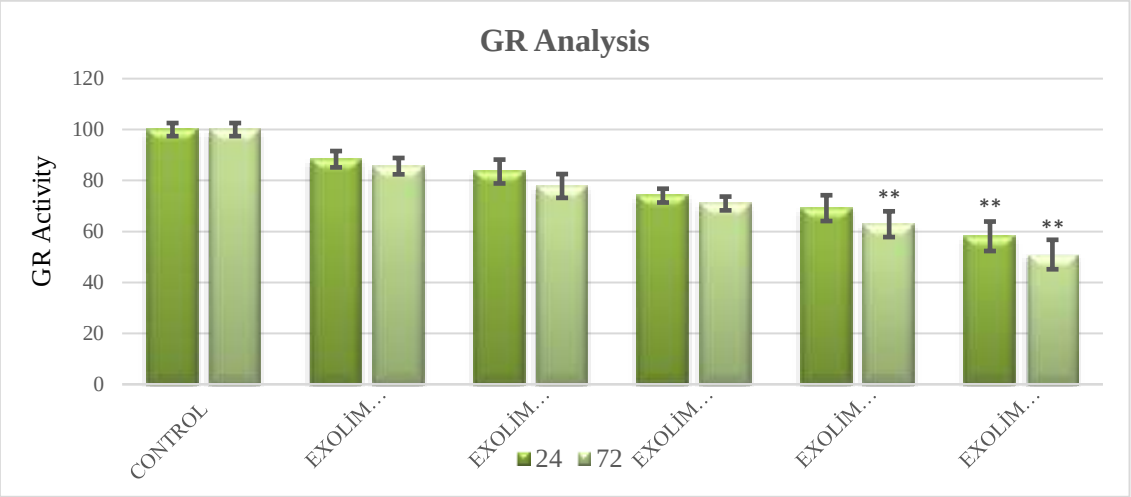

Fig 10 GR results at 2, and 72 hours for Control, Exolimer+TMZ (50 ng/ml, 100 ng/ml, 200 ng/ml, 400 ng/ml, 800 ng/ml groups. (* P<0.05 values are significant, ** P<0.001 values are marked as very important)

Antioxidant Activity Measurement

Total Antioxidant Capacity (TAC) of T98-G Cell

T98-G is given in Fig 11-13. Control and positive control values have close values and have no statistical significance. While the TAC value of the control group was 5.2±0.31

at the end of 24 hours, it was 5.3±0.01 at the end of 72 hours. The lowest value in the positive group belonged to the Polymer+TMZ 200 ng/ml group, and the value was the same in both hours, and was found to be 4.6±0.111, and 4.6±0.032, respectively. No significant difference was found among the controls (P>0.05).

