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Article *in* Medical Oncology · March 2023

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https://doi.org/10.1007/s12032-023-01999-7

ORIGINAL PAPER

Determination of Rutin's antitumoral effect on EAC solid tumor by AgNOR count and PI3K/AKT/mTOR signaling pathway

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Received: 21 February 2023 / Accepted: 15 March 2023

Abstract

Rutin is one of the flavonoids found in fruits and vegetables. The PI3K/AKT/mTOR signaling pathway is critical for the life cycle at the cellular level. In current study, we purposed to demonstrate the antitumoral effect of rutin at different doses through the mTOR-signaling pathway and argyrophilic nucleolar regulatory region. EAC cells were injected subcutaneously into the experimental groups. 25 and 50 mg/kg Rutin were injected intraperitoneally to the animals with solid tumors for 14 days. Immunohistochemical, Real-time PCR and AgNOR analyzes were actualized on the taken tumors. When the rutin given groups and the tumor group were compared, the tumor size increase was detected to be statistically significant ($p < 0.05$). In immunohistochemical analysis, a significant decrease was encountered in the AKT, mTOR, PI3K and F8 expressions especially in the groups administered 25 mg Rutin, in comparison with the control group ($p < 0.05$). AgNOR area/nuclear area (TAA/NA) and average AgNOR number were determineted, and statistically important differences were detected between the groups in terms of TAA/NA ratio ($p < 0.05$). There were significant statistical differences between the mRNA quantity of the PI3K, AKT1 and mTOR genes ($p < 0.05$). In the in vitro study, cell apoptosis was evaluated with different doses of annexin V and it was determined that a dose of 10 µg/mL Rutin induced apoptosis ($p < 0.05$). In our study, it was demonstrated in vivo and in vitro that Rutin has an anti-tumor effect on the development of solid tumors formed by both EAC cells.

Keywords Rutin · Ehrlich ascites carcinoma · Annexin V · PI3K · mTOR

Introduction

The mechanisms by which cancer occurs are not fully understood, and induction of proliferation in cells and oxidative DNA damage are seen as factors for carcinogenesis. This disease is a growing public health problem in both developing and developed countries [1].

Today, various methods which are radiotherapy, surgery and chemotherapy are applied for the cancers therapy [2]. Somewhat synthetic drugs such as antimetabolites are used

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Published online: 27 March 2023

in the treatment of cancer [3]. However, these applied synthetic drugs have many side effects. Gastrointestinal and kidney disorders, bone marrow suppression, fatigue, as well as resistance of cancer cells to common treatments are conditions that can be seen with chemotherapy. For these reasons, developing drugs with high efficacy and poor toxicity is among the priorities of researchers [3, 4].

Free radicals are cognoscible to be the reason of cancer, neurodegenerative diseases, allergies and autoimmune diseases. Human body is surrounded with a strong netting of native antioxidant enzymes. Native produces have been one of the most precious drug wellspring for years owing to their high benefitability, high efficacy and nominal virulence [4, 5]. The anti-cancer, anti-inflammatory and antioxidant effects of Rutin, a bioflavonoid compound, were declared in studies [6, 7]. It is a glycoside derivative of the Rutin polyphenol quercetin [7, 8]. Due to the fact that Rutin has disaccharide sugar molecules as a lateral chain, it has been reported to have a higher bioavailability potential compared to quercetin, and exhibits a more advanced antioxidant property [9, 10]. The phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (AKT)/the mammalian target of rapamycin (mTOR) signaling pathway can trigger tumor growth and metastasis, leading to the progression of many carcinomas. PI3K stimulates and signals tyrosine kinase growth receptors because mutations in AKT protein can adversely influence related proteins and thus stimulate cancer cell widening and improvement. It has been reported that Rutin modulates the response and uses its impact on the AKT/PI3K/mTOR pathway and increases apoptosis in cancerous cells with its antioxidant properties [11, 12].

Rutin's anti-cancer property is associated with regulation of multiple known signaling pathways (Fig. 1). Rutin inhibits increased AKT phosphorylation by disrupting the PI3K-ATP binding site and activation of the mTOR complex 2 (mTORC2). Rutin inhibits Glycogen synthase kinase-3beta (GSK-3β) phosphorylation through PI3K suppression and increases tumor suppressor proteins [13, 14]. Studies indicate that irregular microRNAs (miRNAs) contribute to cancer progression and tumor growth. Rutin has been declared to limit the proliferation of cancer suppressor and oncogenic miRNAs in mouse breast cancer cells by reducing the proliferation [15].

In this study, the antitumoral effect of Rutin with different doses was investigated in Ehrlich ascites carcinoma (EAC) model both in vivo and in vitro. EAC cells proliferate in 2 phases following inoculation into the mice peritoneal cavity. It has been reported in studies that after intraperitoneal injection of 1×10^6 EAC cells [16].

EAC is originally hyperdiploid, with a high rate of transplantability, no regression, rapid proliferation, brief survival, cause 100% mortality, and immunologically lacking tumor-specific transplantation antigens [17]. Nucleolar regulatory regions (NOR) are reported as ribosomal gene regions located on chromosomes. These regions are composed of ribosomal DNA (rDNA) and proteins that develop with argiophilic properties [18].

These regions are replicated one-to-one into ribosomal RNA, which is converted into precursor ribosomes in the

