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T. KISKOVA[1], V. DEMECKOVA[1], Z. JENDZELOVSKA[1], M. KIKTAVA[1], K. VENGLOVSKA[1], M. BOHMDORFER[2], W. JAGER[2], T. THALHAMMER[3]

NOCTURNAL RESVERATROL ADMINISTRATION INHIBITS CHEMICALLY INDUCED BREAST CANCER FORMATION IN RATS

[1]Faculty of Science, Institute of Biology and Ecology, University of Pavol Jozef Safarik in Kosice, Kosice, Slovakia; [2]Department of Clinical Pharmacy and Diagnostics, University of Vienna, Vienna, Austria; [3]Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

Resveratrol (RES) is well known natural polyphenol with proven antioxidant, antiinflammatory and anticarcinogenic properties. Since mode of application may be important for cancer-preventive effects of RES, the aim of this study was to evaluate a possible delay in the initiation and progression of chemically induced mammary carcinogenesis in female Sprague-Dawley rats after the nocturnal administration of RES. Application of a high dose of RES (100 mg/kg body weight), starting 2 weeks before the first N-methyl-N-nitrosourea dose (NMU) (50 mg/kg body weight), reduced tumor incidence and markedly prolonged latency period ($P < 0.01$) in the NMU + RES group in comparison to NMU tumor bearing animals. In addition, the tumor volume decreased significantly ($P < 0.05$) together with tumor frequency ($P < 0.05$). We also observed that food but not water intake was significantly reduced by 17% between weeks 4 and 12 in the NMU + RES group leading to a pronounced reduction in the body mass of about 25% as compared to untreated controls. In addition to direct effects of RES in tumor tissues, this polyphenol did also improve metabolic functions in RES-treated animals since it normalizes hypoproteinemia and urea levels and increases the number of lymphocytes when compared with NMU. Higher level of reactive oxygen species (ROS) in leukocytes and the elevation of proinflammatory plasma cytokines IL-1 and IL-2 may contribute to the observed reduction in tumor development. These results indicate for the first time that nocturnal administration of a high dose of RES significantly affects tumor development *in vivo*. Therefore, we conclude that RES is a promising candidate for cancer chemoprevention. However, it should be noted that the mode of application might significantly affect RES ability to fight cancer.

Key words: *resveratrol, circadian rhythm, carcinogenesis, breast cancer, immunity, reactive oxygen species*

INTRODUCTION

Breast cancer is the mostly diagnosed cancer type in women. Approximately 70% of human's breast cancers are hormone-dependent and estrogen receptor (ER)-positive; hence ER expression is the main indicator of potential responses to hormonal therapy. This basic classification was followed by the progesterone receptor positivity and human epidermal growth factor 2 (HER2) gene alterations (1). Tamoxifen is the best established selective ER modulator (SERM). It has favorable effects on breast cancer control and bone metabolism, but also adverse effects due to its estrogenic activity in other tissues (1).

Resveratrol (RES) is well known natural polyphenol with proven antioxidant, antiinflammatory and anticarcinogenic properties. RES has both estrogenic and antiestrogenic properties when bound to the ER and therefore has characteristics like SERMs (2). Recently we showed that RES acts as a preventive agent for N-methyl-N-nitrosourea (NMU) induced hormone-sensitive breast tumors in combination with melatonin during day time (3). RES alone had no effect in this study. Melatonin alone even increased tumor frequency and incidence (3). It is well established that melatonin has oncostatic effects in breast cancer by disrupting estrogen-mediated cellular pathways (4). The pineal production of melatonin follows a diurnal rhythm, with peak production at about 0 – 2 a.m (5). Studies have shown that circadian disruption, specifically night shift work, is correlated with an increased risk of developing breast cancer (6-8). As melatonin levels are highest during night we hypothesized that RES application during night time at the time of physiologically melatonin secretion might be even more beneficial.