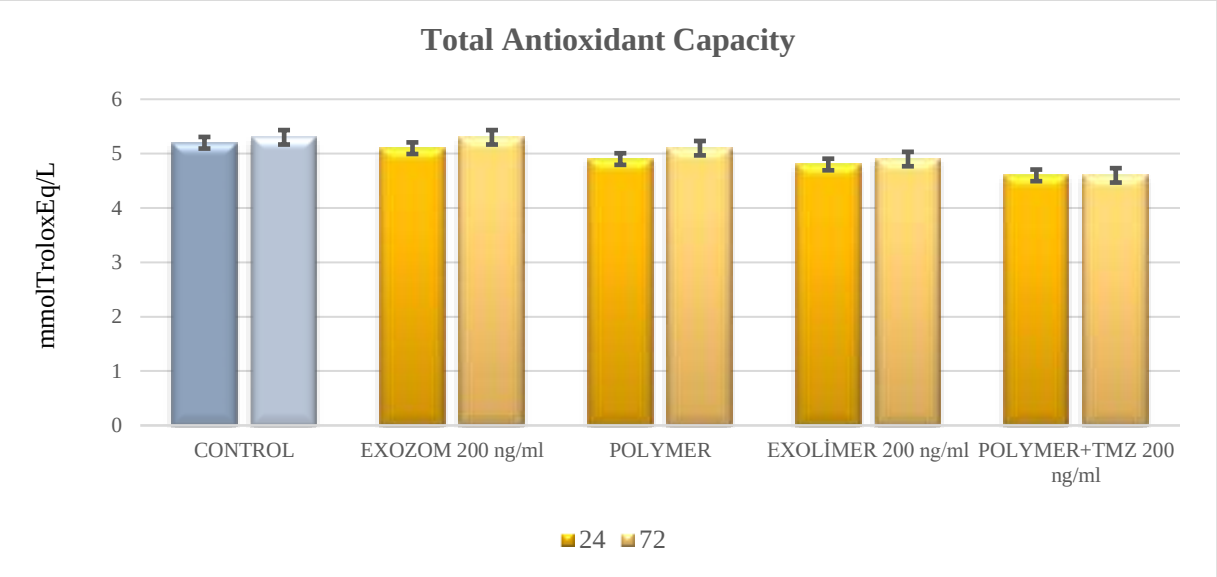

Fig 11 TAC test results of the control and positive control groups. The data in Fig are expressed as values in mmol Trolox Eqiv/L^{-1} compared to the control group. * p<0.05 values are marked as important, ** p<0.001 values are marked as very important

Although the TMZ group created a significant difference on the T98-G cancer line in correlation with MTT, and other cytotoxicity tests, it showed less effect than the Exolimer encapsulation formulation applied at the same dose. When the TMZ 400 ng/ml and TMZ 800 ng/ml values are compared with the control, significant results are obtained. TAC level was found to be 3.8±0.125, and 3.4±0.09 at 24 hours, respectively, and 3.4±0.056, and 3.1±0.21 after 72 hours (p<0.05).

Values obtained at the end of 24 hours from low dose to high dose in the Exolimer+TMZ group, respectively, were 4.8±0.1, 4.4±0.04, 4.1±0.06, 3.1±0.3, and 2.8. Despite this, it was determined as 4.4±0.21, 4.1±0.032, 3.3±0.43, 2.9±0.12, and 2.5±0.076 after 72 hours. According to the results obtained, it was observed that the TAC level decreased almost half as much as the control. The results obtained were statistically significant (p<0.05, and p<0.001).