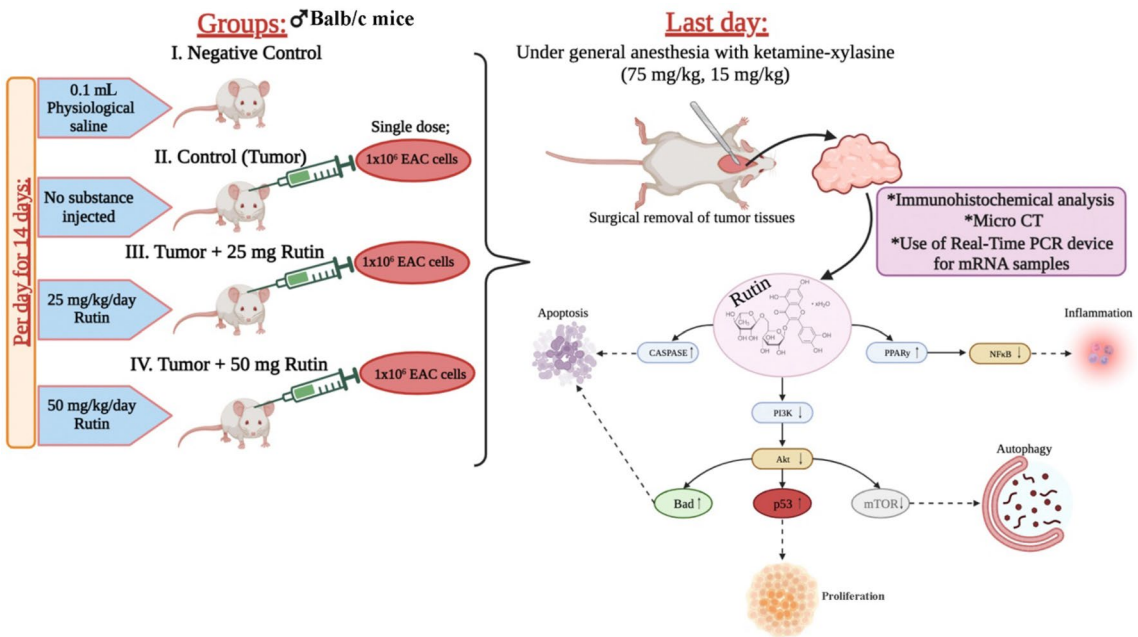

Fig. 1 Experimental design and signaling pathway schematic representation

nucleoli and large ribosomes formed in the cytoplasm. When these areas are active, they are stained with silver. Based on silver, these proteins are called argiophilic NOR (AgNOR)-related proteins, and the stained regions are used for diagnostic purposes [19].

There is no study in the literature showing the suppressor effect of Rutin with different doses on signaling pathway in both in vivo and in vitro EAC tumor model. The goal of current study was to explore the antitumoral effect of Rutin at different doses on experimentally induced EAC in male Balb/c mice.

Materials and methods

Distribution of mice in the study

The stage of the study on experimental animals was carried out in accordance with the decision numbered 20/105 taken from the Animal Experiments Local Ethics Committee of Erciyes University. In the study, 8–10 weeks old male Balb/c mice with an average weight of 25–30 g were used. Mice were housed in specially prepared, automatically heated rooms with a constant temperature of 21°C and 12 h light/dark periods throughout the study. The weights of the animals were measured from the first day of the experiment until the day they were sacrificed. At the same time, the solid tumor area was palpated manually every day, and the tumor diameters were measured with an electronic caliper after the tumor started to form. To measure tumor volumes: Tumor volume (mm^3) = Width × Height × 0.52 formula was used [20].

Formation of in vivo experimental groups

Group 1: Control (−) No cancer was established. Physiological saline (SF) was administered intraperitoneally (i.p) to the subjects for 14 days.

Group 2: Control (+) (Tumor) On day 0, 0.1 mL of ascitic fluid containing 1×10^6 EAC cells was administered subcutaneously (s.c) from the nape region.

Group 3: (Tumor+25 mg Rutin) 0.1 mL of ascitic fluid containing 1×10^6 EAC cells was administered as s.c from the nape area. From day 0 to 14 days rats 25 mg/kg/day Rutin i.p was injected.

Group 4: (Tumor+50 mg Rutin) 0.1 mL of ascitic fluid containing 1×10^6 EAC cells was administered as s.c from the nape area. From day 0 to 14 days rats 50 mg/kg/day Rutin i.p was injected.

All subjects were sacrificed under general anesthesia with ketamine-xylasine (75 mg/kg, 15 mg/kg) on the 14th day and the resulting tumors were took out. The experimental design representation of the study is given Fig. 1.

Creation of stock mice

EAC cells stored at −80 °C were kept at normal room temperature to dissolve. After checking the viability of the dissolved cells, 0.1 mL ascid fluid containing EAT cells was injected intraperitoneally with the help of a sterile injector. Acid tumor formation was observed in the stock mice within 6–7 days. The acid fluid withdrawn from the stock mice was suspended in 0.1 mL of PBS and counted on the Thoma slide. Solid tumors were formed by administering 1×10^6 EAC cells subcutaneously to 8–10-week-old mice.

Histological analysis

After fixation with 10% formaldehyde, tumor samples were passed through 70, 80, 96, and 100% alcohol, then cleared in xylene and embedded in paraffin. Sections taken as 5 µm were taken to normal slides for hematoxylin-eosin staining and to positively charged slides for immunohistochemical staining. EAC cells characterized by hyperchromatic large nuclei and eosinophilic cytoplasm of different shapes and sizes in sections belonging to the experimental groups were displayed under the light microscope.

Immunohistochemical analysis

AKT (Santa Cruz Biotechnology, Inc, sc-271149, CA, USA), mTOR (Santa Cruz Biotechnology, Inc, sc-517464, CA, USA), PI3K (Santa Cruz Biotechnology, Inc, sc-1637, CA, USA) in sections taken from tumor tissues of experimental groups using the Avidin-Biotin peroxidase method and F8 (Bioss, BS10048R) proteins were determined by immunohistochemical analysis [21]. In summary, epitopes were opened with citrate buffer, after paraffin removal from 5 µm tissue sections (pH 6.0; Thermo Fischer Scientific, UK, AP-9003-500). Slides were then placed in a 3% hydrogen peroxide solution in methanol to inhibit endogenous peroxidase activity. Ultra V block solution (Thermo Fischer Scientific, UK, TA-125-UB) was applied to prevent non-specific staining. The sections were then incubated with primary antibodies at 4 °C overnight. Then, they were incubated with biotinylated goat anti-polyvalent secondary antibody (Thermo Fischer Scientific, UK, TP-125-BN) for 40 min in an oven at 37 °C. After washing several times with PBS, they were incubated with streptavidin peroxidase (Thermo Fischer Scientific, UK, TS-125-HR) for 30 min in an oven at 37 °C. The antibody complex was visualized by incubation with diaminobenzidine (DAB) chromogen (Thermo Fischer Scientific, UK, TA-125-HD). Sections were then counterstained with Gill III Hematoxylin (Merck, Germany, 1.05174.1000). They were dehydrated by passing through a series of increasing alcohol and covered with a sealant called entellan. Sections were viewed with an Olympus BX53 light

microscope. Evaluation of immunoreactivity levels was done with Image J Version 1.46 (National Institutes of Health, Bethesda, Maryland).