The time-dependency of RES is complicated, still opened and well discussed issue. The most experiments focused on cancer are carried out during the day because of the simple design of the experiment. However, this polyphenol behaved as an antioxidant during the dark part of the day and as a prooxidant during the light span (9). But the information about the diurnal activity of RES in the process of carcinogenesis still lacks.

The aim of our experiment was to evaluate the cancer initiation and progression after the nocturnal administration of RES in the process of chemically induced mammary carcinogenesis in female Sprague-Dawley rats.

MATERIALS AND METHODS

Animals and conditions

Female Sprague-Dawley rats (n = 31), aged 31 days and weighing 100 – 130 g, were obtained from Velaz (Unetice, Czech Republic). They were adapted to standard vivarium conditions with a temperature of 21 – 24ºC, a relative humidity of 50 – 65% and to an alternating 12:12 h light-dark regimen, with lights on from 10:00 p.m. to 10:00 a.m. (light intensity of 150 lux per cage). In our experiments, RES was given to the animals 4 hours after the starting of dark phase (at 14:00 p.m.) because Per2 as well as melatonin are described to be highest in humans at midnight usually 4 – 6 hours after dark (7, 10).

Animals were fed standard rat pellets (Peter Misko, Snina, Slovakia) and had free access to tap water. RES (Carbosynth, Compton, UK) was dissolved in 10% ethanol (EtOH). The solution in a concentration of 100 mg/kg body weight was always freshly prepared 15 min before the administration (Mon-Fri). RES was administered *per os* with the pipette tip at the base of the tongue at 14:00 h (midnight according to the converted regimen).

All animal experiments were performed according to the principles provided in Law of the Slovak Republic for the Care and Use of Laboratory Animals (Protocol Nr. 1667/13-221/3).

Experimental design

Mammary carcinogenesis was initiated with two intraperitoneal doses of NMU (50 mg/kg body weight each; Sigma, Deisenhofen, Germany) dissolved in a physiological NaCl solution. The first dose was injected on the 43rd postnatal day and the second dose was injected on the 50th postnatal day according to our previous experimental design (11). During this postnatal period the breast is developing and is therefore more sensitive to NMU treatment. Rats with NMU induced mammary tumors were divided into 3 groups: NMU group (n = 8) was used as the control group of mammary cancer progression. Vehicle group (n = 8) received 10% EtOH (NMU + EtOH) and NMU + RES group (n = 9) was treated with RES on daily basis. The intact control group (CONT, n = 6) consisted of healthy control animals. The prevention with RES and EtOH began 2 weeks before the 1st NMU dose and run until the end of the experiment (16 weeks).

The rats were weighed and palpated twice a week to record the presence, number, localization, and size of each palpable tumor. In 4th, 8th and 12th week of the experiment, food and water intake was monitored. At the end of the experiment, the animals were killed by quick decapitation with a guillotine after the last dose of RES. Mammary tumors were excised, characterized as ER positive tumors corresponding to luminal A ductal tumors (12) and tumor size was recorded. Histology inspections of these tumors revealed that 43% were *in situ* carcinomas whereas 57% were invasive carcinomas with no differences between NMU, NMU + RES and NMU + EtOH groups. The basic parameters evaluated included tumor incidence, latency period, tumor frequency and tumor volume as described previously (3). The tumors and healthy mammary glands were resected and samples immediately frozen and stored at –80ºC for reverse transcription-polymerase chain reaction (RT-PCR) and high-performance liquid chromatography (HPLC) analysis. Part of the tissues was fixed in formaldehyde and embedded in paraffin for immunohistochemistry (IHC). In addition, blood was collected for the total blood analysis by using the MindrayBC 2800VET (China) autoanalyzer. Biochemistry analysis was performed using the ELLIPSE, AMS SpA, (Italy) analyzer.

