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Investigation of Effect of Paclitaxel on Netrin 1 and Factor 8 in Ehrlich Solid Tumors

Investigación del Efecto del Paclitaxel en Netrina 1 y Factor 8 en Tumores Sólidos de Ehrlich

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KAYMAK, E.; ERTEKIN, T. & ÖZDAMAR, S. Investigation of effect of paclitaxel on netrin 1 and factor 8 in Ehrlich solid tumors. *Int. J. Morphol., 39(2)*:564-570, 2021.

SUMMARY: Cancer known as a malignant tumor, is a class of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. The Ehrlich tumor is a mammary adenocarcinoma of mice developed in solid and ascitic forms. This study was aimed to investigate the effects of paclitaxel on Netrin 1 and Factor 8 expression and also in tumor cell proliferation, apoptosis, angiogenesis, and development of tumor in Ehrlich solid tumors treated with paclitaxel. In this study, 26 adult Balb/C male mice were used. 6 of them were used as stock. Ehrlich ascites cells taken from animals in stock were injected subcutaneously from the neck area to all animals. The mice were randomly assigned to two groups of ten rats per group. Paclitaxel treatment group 10 mg/kg were administered to mice intraperitoneally (i.p.) 4,9, and 14th days. 15th day the animals were sacrificed and tumor tissues were taken. Paraf fin-embedded solid tumor sections were stained Hematoxylin & Eosin, Masson's Trichrome. Also solid tumor sections were stained immunohistochemically with Netrin1 and Factor 8. Tunel method was applied to determine apoptosis. Paclitaxel applied as a therapeutic Ehrlich solid tumor reduced the volume of tumors in the treatment groups. At the end of the experiments, in the treatment groups' significantly reduced the Netrin 1 expression and microvessel density compared to the group control. Also paclitaxel in the treatment group increased the number of apoptotic cells. We suggest that decreasing the expression of Netrin 1 would be reduced vessel density and increased apoptosis.

KEY WORDS: Ehrlich Solid Tumor; Paclitaxel; Netrin 1; Factor 8.

INTRODUCTION

It is known all over the world that cancer is a disease that causes and destroys death despite serious advances in medicine in the diagnosis and treatment of cancer (Graham *et al*., 2000) The most commonly used methods in cancer treatment are surgical removal of the tumor, chemotherapy, and radiotherapy (Mimeault *et al*., 2007). Abnormal blood vessel formation and formation of vascular endothelial cells from previous vessels are important in tumor growth (Dvorak, 2005).

Transplantable tumor models are derived from suspensions and spontaneous tumors. These tumors are similar to human cancer types in terms of kinetic features. Ehrlich acid tumor (EAT) is one of the widely used tumor models among transplantable tumors. EAT is derived from Ehrlich Mouse Carcinoma, extracted from the mammary gland of a mouse by Ehrlich and Apolant in 1907, and it is a tumor model that is unique to mice and has survived to the present day (Zeybek, 2013).

Netrin is derived from Sankrist Netr, which means guide. UNC-6, which is the first recorded netrin, was identified in Caenorhabditis elegans, a nematode worm for screening proteins that regulate neural development (Ishii *et al*., 1992). Netrins also play a role in cell migration, adhesion, differentiation, survival and angiogenesis in the biological process (Sun *et al*., 2011). Netrin 1 acts as a regulator in the morphogenesis, tumor growth, and inflammation of endothelial and vascular smooth muscle cells. Furthermore, Netrin is important in the reorganization of the cytoskeleton (Arakawa, 2004; Bouvrée al., 2008; Fitamant *et al*., 2008).

Factor 8 is an important protein in endothelial cells. Factor 8 is used to determine whether benign and malignant lesions have an endothelial origin (DeLisser *et al*., 1997).

Paclitaxel is an alkaloid which is isolated from the tree called Taxus brevifolia grown in the USA and used in

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the treatment of ovarian, breast, and non-small cell lung cancers. The anticancer effect of paclitaxel was discovered in 1970 (Karahan, 2010). It shows its anti-tumor feature through microtubules. It binds to microtubules with a high affinity and prevents them from binding and depolarizing them by stabilizing them (Türkyılmaz, 2008).