AgNOR staining

Tissues fixed in 10% neutral formalin and embedded in paraffin were taken to slides. Sections were deparaffinized and stained. AgNORs, Ploton et al. Stained according to the method recommended by the International AgNOR Quantitative Committee revised by [16, 22]. Silver-stained tissues were appraised with a light microscope (Eclipse E-600, Nikon, Japan) using an image analysis system (NIS Elements Nikon, Japan). 50 nuclear AgNOR protein images were appraised per tissue. The average number of AgNORs and the ratio of total AgNOR area to total nuclear area (TAA/TNA) were calculated for each nucleus.

Real-time PCR analysis

PureZole reagent (Biorad, USA, Cat. No: 732-6890) was used for RNA isolation from tumor tissues. iScript Reverse Trancription Supermix (Biorad, USA, Cat. No: 170884) was used for reverse transcription. *PI3K*, *AKT1* and *mTOR* expression analysis was performed by Real-time PCR method using the Rotor Gene-Q (Qiagen, Hilden, Germany) and SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Biorad, USA). Oligomere Biotechnology (Ankara, Turkey) designed oligonucleotide primers: *Rat-PI3K-F:* AACACAGAAGACCAATACTC, *Rat-PI3K-R:* TTCGCCATCTACCACTAC, *Rat-AKT1-F:* GTGGCA AGATGTGTATGAG, *Rat-AKT1-R:* CTGGCTGAGTAG GAGAAC, *Rat-mTOR-F:* GACAACAGCCAGGGCCGC AT, *Rat-mTOR-R:* ACGCTGCCTTTCTCGACGGC, *Rat-Gapdh-F:* GAGGACCAGGTTGTCTCCTG, *Rat-Gapdh-R:* GGATGGAATTGTGAGGGAGA.

Cell culture

The cells used in the study were obtained from Erciyes University, Department of Anatomy, Cell Culture Laboratory (Kayseri, Turkey). Cells were cultured in 24-well plate. Dulbecco's Modified Eagle Medium (DMEM) containing 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture was used as the cell culture medium (Gibco™ DMEM is manufac-tured at a cGMP compliantfacility, located in Paisley, Scotland, UK/Catalog no: 41966029) [23]. In the experiment, the antitumoral effect of Rutin on EAC cells was tested at different doses in the range of 10, 30, 50, 70 µg/mL. In addition, this effect was compared with the control group. Only EAC cells were cultivated in the control group and no therapeutic agent was used. Cells were taken to the incubator at 37 °C with 95% humidity and 5% CO_2 for 24 and 48 h. Experiment groups; Control group (EAC cells + Medium), R10 group (EAC cells + Medium + 10 µg/mL Rutin), R30 group (EAC cells + Medium + 30 µg/mL Rutin), R50 group (EAC cells + Medium + 50 µg/mL Rutin), R70 group (EAC cells + Medium + 70 µg/mL Rutin).

Annexin V and dead cell assay

EAC cells were cultivated in 24 well-plates with 1×10^6 cells per well and left to incubate for 24 and 48 h. At the end of the incubation periods, the protocol we used in our previous studies was applied to evaluate the apoptosis status [23].

Statistical analysis

The data obtained at the end of the experiment were statistically analyzed in GraphPad Prism (version 8.0, GraphPad Software Inc., San Diego, California) and the data were presented as mean ± SD. The data were analyzed using one-way ANOVA analysis of variance and Dunnett's multiple comparison test was applied as a post-hoc test, $p < 0.05$ was considered statistically significant. All the genetic analyzes were performed using REST 2009 V2.0.13 where $p < 0.05$ is deemed to represent a statistically significant result [24].

Results

Morphological alteration of the experimental groups

Considering the data on the daily body weights of the animals belonging to the groups during the experiment (14 days), it was defined that there was a rise in the weights of the tumor control group and the groups given Rutin in the study, while it was observed that this rise was higher in the control (tumor) group than in the other groups ($p < 0.05$) (Fig. 2).

Tumor volume changes of the experimental groups

While tumor measurement was started from day 6 in the control (tumor) group, these measurements could be measured from day 7 in the treatment groups. On the last day of the experiment, tumor volumes were measured as 2241 mm^3 in the control (tumor) group, 917.3 mm^3 in the tumor + 25 mg Rutin group and 1149 mm^3 in the tumor + 50 mg Rutin group. All tumor sizes in all animals were compared among themselves in terms of volume. When the treatment groups and the control (tumor) group were compared, the tumor volume increase was found to be statistically significant