Enzyme-linked immunosorbent assay (ELISA) for cytokines in serum

For quantitative measurement of the amount of 12 cytokines (IL-1A, IL-1B, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IFN-γ, TNF-α, GM-CSF, RANTES) in blood serum, a multi-ELISA kit was used (Rat Inflamatory Cytokines Multi-Analyte ELISArray™ Kit Qiagen, Germany) following manufacturer´s guidelines. Briefly, frozen serum samples were thawed at room temperature. 50 µl sample was added to 50 µl assay buffer into each well of ELISArray plate. After washing, 100 µl of detection antibody solution was added for 1 hour. Repeated washing was followed by a 1 hour incubation in 100 µl avidin-HRP solution and then by 15 min incubation in 100 µl development solution in the dark. By adding 100 µl stop solution, the reaction was stopped and the absorbance was measured immediately at the 450 nm.

Measurement of reactive oxygen species in blood leukocytes

100 µl of total blood obtained from rats by decapitation were drawn into heparin-treated tubes. Each sample was divided into halves and treated as described previously (11). Briefly, samples were lysed using a red blood cells lysis buffer, centrifuged, and washed in PBS. Thereafter 10 µM dihydrorhodamine-123 (Fluka, Buchs, Switzerland) was added. Following 20 min incubation at room temperature, the samples were measured using a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The reactive oxygen species (ROS) level is expressed as a ratio of dihydrorhodamine-123 fluorescence median to autofluorescence.

Analytical assay for resveratrol and its metabolites

Frozen plasma samples stored at –80ºC were quickly thawed and centrifuged at 13,000g for 5 min at room temperature. For the HPLC analysis, 200 µl of methanol was added to 100 µl of plasma. Subsequently, the samples were centrifuged again at 13,000 g for 5 min and 80 µl of each supernatant was injected onto the HPLC column as described previously (13). Selected tumor tissues (400 mg) were homogenized in 800 µl of 50 mM potassium phosphate buffer (pH 7.4) as described previously (11). Briefly, the cells were lysed by repeated freezing and thawing. Methanol was added and after centrifugation, 80 µl of the supernatant was analyzed for RES and RES metabolite content. Linear calibration curves were performed using external standards of RES and RES metabolites (concentration range of 5 ng/ml to 1 µg/ml), yielding correlation coefficients (r2) of at least 0.99.

Resveratrol (3,4',5-trihydroxy-trans-stilbene) was obtained from Sigma-Aldrich (Munich, Germany). Resveratrol-3-O-glucuronide, resveratrol-3-O-sulfate and resveratrol-3-O-4'-O-disulfate were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

RNA extraction and semi-quantitative RT-PCR for Per2

To assess *Per2* expression, total RNA was extracted from frozen breast tissue samples (obtained from tumors excised 4 hours after onset of darkness) using TRIReagent® (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's recommendations. In addition, RNA was also isolated from the tumor tissues from the 'day experiment' (NMU*) (11) to verify the success of light-dark regimen conversion. The concentration (absorption maximum 260 nm) and purity (260/280 and 260/230 ratios) were determined by assessing UV absorbance. Integrity of RNA samples was evaluated by gel electrophoresis using non-denaturing 1% agarose gel containing 1 × GelRed (Biotium Inc., Hayward, CA, USA) in 1 × Tris-Acetate-EDTA buffer (TAE;

Fermentas, Thermo Fisher Scientific, Vilnius, Lithuania). Subsequently, the samples were normalized to equal amounts of RNA. For reverse transcription (RT), a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) was used according to the manufacturer's instructions. cDNA was synthesized from 2 µg of total RNA using random hexamer primers in a 20 µl solution. Afterwards, cDNA samples were subjected to PCR amplifications with primers selective for *Per2* gene and Actb gene as internal loading control. The primers used are indicated in *Table 1*. Primer specificities were verified using Primer-BLAST (NCBI). Each PCR mixture contained cDNA derived from 40 ng of RNA; deoxynucleoside triphosphates (dNTP Mix; Fermentas) at a concentration of 200 µM; 0.2 µM of each primer; and 1 unit of BioThermAB™ Hot Start Taq DNA Polymerase (GENECRAFT, Koln, Germany) in a total volume of 25 µl. The PCR was performed on a Mastercycler pro S (Eppendorf, Hamburg, Germany) and the reaction conditions were as follows: initial denaturation at 94ºC for 2 min, followed by an appropriate number of cycles (*Table 1*) of denaturation at 94ºC for 30 s, annealing at the annealing temperature (Ta) for 30 s and elongation at 72ºC for

Table 1. Primers used in RT-PCR.