Ehrlich solid tumor, which is used as an experimental cancer model, is used by many researchers today. There are many studies examining the relationship between netrin 1 expression, angiogenesis, paclitaxel, and cancer. However, the effects of these proteins and paclitaxel have not been studied together on the Ehrlich solid tumor. In this study, the relationship between paclitaxel on solid tumor development, Netrin 1 expression, apoptosis, and vascularization was investigated using a tumor model by implanting Ehrlich acid tumor cells into mice.

MATERIAL AND METHOD

Experimental animals. This study was planned and applied in the Erciyes University Faculty of Medicine Histology-Embryology Department in line with the approval of the Ethics Committee of Erciyes University Experimental Animals Ethics Committee. All procedures were carried out in accordance with the Universal Declaration of Animal Rights, with the approval of the Ethical Committee of Erciyes University Experimental Animals (Date: 14.01.2015, Decision no: 15/03) under the control of the veterinarian. The experimental stage of the study was carried out in Erciyes University Experimental Research Application and Research Center (DEKAM). This project was supported by Erciyes University Scientific Research Projects Unit (TYL-2015-5966).

In our study 6 stock mice (weight 30 g, 2 months) carrying liquid EAT were used in the peritoneum obtained from Erciyes University Faculty of Medicine, Department of Anatomy in the creation of solid tumors. As for the experimental group, 20 Balb / C mice in 8-10 weeks old were obtained from the Experimental and Clinical Research Center of Erciyes University Hakan Çetinsaya.

Acid fluid from 6 stock animals with the aid of the injector was suspended in 0.1 ml PBS to form a solid tumor. 0.1 ml of acid fluid with 1x106 EAT cells was injected subcutaneously from the neck area of each mouse. In our experiment consisting of two groups, n = 10 in the control group and n = 10 animals in the treatment group, paclitaxel intraperitoneally was applied to the treatment group on days 4, 9, and 14, 10 mg/kg (Park *et al*., 2015). In the same way, saline was given to the untreated group. On day 15, all animals were sacrificed under ketamine - xylazine anesthesia. Formula (O'Reilly *et al*., 1997; Yılmaz *et al*., 2019) used to measure tumor volumes:

$$\text{Tumor volume (mm3)} = \text{width } 2\times \text{ length x } 0{,}52$$

Histopathological evaluation. Tumor sections of 5-6 mm from paraffin blocks were left in the oven for a certain period of time using histological methods, then paraffin was removed with xylene and passed through graduated alcohol series and diluted. To see the general histological structure, hematoxylin and eosin (HE) and Masson trichrome staining were performed.

Immunohistochemistry. Netrin 1 and Factor 8 expression were detected immunohistochemically using by streptavidin-biotin-peroxidase technique. The sections were deparaffinized in xylene, rehydrated through graded alcohols, and washed in deionized water. Antigen retrieval was performed by microwave treatment in 0.01 M sodium citrate buffer, pH 6.0, at 270° C for 5 min. The slides were cooled and held at room temperature for 10 min. Sections were washed with PBS. Endogenous peroxide activity was inhibited by immersion in 3 % (w/v) H2O2 for 12 min. Lab Vision™ UltraVision™ Large Volume Detection System (TP-125-HL; Thermo Fisher Scientific, Waltham, MA) was used. All sections were washed with distilled water, then Ultra V block was applied for 10 min at room temperature to block background staining. Sections then were incubated overnight at 4º C with a Factor 8 (Abcam: ab9378) antibody, and after washing with PBS, sections were incubated with biotinylated goat anti-Polyvalent secondary antibodies (TP-125-BN) (TP-125-HL; Thermo Fisher Scientific). The Santa Cruz (sc2053) kit was used to dye the Netrin 1 antibody. The serum block was dropped and kept at room temperature for 20 minutes. Netrin 1 (Santa Cruz: sc9292) antibody prepared after it was dropped into sections and kept at 4 ° C for 1 night. The immunoreaction was amplified using the streptavidin-avidin-peroxidase complex and visualized using 3,3' p-diaminobenzidine tetrahydrochloride (TA-060-HDX; Thermo Fisher Scientific). After counterstained with Gill's hematoxylin, sections were washed 3 times with deionized water. Then, the sections were dehydrated through rising alcohols, cleared in xylene, and mounted with Entellan. Images were taken using a light microscope (Olympus BX51; Olympus). At least 5 randomly chosen fields in each slide were counted at the original 40 magnification (Karabulut *et al*., 2016).