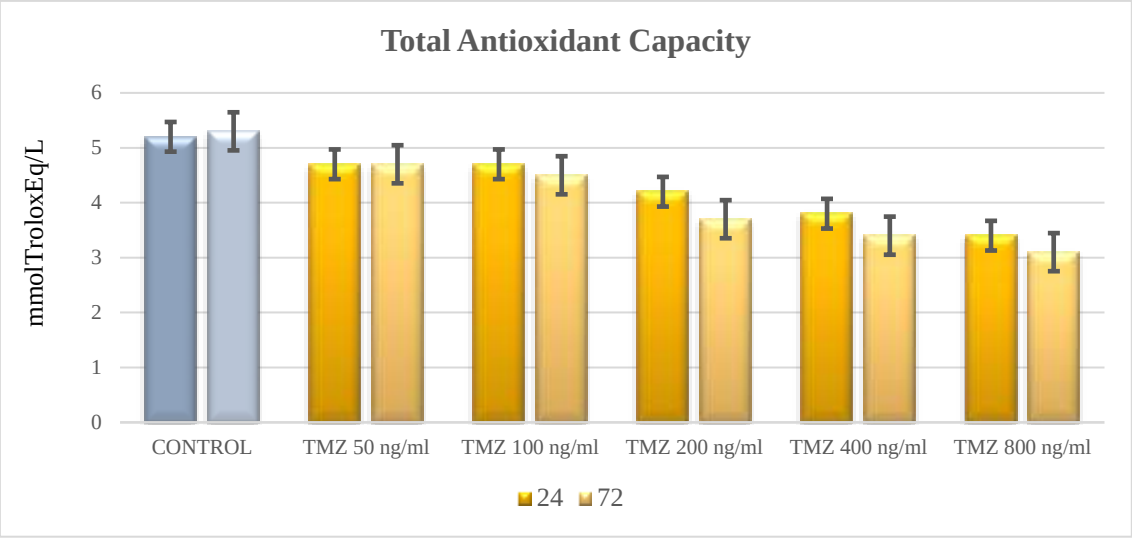

Fig 12 TAC results of the control, and TMZ group. The data in Fig. are expressed as values in mmol Trolox Eqiv/L^{-1} compared to the control group. * $p<0.05$ values are marked as important, ** $p<0.001$ values are marked as very important

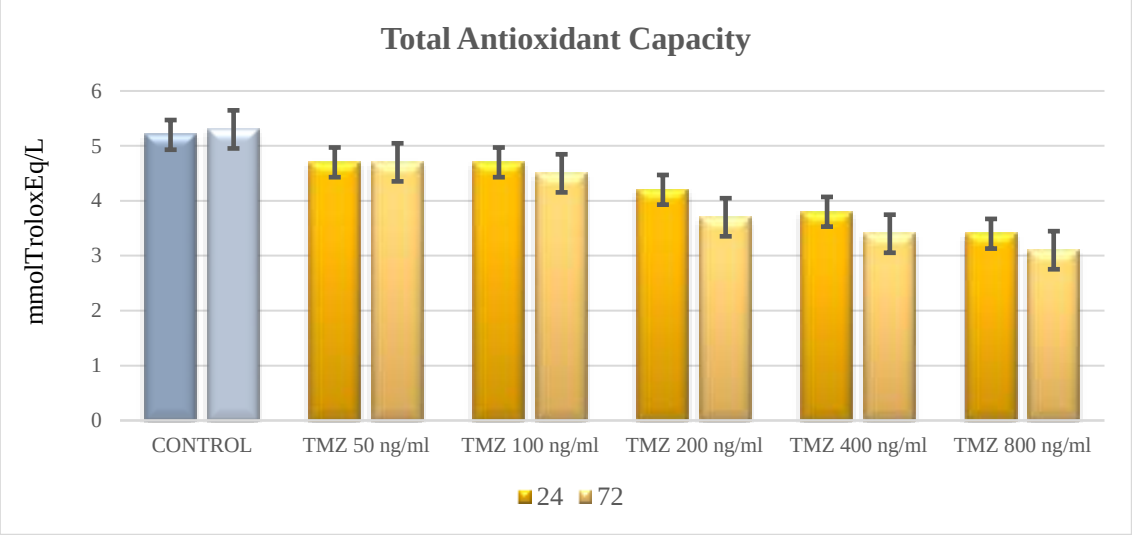

Fig 13 TAC results of the control, and Exolimer+TMZ groups. The data in Fig. are expressed as values in mmol Trolox Eqiv/L^{-1} compared to the control group. * $p<0.05$ values are marked as important, ** $p<0.001$ values are marked as very important

TOS values of T98-G cells

Compared to the control group, exosome 200 ng/ml, polymer, and Exolimer 200 ng/ml groups increased oxidative stress in T-98G cell line at both 24, and 72 hours, but no significant difference was observed.