	Ta	Primer sequence (5'-3')	No. of cycles	Product length (bp)
Per 2 F	61ºC	AAGCAGGTGAAGGCTAATGAGGA	30	151
Per 2 R		CCACAGCAAACATGTCCGAGTT		
ACTB F	55ºC	TCTCTTCCAGCCTTCCTTCCT	25	100
ACTB R		GAGGTCTTTACGGATGTCAACG		

Primers were designed according to previously published nucleotide sequences available in the NCBI (National Center for Biotechnology Information). The specificity of the proposed oligonucleotide was verified using the application Primer-BLAST (NCBI).
F, forward; R, reverse.

Table 2. Food and water intake in healthy and NMU-treated rats.

	week	**CONT**	**NMU**	**NMU + RES**	**NMU + EtOH**
Food intake	4	17.54 ± 1.12	17.25 ± 0.23	14.50 ± 0.83*	16.09 ± 0.37
(g)	8	19.50 ± 0.80	17.90 ± 1.11	16.10 ± 0.32*	17.86 ± 0.59
	12	18.29 ± 0.44	17.25 ± 0.23	14.50 ± 0.34**	16.09 ± 0.23
Water intake	4	22.95 ± 1.56	20.44 ± 0.31	20.73 ± 1.23	19.75 ± 0.52
(ml)	8	21.17 ± 0.55	20.66 ± 0.77	19.58 ± 0.48	20.09 ± 0.32
	12	21.63 ± 1.08	20.22 ± 0.33	20.73 ± 1.23	19.75 ± 0.52

Mean food and water intake measured in the 4th, 8th and 12th experimental weeks. Data are expressed as mean ± SD. Significance versus NMU: *P < 0.05 and **P < 0.01, respectively.