TUNEL staining. Apoptotic cells in the sections taken from the subjects were determined using the Roche brand In Situ Cell Detection Apoptosis Fluorescein Kit. The staining was

done according to the kit procedure. Tumor sections taken at a thickness of 5 µm were deparaffinized and then rehydrated and washed twice with PBS for 5 minutes. Then, 270° C in a microwave oven in 0.01 M 5 % sodium citrate buffer for antigen recovery was left for 5 minutes, then it was allowed to cool at room temperature for 10 minutes. Tissues washed with PBS for 3X5 minutes were placed in the moisture chamber at 37 0C with the TUNEL reaction mixture coming out of the kit and incubated in the oven for 75 minutes. Tissues washed 2 times for 5 minutes with PBS were contrasted with 4 ', 6-diamidino-2-phenylindole (DAPI). Tissues sealed with glycerol closure solution were visualized on the Olympus BX51 fluorescent microscope at a wavelength of 450-500 nm. For the apoptotic index, apoptotic cells in ten different areas were counted in 40X objective from each section (Öztürk *et al*., 2020).

Statistical analysis. Image J program was used to evaluate the results immunohistochemically an to measure the immune reactivity intensity. While the vessel density was being measured, 10 fields were counted from each preparation, and the mean value was taken. For the measurement of vascular density in the control and treatment groups, 10 fields from each preparation were counted first, each preparation was averaged, then each group was averaged and evaluated. In immunohistochemical evaluation, cells with a clear nucleus were considered. Necrotic fields were not subjected to this measurement. All statistical analyzes were carried out using SPSS statistical software (SPSS for Windows, SPSS Inc, Chicago, IL, version 24.0), and graphs were drawn by using Graphpad Prism 8.0 software. Independent sample T-test was used to compare tumor volume, weight, Factor 8, and Netrin 1 between the groups. The data were presented as the mean ± standard deviation (SD). p-value <0.05 was considered as statistically significant.

RESULTS

Body weight and Tumor volume changes in ascites tumor groups throughout the experiment. When the mouse weights were measured at the end of the experiment, an increase in weight was observed in the control group compared to the paclitaxel group. In mice injected with 0.1 ml of acid fluid with EAT cell, tumor mass reached palpable size by hand on the fifth day. When we look at tumor volume dimensions, the tumor volume of the paclitaxel group decreased significantly compared to the control group (ap<0.05). These results are shown in Table I and Figure 1.

Table I. Comparison of body weight and tumor volume between groups.

Groups	Control	Paclitaxel	*p*
Body	33.87±2.86	31.84±1.11	0.065
Tumor	5440±2316	1772±620[a]	0.007

Data are expressed as mean±SD. Significancy among groups was considered when p < 0.05. a : Significantly different from control group.

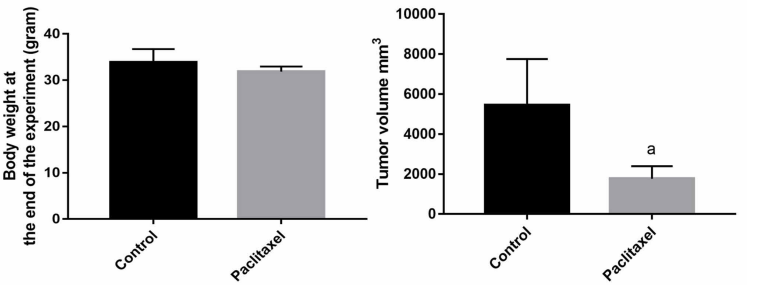

Fig. 1. Comparison of body weight and tumor volume between groups. Significancy among groups was considered when p < 0.05. a : Significantly different from control group.

Histopathological results. In our study, H&E and Masson trichrome staining (MT) were applied to the control and the treatment groups for light microscopic examination. When we looked at the general image of the solid tumor as a result of staining, a lobulated-like tissue image and image of adipose cells and EAT cells were obtained in both groups. A large number of EAT cells with prominent nuclei were seen in the control group. In the H&E staining in Figures 2A-B, necrotic areas were shown in Figure 2B. In the MT staining, EAT cells were seen in a large number in the control group. The amount of fat cells increased in the paclitaxel group. EAT cells and adipose cells are shown in Figures 2C-D.