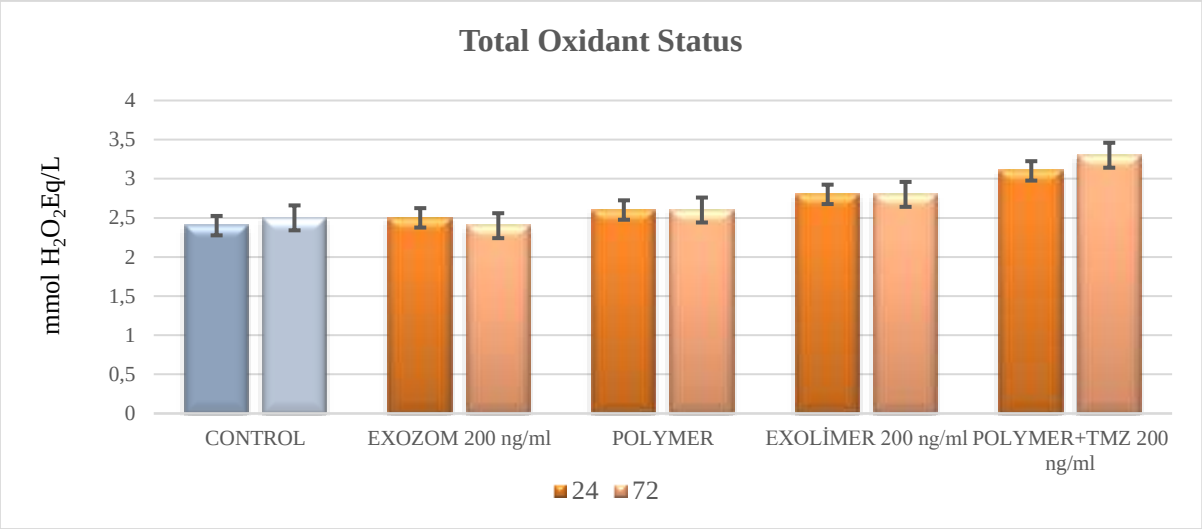

Fig 14 TOS test results of control, and positive control groups. The data in Fig. are expressed as values in mmol H_2O_2 Eqiv/L^{-1} compared to the control group. * $p<0.05$ values are marked as important, ** $p<0.001$ values are marked as very important

It was concluded that TMZ groups (50, 100, 200, 400, and 800ng/ml) compared to the control group increased oxidative stress at both 24, and 72 hours depending on the increasing dose. Compared to the control group, the most significant result after 72 hours was detected in the TMZ 400, and TMZ 800ng/ml groups, which increased oxidative stress by 1.64, and 1.84 times, respectively.

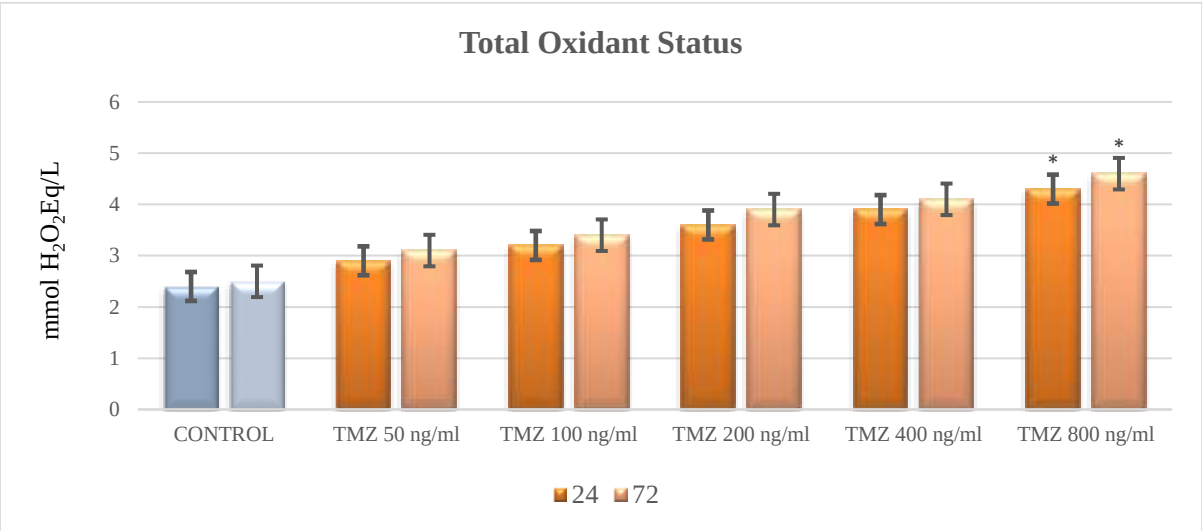

Fig 15 TOS test results of the control, and TMZ groups. The data in Fig. are expressed as values in mmol H_2O_2 Eqiv/L^{-1} compared to the control group. * $p<0.05$ values are marked as important, ** $p<0.001$ values are marked as very important