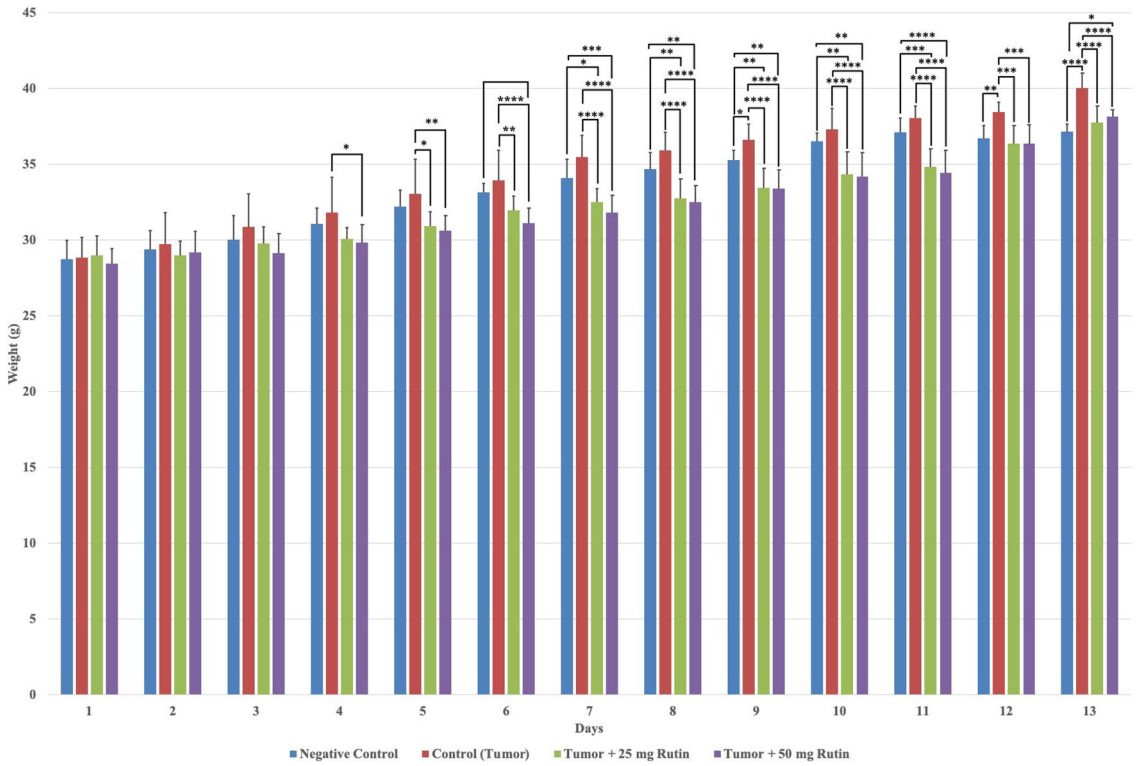

Fig. 2 Average weight (g) changes in control and experimental groups. Data were expressed as mean ± sd (mean ± standard deviation). *$p < 0.05$, was considered statistically significant (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

($p < 0.05$) and the findings of this comparison are given in Table 1.

Histological results

When tumor sections stained with hematoxylin and eosin were examined under the light microscope (Fig. 3), in the Ehrlich ascites carcinoma control (tumor) group, hyperchromatic large-nucleated cells and eosinophilic dense cytoplasm of different shapes and sizes were observed. Histopathology observed decreased in the groups that were administered 25 and 50 mg Rutin.

Immunohistochemical analysis results

According to the immunohistochemical analysis findings, there was an increase in the expression of AKT, mTOR, PI3K and F8 in large nucleated cells in the control (tumor) group. A significant decrease was observed in AKT, mTOR, PI3K and F8 expressions especially in the groups administered 25 mg Rutin compared to the control group (*$p < 0.05$). AKT expression was 3.52-fold, mTOR expression 1.3-fold, PI3K expression 2.61-fold, and F8 expression 1.67-fold decreased in the group that was administered 25 mg Rutin compared to the control (tumor) group. It was

Table 1 Tumor volume changes of the experimental groups

Day	Control (Tumor) Median (Min.–Max.)	Tumor + 25 mg Rutin Median (Min.–Max.)	Tumor + 50 mg Rutin Median (Min.–Max.)	*p*
7	287.66 (106.3–281.4)	–	–	–
8	526.5 (142–3536)	175.8 (50.25–1020)*	158.5 (78.01–270.8)**	0.0053
9	632.9 (156.1–6942)	384.9 (123–1121)	305.3 (133–618)*	0.0411
10	1410 (429.5–12571)	604.5 (108.6–1540)*	484.6 (177.6–946.2)*	0.0126
11	1703 (282.8–15120)	685.1 (466.1–1423)	767.3 (128–2427)	0.1449
12	1916 (1058–34725)	763.4 (492.7–1653)**	745 (320.4–1585)**	0.0004
13	2241 (1278–22899)	917.3 (333.1–1211)***	1149 (258.1–1758)**	<0.0001

Values measured in mm^3. Compared with tumor control group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$

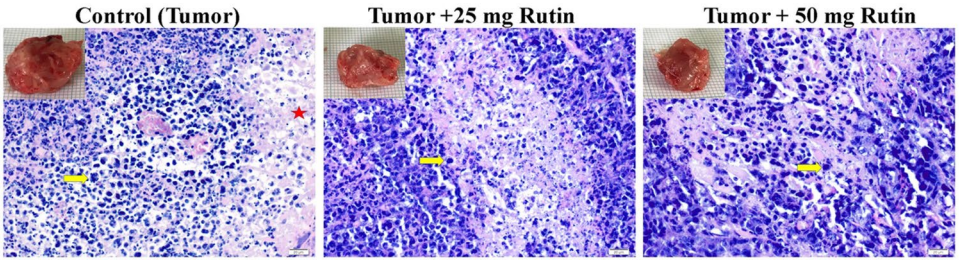

Fig. 3 Hematoxylin-eosin staining images of tumor sections belonging to the experimental groups. Yellow arrow indicates hyperchromatic large-nucleated predicted Ehrlich ascites carcinoma cells, red star indicates eosinophilic cytoplasm. Magnification: ×40, bar= 20 µm

determined that there was a significant decrease in PI3K and F8 expressions in the groups administered 50 mg Rutin compared to the control group (Figs. 4, 5; $*p < 0.05$). PI3K expression was decreased 1.55-fold and F8 expression was decreased 2.69-fold compared to the control (tumor) group in the 50 mg Rutin group (Fig. 4).

AgNOR staining results

A sample of AgNOR stained cells is shown in Fig. 6. TAA / NA ratio and mean AgNOR number were determined in the treatment (25 and 50 mg/kg) groups and control (tumor) (Table 2). Significant statistical differences were found between the three groups for the TAA/NA ratio (x2 = 82.321, $p = 0.000$) and the mean AgNOR number (x2 = 114.360, $p = 0.000$) (Table 3). In pairwise comparison of groups, TAA / NA ratio control (tumor) − Tumor + 25 mg Rutin ($Z = -2.701$, $p = 0.004$), control (tumor) − Tumor + 50 mg Rutin ($Z = -5.621$, $p = 0.003$), Tumor+25 mg Rutin − Tumor + 50 mg Rutin ($Z = -6{,}348$, $p = 0.001$) was significant for the TAA/NA ratio in all groups (Table 4).