Immunohistochemistry. The average intensity of netrin 1 expression was measured as 96.47 in the control group. In the treatment group, Netrin 1 intensity was measured on average 89.72. As seen on the Table II. Netrin 1 density decreased significantly in the paclitaxel group compared to the control group (ap<0.05) (Figs. 3A-B). Staining was observed in the vascular endothelium in the sections belonging to the control and treatment groups with factor 8 immunohistochemistry staining (Figs. 3C-D). The vascular density between the groups is shown in Table II. While the mean vascular density was calculated as 4.93 in the control group, the mean vascular density in the paclitaxel group was 3.20 and showed a statistically significant decrease (ap<0.05).

Apoptotic results. It was observed that apoptotic cells were distributed throughout the tissue in the EAT tissue sections (Figs. 4A-B and Table III). As a result of the statistical

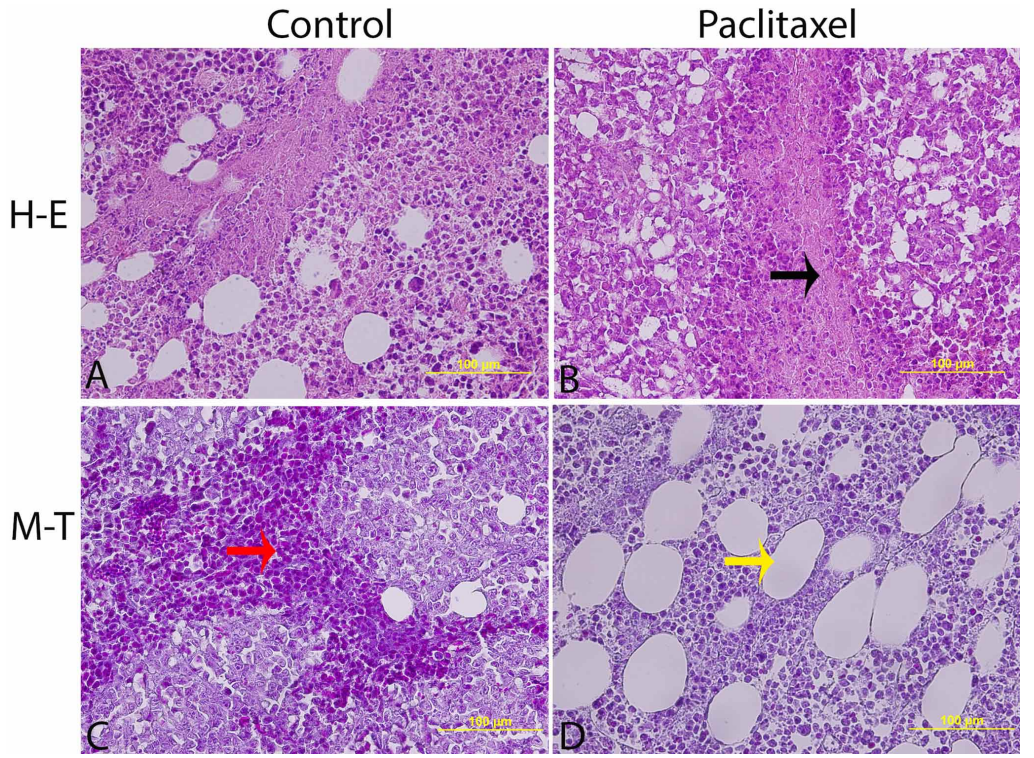

Fig. 2. Hematoxylin-eosin (H&E) (2A-2B) and Masson's Trichrome (2C-2D) staining. X400.Necrotic areas are indicated by a black arrow. EAT cells are indicated by the red arrow Adipose cells are indicated by the yellow arrow.