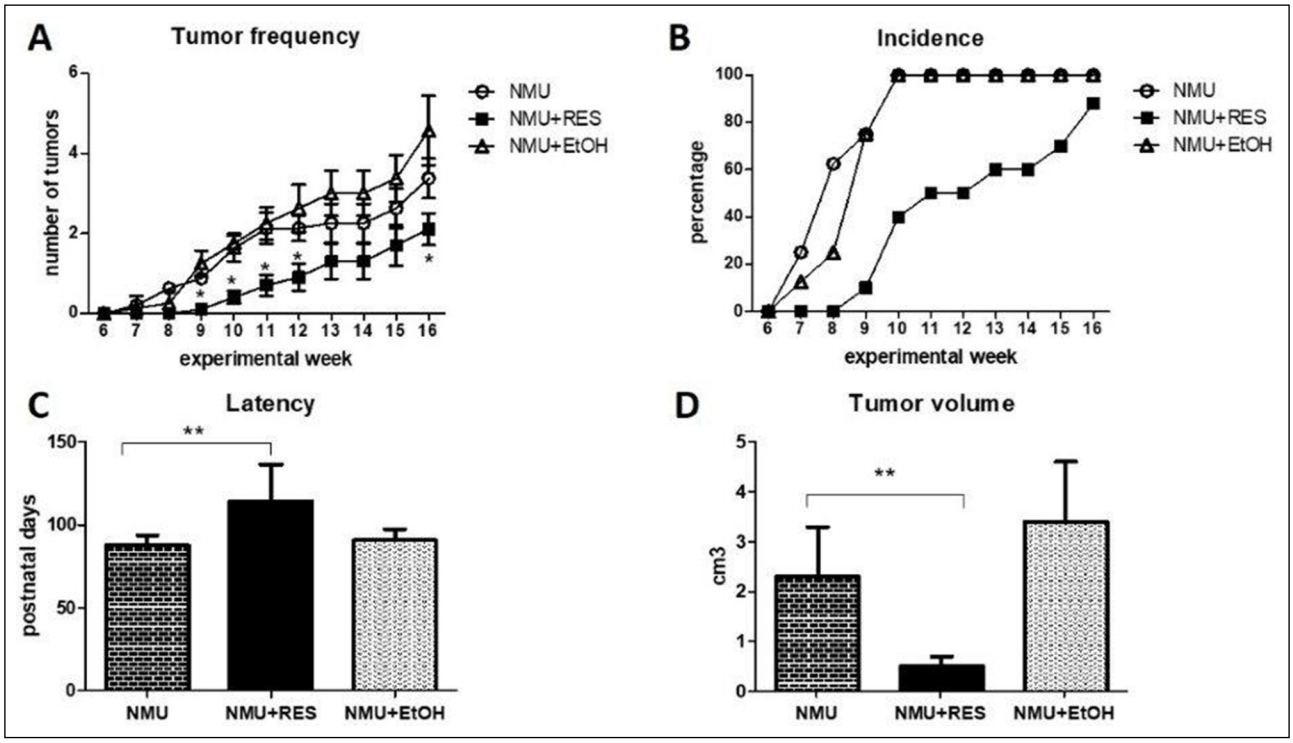

Fig. 1. Carcinogenesis in NMU-treated rats. (A) tumor frequency: average tumor number per group (means ± SD); (B) incidence: percentage of tumor bearing animals compare to animals per group monitored over the experimental period, (C) latency: day, when the first tumor appeared (means ± SD); (D) tumor volume in cm^3 (means ± SD). Significance versus NMU: *P < 0.05 and **P < 0.01, respectively.

45 s, with a final extension at 72ºC for 10 min. The amplified products were loaded onto an agarose gel in 1 × TAE buffer. The specific bands were visualized with 1 × GelRed and photographed under UV light using a ChemiDoc™ XRS + Imaging System (Bio-Rad Laboratories). For semi-quantitative analysis, PCR products were normalized to ACTB by the mean optical density value of specific bands using Image J software (NIH, Bethesda, MD, USA).

Immunohistochemical staining

Formalin fixed and paraffin embedded tissues were sectioned. 4 µm sections were washed 3 × in xylene and 1 × in absolute EtOH. Thereafter, slides were boiled in microwave for 10 min in DEPP 9 buffer prepared according to manufacturer´s recommendations (DEPP 9 buffer with distilled water 1:20; EB-depp9-250, Eubio, Vienna, Austria). After 25 min cooling, followed by washing in PBS Tween 0.1% (3 × 3 min), slides were blocked by UltraVision Protein Block (Thermo Scientific) for 7 min. After drying, slides were incubated with primary antibodies (ERα ab2746, ERβ H-150: sc-8974; Santa Cruz Biotechnology Inc. Heidelberg, Germany, Europe) in BSA in ratio 1:100 (ERα) and 1:200 (ERβ) for 60 min. After washing in PBS Tween, AB Enhancer (Thermo Scientific) was used for 10 min, followed by 15 min incubation with HRP-polymer (Thermo Scientific). The slides were washed and the antigen was visualized using DAB Plus chromogene (Thermo Scientific) and washed 4 × in distilled water. After 1 min incubation in hematoxylin, washing with the tap water was used. After drying, slides were mounted with Fluoromount-G™ (Southern Biotech). For quantitative analysis of IHC-stained antigens, HistoQuest software (TissueGnostics, Vienna, Austria) was utilized.