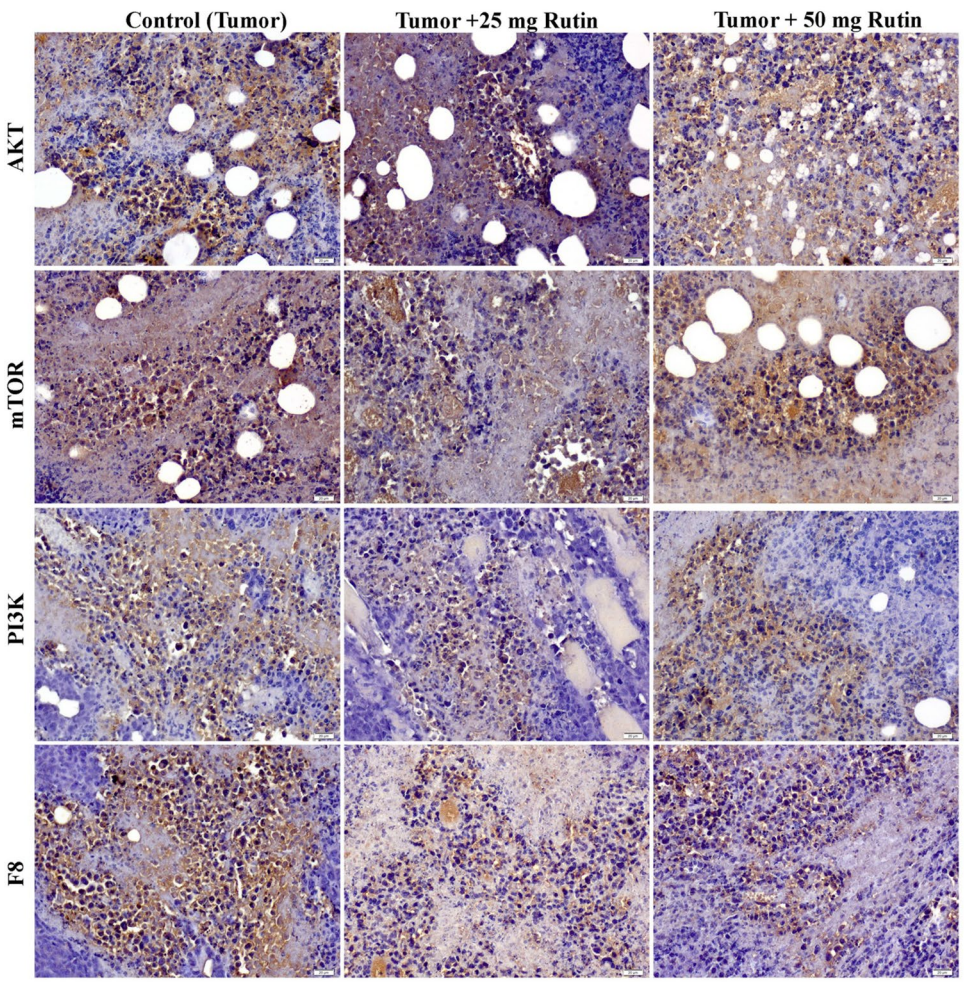

Fig. 4 AKT, mTOR, PI3K and F8 immunostaining images in tumor sections of the experimental groups. Magnification: ×40, scale bar= 20 µm

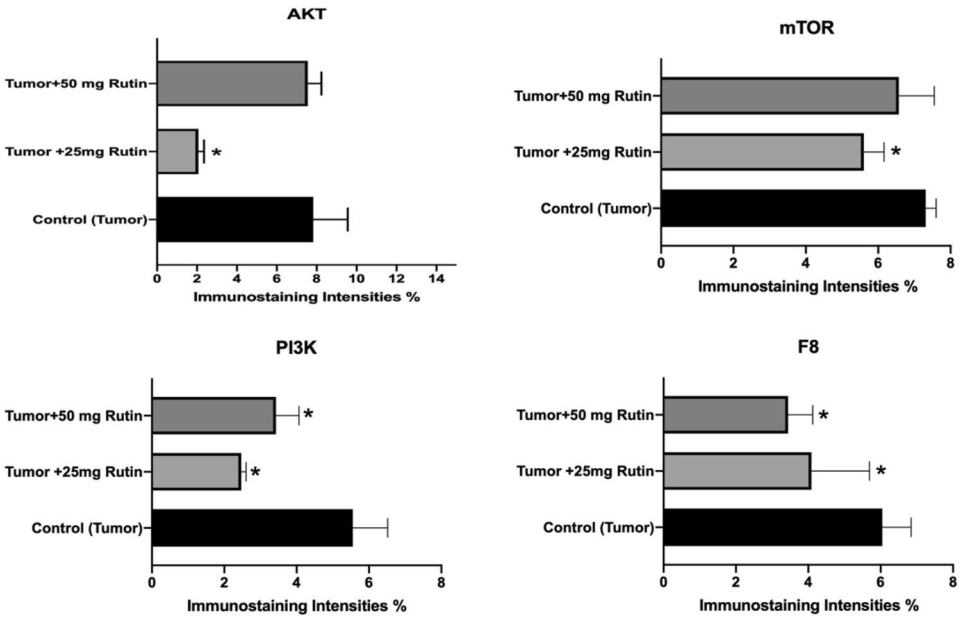

Fig. 5 Histogram plots showing the intensities of AKT, mTOR, PI3K and F8 immunostaining. Data shown in the histogram plot are expressed as mean ± sd. One-way analysis of variance and Dunnett's multiple comparison test were applied. *$p < 0.05$ represents different from the control (tumor) group

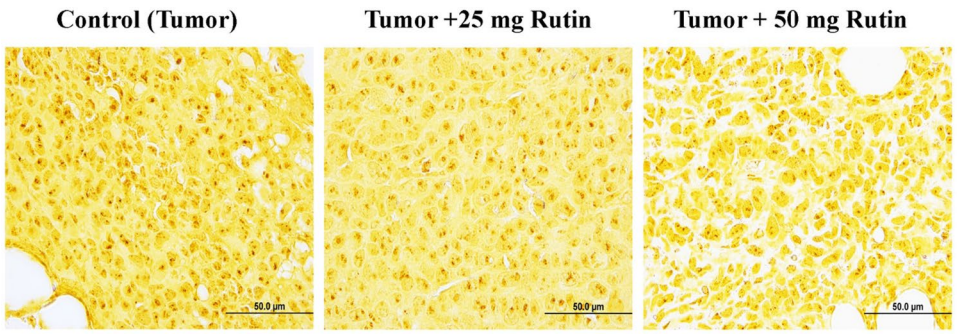

Fig. 6 Examples of the AgNOR staining cells in groups. Scale bar: 50.0 µm

PI3K, AKT1 and mTOR gene expression levels in tumor tissues

PI3K, AKT1 and mTOR expression levels in control tumors and tumor tissues exposed to different doses of rutin (25 and 50 mg/kg) were analysed by Real-time PCR method. *PI3K, AKT1* and *mTOR* mRNA levels of each group tumors were determined according to the mRNA levels of *PI3K, AKT1 and mTOR* expressed in control tumors. *GAPDH* is used as a reference gene for normalization.