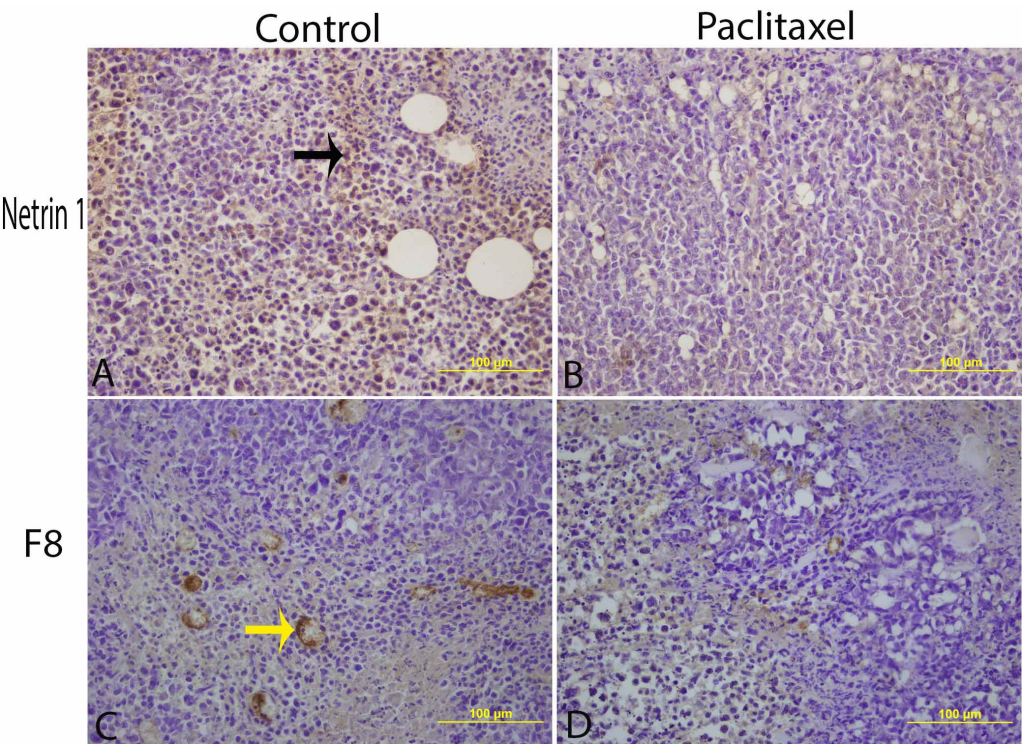

Fig. 3. Netrin 1 (3A-3B) and Factor 8 (3C-3D) immunohistochemical staining. (arrow =Netrin 1 and Factor 8 positive cells). X400. EAT cells are indicated by a black arrow. Endothelial cells are indicated by a yellow arrow.

analysis of the counts taken from the sections, a statistically significant increase was observed in the number of apoptotic cells (4.820 ± 0.43) in the paclitaxel group compared to the control group (1.798 ± 0.21) (ap<0.05).

Table II.Comparison of vascular density of Factor 8 and Netrin 1 expression between groups.

Groups	Control	Paclitaxel	*p*
Netrin 1 immunoreactivity	96,47 ± 5,07	89,72 ± 3,07[a]	0.002
Vascular density (Factor 8)	4,93 ± 1,33	3,20 ± 0,85[a]	0.003

Data are expressed as mean±SD. Significancy among groups was considered when p < 0.05. a : Significantly different from control group.

Table III. Comparison of apoptotic index between groups.

Groups	Control	Paclitaxel	*p*
Tunel Positive cell count (n=100)	1.798±0.21	4.820±0.43[a]	0.0001

Data are expressed as mean±SD. Significancy among groups was considered when p < 0.05. a : Significantly different from control group.