Fig 16 showed that the Exolimer + TMZ (50, 100, 200, 400, and 800 ng/ml) groups increased oxidative stress at 24, and 72 hours depending on the increasing dose, negatively affected T-98G cell viability, and led to cellular death. These results we

obtained reveal that the Exolimer positively increases the TMZ efficiency depending on the increasing dose. The most significant result compared to the control group was found in the Exolimer + TMZ 800ng/ml group, which increased oxidative stress by 2.04 times at the end of the 72nd hour.

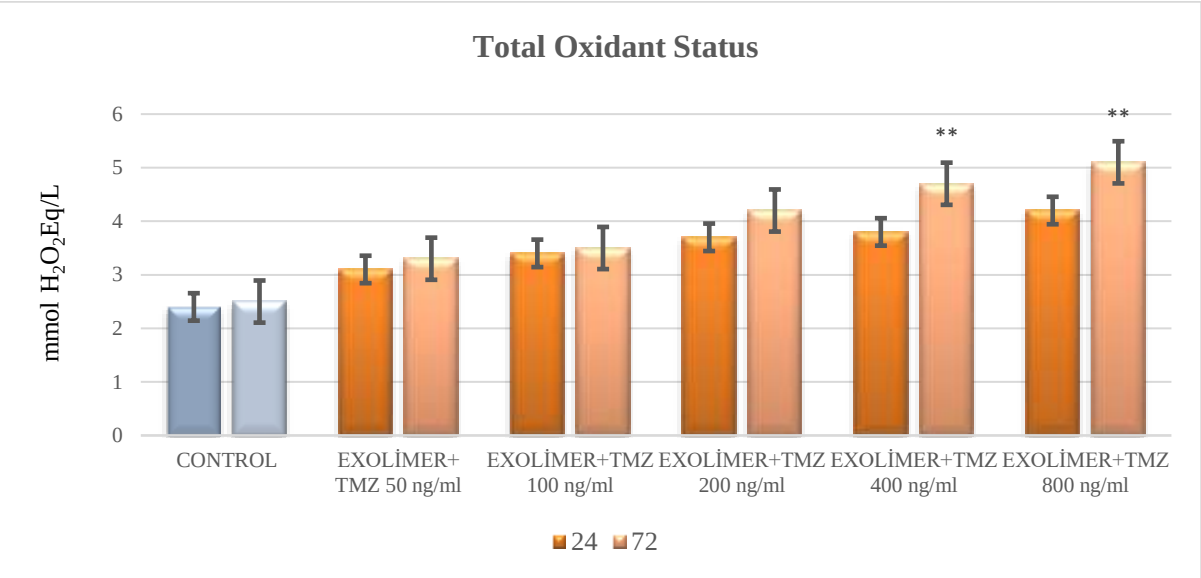

Fig 16 TOS test results of the control, and Exolimer+TMZ groups. The data in Fig. are expressed as values in mmol H_2O_2Eqiv/L^{-1} compared to the control group. * $p<0.05$ values are marked as important, ** $p<0.001$ values are marked as very important

Considering its effectiveness on T98-G, encapsulation of the drug with the Exolimer offered many advantages together as it was predicted. These two systems, which also have many disadvantages, came together, and increased each other's power, thus increasing the effectiveness of the drug in parallel.