PI3K mRNA level was increased in R25 group compared to the control tumors (3.9, fold regulation value). Whereas, *PI3K* mRNA level was decreased in R50 group compared to the control tumors (1.17). *AKT1* mRNA levels were increased in R25 and R50 groups compared to the control tumors (Fig. 7) (1.57, 1.57, respectively). *mTOR* mRNA levels were increased in R25 and R50 groups compared to the control tumors (2.2; 3.4, respectively, $p < 0.001$) (Fig. 7).

Annexin V and dead cell assay results

Data were taken from the graphs output from the Muse Cell Analyzer instrument (Fig. 8I). The viability and early/late/total apoptosis status of EAC cells were evaluated.

When the live cell count were measured, there was a significant reduce ($p < 0.05$) in the R10 group compared to the control group at the 24-h incubation. Live cell count was lower in R50 group compared to control group however, there was no statistically significant difference ($p > 0.05$) (Fig. 8II). In 48-h incubation, cells viability of the R30 and R70 groups significantly higher ($p < 0.05$) than the control group (Fig. 8II).

Early apoptotic cell count was significantly lower in R10 group compared to the control group ($p < 0.05$) in 24-h. In 48 h, there was no significant difference in terms of early apoptotic cell count in the all groups compared to the control group ($p > 0.05$) (Fig. 8II).

When the 24-h data were appraised, there was significant increase with regard to late apoptotic cell count in the R10

Table 2 TAA/NA and AgNOR number values of the experimental groups

Groups	TAA/NA	AgNOR number
Control (Tumor) 1	0.35 ± 0.07	7.40 ± 1.02
Control (Tumor) 2	0.39 ± 0.09	6.10 ± 1.08
Control (Tumor) 3	0.38 ± 0.07	4.10 ± 1.12
Control (Tumor) 4	0.32 ± 0.10	5.35 ± 1.37
Control (Tumor) 5	0.35 ± 0.09	5.40 ± 1.43
Control (Tumor) 6	0.35 ± 0.09	5.90 ± 1.23
Control (Tumor) 7	0.40 ± 0.05	5.00 ± 1.77
Control (Tumor) 8	0.41 ± 0.08	7.90 ± 1.33
Tumor + 25 mg Rutin 1	0.36 ± 0.08	5.92 ± 1.40
Tumor + 25 mg Rutin 2	0.30 ± 0.05	4.18 ± 1.67
Tumor + 25 mg Rutin 3	0.35 ± 0.03	4.40 ± 1.53
Tumor + 25 mg Rutin 4	0.30 ± 0.04	3.50 ± 1.56
Tumor + 25 mg Rutin 5	0.36 ± 0.07	4.50 ± 1.58
Tumor + 25 mg Rutin 6	0.47 ± 0.07	4.40 ± 1.60
Tumor + 25 mg Rutin 7	0.32 ± 0.04	4.60 ± 1.21
Tumor + 25 mg Rutin 8	0.26 ± 0.03	4.50 ± 1.12
Tumor + 50 mg Rutin 1	0.23 ± 0.04	4.20 ± 0.88
Tumor + 50 mg Rutin 2	0.15 ± 0.02	2.70 ± 0.77
Tumor + 50 mg Rutin 3	0.16 ± 0.04	3.50 ± 0.92
Tumor + 50 mg Rutin 4	0.13 ± 0.03	3.50 ± 1.33
Tumor + 50 mg Rutin 5	0.16 ± 0.04	3.40 ± 1.18
Tumor + 50 mg Rutin 6	0.20 ± 0.03	3.40 ± 0.57
Tumor + 50 mg Rutin 7	0.18 ± 0.06	3.40 ± 0.68
Tumor + 50 mg Rutin 8	0.16 ± 0.05	3.20 ± 0.75

TAA/NA Total AgNOR Area/Nuclear Area

Table 3 Comparison of the three groups for mean AgNOR count and TAA/NA

Groups	TAA/NA	AgNOR number	*p*	X^2
Control (Tumor)	0.36 ± 0.08	5.89 ± 1.29	0.000*	82.321*
Tumor + 25 mg Rutin	0.34 ± 0.05	4.05 ± 1.47	0.000	
Tumor + 50 mg Rutin	0.17 ± 0.03	3.41 ± 0.08	0.000	114.360

TAA/NA Total AgNOR Area/Nuclear Area

group compared to the control group ($p < 0.05$) (Fig. 8II). At the end of 48 h, there was significant rise with regard to late apoptotic cell count in the R50 and R70 ($p < 0.05$) groups compared to the control group (Fig. 8II).

A significant decrease was sighted in the total apoptotic cell count of the R70 group compared to the control group ($p < 0.05$) in 24 h (Fig. 8II). At the end of 48 h, there was significant decrease with regard to total apoptotic cell count in the R30 and R70 groups compared to the control group ($p < 0.05$) (Fig. 8II).