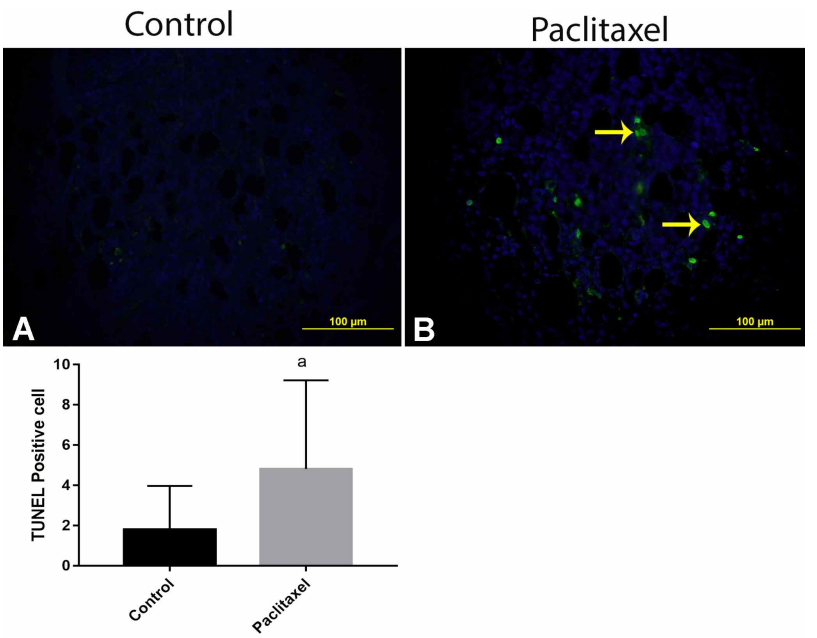

Fig. 4. Apoptotic cells belonging to the experimental groups. Apoptotic cells are indicated by arrows. X400. Significancy among groups was considered when p < 0.05. a : Significantly different from control group.

DISCUSSION

Cancer is defined as the uncontrolled growth of tumor-forming cells called neoplasms (Ogden, 1998). In cancer treatment, methods such as chemotherapy, radiotherapy, surgery, and immunotherapy are generally used. Depending on the individual characteristics and disease conditions of patients diagnosed with cancer, at least one or more of these methods are used in the treatment (Kızılcı, 1999). Different cancer studies have examined anti-cancer drugs such as Netrin 1 expression, F8-linked angiogenesis, or paclitaxel. In this study, we investigated tumor development and vascularization using the EAT model in which tumor growth was observed by injecting mice as a different model.

Joseph *et al*. (2014) stated in their model that tumor volumes reached a measurable level within 8 days. Due to this rapid proliferation of the tumor, features such as growth, vascularization, and invasion in tumor tissue can be explicitly studied. In our study, we observed that the tumor tissue was palpated manually on the fourth day after the injection, and on the sixth day, we found that the tumor volumes reached a measurable level.

Another study reported that there were necrotic areas in the central parts of the solid tumor formed one week after the subcutaneous transplantation of Ehrlich tumor cells, and the size of tumor cells varied (Ekinci, 2000). In our study, when we evaluated histopathologically, we observed necrotic areas and adipose tissue. The presence of necrotic cells in the tumor mass, especially in the central regions and mitotic cell areas in the periphery shows that rapid growth of the tumor is not accompanied by vascularization, and cell deaths can occur. When the effect of an anti-carcinogenic thymoquinone on Ehrlich solid tumor was examined, a decrease in tumor tissue volume was observed. In addition, cytoplasmic vacuolization, eosinophilic staining, increased necrotic areas, and increased pleomorphic nuclei have been observed (Areida *et al*., 2015). In our study, we showed that the paclitaxel applied group decreased compared to the tumor volume in the control group.

In the studies on lung (Delloye-Bourgeois *et al*., 2009) and ovarian (Papanastasiou *et al*., 2011) cancer, it has been reported that excess netrin expression contributes to the growth of tumor tissue by increasing the proliferation of tumor cells. Similarly, Liu *et al*. (2014) reported that Netrin 1 expression increased in patients with bladder cancer and showed a positive correlation with histological grade, tumor stage, metastasis, and low prognosis. Gong & Feng (2015) suggested that Netrin 1 contributes to the development of tumor tissue and it is associated with the survival rate of

patients, and it may be important in the prognosis and diagnosis of the disease as a result of their research on patients with renal cancer. In the studies on tumor tissue, it is stated that the effect of Netrin occurs in two different ways one of which is to promote angiogenesis and the other is to prevent apoptosis. The proangiogenic effect of Netrin 1 is achieved by the receptors of neogene in vascular smooth muscle cells. When neogene is blocked, migration and adhesion in vascular smooth muscle cells have been shown to be inhibited (Park *et al*., 2004) so that no new vascularization can occur. Netrin 1 has been shown to prevent apoptosis in cancer cells and exert this effect through netrin-dependent receptors. In this way, the viability of tumor cells is maintained by inhibiting apoptosis (Castets *et al*., 2009). In this study, it was observed that Netrin 1 expression in the treatment group decreased statistically significantly compared to the control group. Similarly, the apoptotic index was higher in the treatment group compared to the control group, and it was found statistically significant. The data obtained between the variability in Netrin 1 expression and apoptosis show significance with the results in other studies.