Discussion

Exosomes take an active role in different cancer physiopathology. Exosomes released from GBM cancer play a role in many important tasks such as suppression of the immune response against cancer, cancer spread, and metastasis. Today, with the advancement of biotechnology, the use of exosome-based drug delivery systems against cancer is increasing rapidly [19-21]. For this reason, the use of these vesicles as carriers has gained importance in new studies. However, the low carrying capacity of exosomes, and the difficulty of elimination of various bioactive substances they carry limit the effective use of these vesicles. In our study, we produced new carrier molecules, which we call Exolimers, by combining these vesicles with new nanotechnological methods. In order to investigate the anticancer activity of these carrier nanocargoes, after characterizing the

particles obtained at different stages, we designed *in vitro* experiments to evaluate them in terms of anticancer activity. The aim of our study is to target cancer cells such as trojan horse of the Exolimers produced. Thus, we plan to increase the potency of the TMZ drug, which is widely used in the classical treatment of GBM, by ensuring that it is more effectively transported to cancer cells. For this purpose, we examined the effectiveness of our encapsulated drug with cytotoxic analysis, oxidant, and antioxidant analyzes at the end of the experiment.

Its use as an exosome transporter has been shown in different studies, and it has been used especially as a miRNA transporter in these studies. Jianxing Yin et al.[22] used exosomes to reduce drug resistance to temozolomide in their study in 2019. In this study, cancer cells were transfected by loading the exosomes obtained from the TMZ-resistant glioblastoma line. Their findings showed that as a result of transfection, the amount of mir1238 increased in target cells, and became sensitive to TMZ drug. Thanks to the data obtained, it has been proven that the Exosome transport system effectively penetrates the cancer microenvironment, and cancer cell more easily [22]. In our study, a mixture of polymer, and exosome was used instead of the pure form of different exosomes. In this way, the content of exosomes is discharged, and the carrying capacity is increased. In addition, the small amount of pure exosome obtained would cause the obtained material to be limited, so a higher amount of carrier material was obtained with the newly formed encapsulation with the polymer. When we look at the MTT result graphs in our experiment, the pure TMZ effect is weaker than the Exolimer-TMZ. The reason for this is that TMZ transported by the Exolimer is more effectively transported into the cell.

Munoz JL et al. [23] showed in their study in 2014 that high doses of TMZ led to an increase in LDH levels, and a decrease in GR values in cancer cells, leading to cell death. However, studies have also shown that resistance to TMZ causes a decrease in cell death rate, and LDH levels. In our study, the highest LDH activity in the TMZ group was observed between the TMZ 400-800 ng/ml groups. The 24-hour result was 58.9±1.37, and 68.8±1.68, while the 72-hour result was 75.5±6.02, and 85.3±6.88. At the highest dose, LDH activity increased almost 6-fold. However, it was determined that the absence of resistance in our Exolimer groups increased cytotoxicity, and cellular stress.

In the treatment of glioblastoma, oxidative stress caused by the damage of anticancer drugs in cancer cells has been shown in many studies. In their study conducted in 2018,

Emsen B et al. [24] showed that the total oxidant , and antioxidant ratios of anticancer drugs were correlated with the mortality rate. In addition, Tuzgen S et al [25] investigated total antioxidant levels in their study in glioblastoma patients in 2007. The data obtained in both studies reported that the oxidant level increased, and the antioxidant capacity decreased depending on the effectiveness of the treatment against cancer cells. The data obtained in both studies reported that the oxidant level increased, and the antioxidant capacity decreased depending on the effectiveness of the treatment against cancer cells [25]. Similar results were obtained in our study. However, in this study, the use of pure drug was compared with Exolimer+TMZ , and it was foun that the oxidant level increased , and the antioxidant level decreased in the Exolimer+TMZ groups compared to the equivalent dose.

Recently, targeted studies in cancer treatment have become widespread. Exosomes have an important role in cellular signal transmission, thanks to the biomarkers they carry. With these features, it is seen as a new hope in the treatment of many diseases, including cancer.

Abbreviations

GBM: Glioblastoma Multiforme; GR: Glutathione Reductase; LDH: Lactate Dehydrogenase; MTT: (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) Analysis; RES: Reticuloendothelial System; MPS: Mononuclear Phagocyte System; TAC: Total Antioxidant Capacity (TAC); TOS: Total Oxidant Number, TMZ: Temozolomide TEM: Transmission Electron Microscope.

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