Discussion

Cancer is a growing condition worldwide, and damage to the microenvironment, focal adhesion kinases, membrane and extracellular matrix is related to integrins. The increase in cancer cells is closely related to epithelial-mesenchymal transition and metastasis. Antioxidative reagents have been applied in the treatment of many types of cancer [25]. Rutin, also known as quercetin-3-rutinoside, is a natural plant-derived antioxidant [26]. In this study, we created a solid tumor cell model of EAC in an experimental mice model and determined the antioxidant property of Rutin on solid tumor cells. Next, we constructed a cell model under stimulation of EAC and investigated the in vitro effect of rutin applied at different doses. We also showed that rutin significantly suppressed the activity of the PI3K/AKT/mTOR signaling pathway in a solid tumor model. mTOR is an important component of signaling pathways that include PI3K/AKT as an effective therapeutic target in breast cancer [27]. Previous studies have reported that dysregulation of mTOR signaling plays a role in various diseases such as obesity and neurodegenerative disorders, including cancer. AKT is generally referred to as serine/threonine kinases, which belongs to the subfamily of protein kinases, and is involved in the regulation of AKT through the increase in cell growth and the interaction of mTOR and tuberous sclerosis complex (mTORC1, TSC1, TSC2) [28]. PI3K is a lipid kinase divided into three different classes based on substrate specificity. Class 1, PI3K is often associated with cancer [29]. It has been reported that antioxidants given in studies in the literature downregulate the mTOR-signaling pathway. Wen et al. (2019) investigated whether eribulin

Table 4 Pairwise comparison between groups

Groups	TAA/NA		AgNOR number	
	p	*Z*	*p*	*Z*
Control (Tumor) vs. Tumor + 25 mg Rutin	0.004	−2.701	0.758	−0.155
Control (Tumor) vs. Tumor + 50 mg Rutin	0.003	−5.621	0.170	−2.426
Tumor+25 mg Rutin vs. Tumor+50 mg Rutin	0.001	−6.348	0.005	−3.142

TAA/NA Total AgNOR Area/Nuclear Area, *vs.* versus

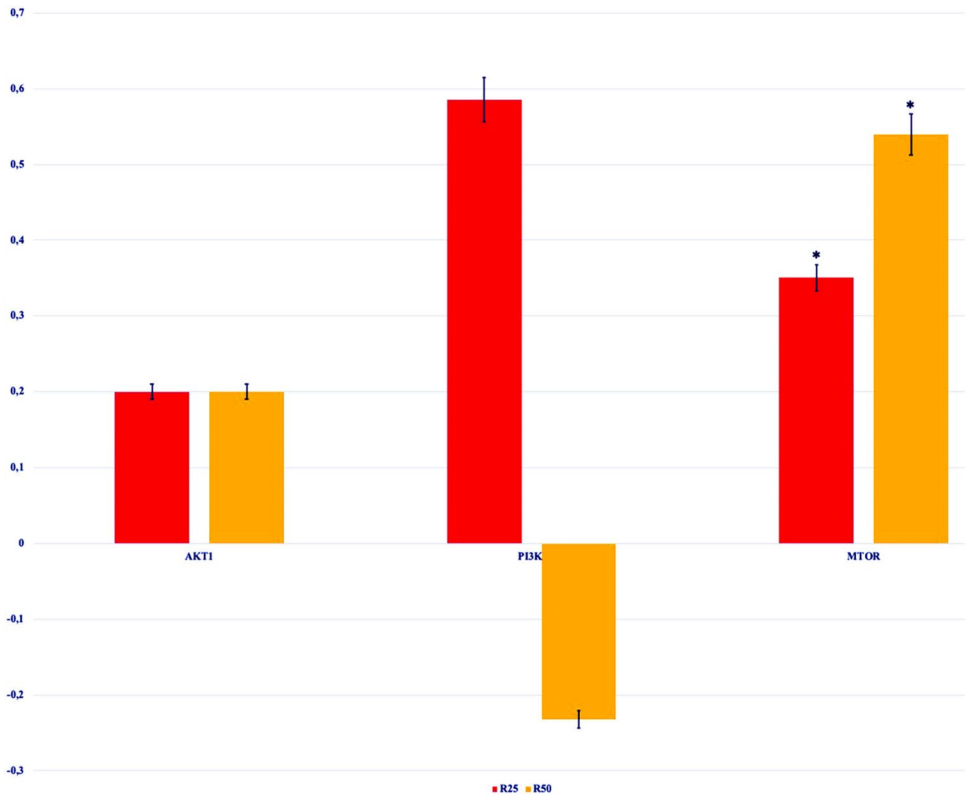

Fig. 7 Relative mRNA expression of PI3K, AKT1 and mTOR in tumor tissues exposed to 25 and 50 mg/kg rutin were given as fold regulation levels. GAPDH is reference gene for normalization. * Represents the significance of $p < 0.001$

increases the anticancer activity as an mTOR inhibitor and stated in their study that the treatment with eribulin inhibited the phosphorylation of AKT in vivo and in vitro [30]. Vladić et al. (2020) investigated the antiproliferative and antioxidant activity of Satureja montana extracts in their study in an EAC model in vivo and reported that the extract concentrations inhibited tumor growth [31]. Ahmed et al. (2022) reported that they determined improved liver p53 levels in the experimental groups administered Rutin and Quercetin [32]. In another study conducted with Rutin, cytotoxic activity due to proliferation of cervical cancer cell line (SiHa) and mRNA expression were evaluated by Real-time PCR analysis and it was shown that rutin increases anticarcinogenic efficacies by inducing modulation in the expression of oncogenes besides the tumor suppressor genes [33]. High levels of von willebrand factor (VWF) and factor VIII (FVIII) are defined as acute phase reactants that can indicate endothelial dysfunction and inflammation in different settings, including chronic autoimmune diseases. It has been reported that tumor-associated macrophages and inflammatory cell groups in the tumor microenvironment contribute to the high FVIII levels seen in cancer patients [34, 35]. Quercetin in literature, given at different doses, suppresses increased angiogenesis in the EAC solid tumor model [10]. Diverse species of proteins related with the silver-binding ribosome can form AgNORs. The specification of the activities of these proteins is used as a biomarker revealed by the proliferation and metabolic activity of the cells. The detection of NOR proteins is used to obtain information about the anticancer effect of phytotherapeutic agents and to determine the reliable dose for therapeutic uses. Studies have reported that antioxidant agents have cancer-protective effects because NOR proteins show the proliferation rate of cells [10, 35–37].

Firouzai-Amandi et al. (2022) reported that in treated MDA MB-231 cells with RUT-loaded PCL-PEG, there was a significant up regulation of caspase-8, -9, -3, and Bax [38]. In another study, Rutin treatment showed decreased cell viability with increased cell accumulation in the G0/G1 phase of the cell cycle in HeLa cell lines [39]. Khan et al. (2021) emphasized that rutin cause a dose-dependent antiproliferative effects on Caski cervical cancer cells. And they reported that DAPI and Mitotracker red staining revealed that rutin induced significant apoptotic effects via caspase-3/9 activation, ROS generation, and variance in Bax/Bcl2 mRNA expression [40].