Factor 8 is an important protein expressed in endothelial cells and is frequently used to assess the presence of benign and malignant lesions with the endothelial origin (DeLisser *et al*.). The expression intensity of factor 8 determines the degree of tumor vascularization (Hlatky *et al*., 2002). Angiogenesis is also essential for the proliferation and growth of cancer cells and is known to induce and promote vascular development of tumor tissues (Kajdaniuk *et al*., 2011). Therefore, vascularization is prevented by using antiangiogenic substances as a way of cancer treatments (Inoue *et al*., 2000; Chabannes *et al*., 2001). In our study, it was observed that angiogenic activity was high in the control group by looking at the F8 intensity. In the treatment group where we applied paclitaxel, angiogenic activity decreased and necrotic areas occupied more area than controls. In our study, we used Factor 8 to determine the connection and correlation of micro vascularization with the development of a solid tumor. In this study, it was seen that the expression density of F8 in all tumor masses was quite low in necrotic areas and relatively high in peripheral areas. This difference in vascular density may explain why vascularization occurs in Ehrlich tumors. The growth rate of the tumor caused by regional differences in tumor tissue and vascularity in the necrotic areas and other areas are not the same. As stated in another study, paclitaxel can help control the disease by decreasing the vascularization of tumors in our study (Pamir, 2005).

When we compared the control and treatment groups of our study, the high apoptotic index in the treatment group showed that paclitaxel can also be effective on Ehrlich solid tumor. In this apoptotic process, during treatment with paclitaxel, members of the bcl2 family are upregulated; antiapoptotic proteins are phosphorylated and downregulated (Whitaker *et al*., 2019).

CONCLUSION

We used EAT in our study to be an example of tumors that developed in vivo. In Ehrlich solid tumor tissue which we created by subcutaneous injection, we observed that Netrin 1 expression occurs in tumor cells, F8 is expressed in endothelial cells, and angiogenesis is observed in endothelial cells. With these features, we provided the evaluation of Ehrlich solid tumor with Netrin 1 and F8 expression. We have shown that it can be an effective research tool in the fight against cancer by applying paclitaxel treatment used in the clinic to such cancer models.

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RESUMEN: El cáncer, conocido como tumor maligno, es una clase de enfermedad que involucra un crecimiento celular anormal con potencial de invadir o diseminarse a otras partes del cuerpo. El tumor de Ehrlich es un adenocarcinoma mamario de ratones desarrollado en formas sólidas y ascíticas. Este estudio tuvo como objetivo investigar los efectos del paclitaxel en la expresión de Netrin 1 y Factor 8 y también en la proliferación de células tumorales, apoptosis, angiogénesis y desarrollo de tumores sólidos de Ehrlich tratados con paclitaxel. En esta investigación se utilizaron 26 ratones machos Balb / C adultos. Seis de ellos se utilizaron como stock. Se inyectaron por vía subcutánea células de ascitis de Ehrlich tomadas de animales en la zona del cuello. Los ratones se asignaron aleatoriamente a dos grupos de diez ratas por grupo. Se administraron 10 mg/kg del grupo de tratamiento con paclitaxel a ratones por vía intraperitoneal (i.p.) 4, 9 y 14 días. El día 15 se sacrificaron los animales y se extrajeron los tejidos tumorales. Las secciones de tumor sólido incluidas en parafina se tiñeron con hematoxilina y eosina y tricrómico de Masson. También se tiñeron inmunohisto-químicamente secciones de tumor sólido con Netrin1 y Factor 8. Se aplicó el método Tunel para determinar la apoptosis. El paclitaxel aplicado como tumor sólido terapéutico de Ehrlich redujo el volumen de tumores en los grupos de tratamiento. Al final de los experimentos, en los grupos de tratamiento se redujo significativamente la expresión de Netrin 1 y la densidad de microvasos en comparación con el grupo control. Además, el paclitaxel en el grupo tratamiento aumentó el número de células apoptóticas. Sugerimos

que la disminución de la expresión de Netrin 1 reduciría la densidad de los vasos y aumentaría la apoptosis.

PALABRAS CLAVE: Tumor sólido de Ehrlich; Paclitaxel; Netrin 1; Factor 8.

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