Statistical analysis

For statistical analyses a GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA) software package were used. The values were expressed as mean ± SD and the Student's t-test and ANOVA with Tukey´s post-test were used to compare differences between control samples and treatment groups. Statistical significance level was set to $P < 0.05$.

RESULTS

Food and water intake

During the whole experiment, water and food intake was measured. While water intake remained constant during the experiment, food intake was significantly reduced by

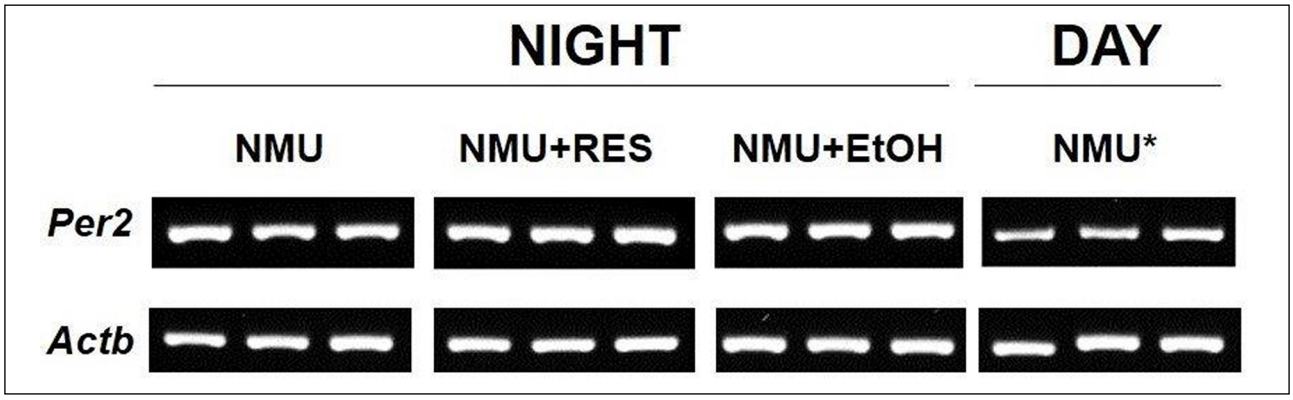

Fig. 2. Per2 mRNA level in tumors in three representative tumors in NMU-treated rats. Night samples were taken at the dark period and day samples during the light period (see Materials and Methods).