Conclusion

Rutin is known to contribute to very powerful biological actions, including anticarcinogenic effects. It has been demonstrated in studies that Rutin uses many mechanisms (apoptosis, inflammation, angiogenesis and autophagy) to inhibit

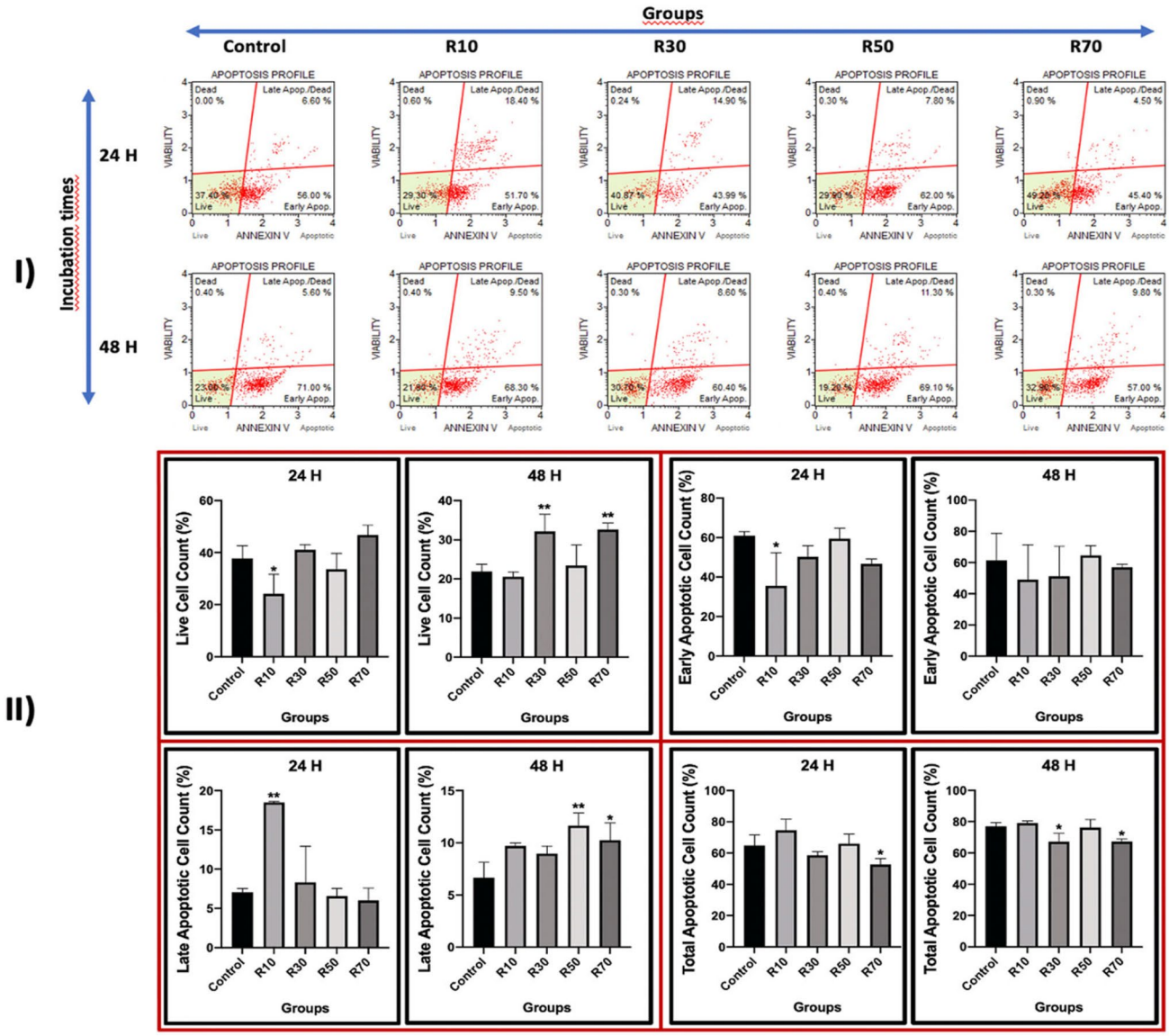

Fig. 8 **I** Raw graphic taken from the Muse Cell Analyzer (Annexin V and Apoptosis Profile). **II** Data of the Annexin V & Dead Cell Assay at the end of 24 and 48 h (24 H and 48 H) of incubation. *$p < 0.05$ was considered statistically significant. Data are expressed as mean ± SD (Standard Deviation). Control (Tumor) group: Control, Rutin groups: R10, R30, R50, R70 (10, 30, 50, 70 µg/mL Rutin)

cancer induction and progression by modulating several dysregulated signaling pathways.

Particularly, the anticancer effect of rutin has been associated with the regulation of multiple signaling pathways, including NF-κB, PI3K/AKT/mTOR, Nrf2, ERK, JNK and p38 MAPK. In our study, we determined the effects of rutin at different doses on solid EAC tumor cells using the mTOR signaling pathway. At the same time, we determined that tumor cells went to apoptosis in our in vitro experimental groups.

With the study, both the effects of rutin on the signal pathway and the results of the TAA / NA ratio give information about the choise of a safe dose for cancer therapy.

Acknowledgments Thanks to Yozgat Bozok University Scientific Research Projects Coordination Unit for financial support for the project.

Authors Contribution Material preparation, conceptualization and project administration were performed by [SY] and [ZD]. Data collection and analysis, validation were performed by [AOO], [ŞA] and [ESAS]. Writing—Review & Editing were performed by [MN] and [AAF]. All authors read and approved the final manuscript.

Funding This project was financially supported by Yozgat Bozok University Scientific Research Projects Coordination Unit (Grant Number: 6602c-TF/21-480).

Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest None.

Ethics Approval The stage of the study on experimental animals was carried out in accordance with the decision numbered 20/105 taken from the Animal Experiments Local Ethics Committee of Erciyes University (Decision date: 12.08.2020).

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