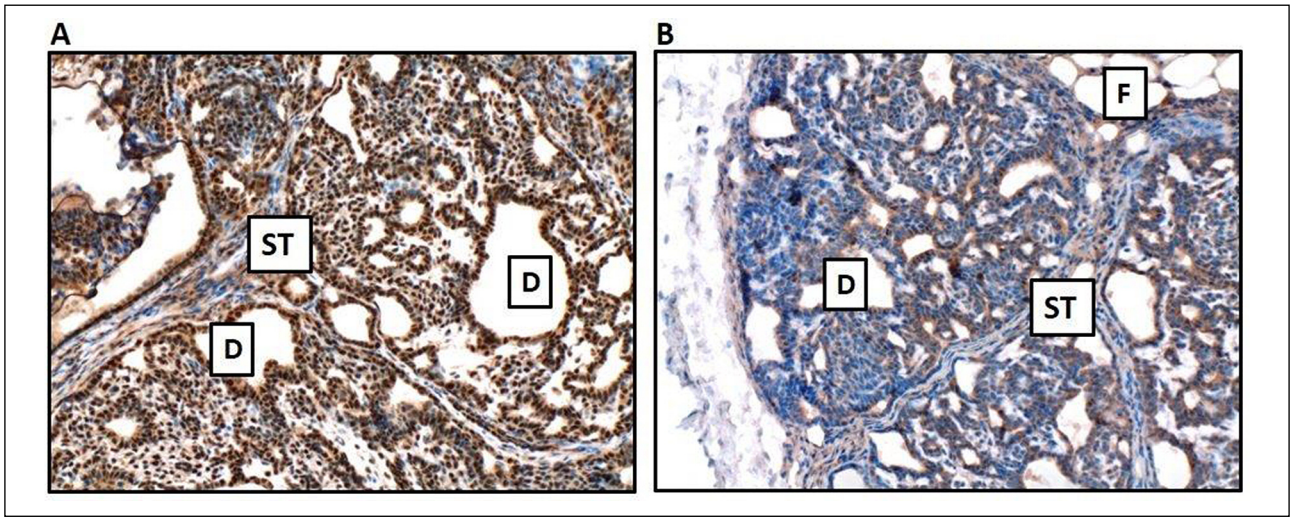

Fig. 3. Immunohistochemical staining for estrogen receptor ERα (A) and ERβ (B) in representative specimen from ductal carcinoma *in situ*. Paraffin-embedded specimens from breast cancer were chosen from the NMU group. Brown stained nuclei indicate presence of the estrogen receptor isoform in cells.
D, mammary duct; ST, stroma; F, fat

approximately 17% between weeks 4 and 12 in the NMU + RES group (*Table 2*). NMU + RES intake therefore led to a more pronounced reduction in the body mass of about 25.1 ± 3.8% than NMU and NMU + EtOH (21.6 ± 2.9% and 22.4 ± 3.5%, respectively) as compared to untreated controls.

Process of carcinogenesis

Following NMU administration, the tumors appeared in NMU group already in 7th experimental week. However, in NMU + RES group, the first tumors were palpable in 9th week. During the whole experiment, tumor frequency was decreased in NMU + RES group up to 40% when compared with NMU (*Fig. 1A*). Moreover, tumor incidence was lower in the NMU + RES group, especially from 7th to 14th week of the experiment (*Fig. 1B*). Additionally, RES significantly increased tumor latency (114.6 ± 22.0 versus 87.5 ± 6.2 postnatal days; $P < 0.01$) (*Fig. 1C*) and decreased tumor volume compared with NMU animals (0.51 ± 0.2 versus 2.34 ± 0.61 cm^3; $P < 0.01$) (*Fig. 1D*). The NMU + EtOH group showed no differences in the numbers of tumors and in the tumor frequency compared with NMU animals (5% error). However, EtOH increased tumor volume as shown in *Fig. 1D* (3.40 ± 0.94 versus 2.34 ± 0.61 cm^3).

Per2 expression in tumor samples

In order to determine whether or not the rats were adapted to the day and night cycle, *Per2* mRNA levels were analyzed. As shown in *Fig. 2*, mRNA levels of *Per2* indeed increased during night by approximately 38% in NMU, NMU + RES and NMU + EtOH animals as compared to NMU*-treated animals during day time.

Table 3. The effect of RES on whole blood parameters in NMU-induced mammary carcinogenesis in rats.

Parameters	SI unit	**NMU**	**NMU + RES**
Total WBCs	10^9/L	12.3 ± 6.9	8.4 ± 4.6
Lymphocytes	10^9/L	7.0 ± 2.7	**8.5 ± 2.2***
Lymphocytes	%	57.1 ± 11.0	**72.8 ± 10.9***
Monocytes	10^9/L	0.43 ± 0.38	0.38 ± 0.28
Granulocytes	10^9/L	6.0 ± 4.5	2.0 ± 1.6
RBC	10^{12}/L	6.2 ± 1.6	6.6 ± 0.4
Hematocrit	%	37.5 ± 6.2	40.4 ± 2.7
HGB	g/L	136.5 ± 2.3	137.9 ± 1.57
MCV	fL	62.1 ± 8.2	61.0 ± 3.8
RDW	%	13.2 ± 1.6	12.9 ± 1.7
Platelets	10^9/L	635.4 ± 226.9	521.1 ± 228.5
PDW	%	15.88 ± 0.33	15.91 ± 0.38
PCT	%	0.48 ± 0.16	0.41 ± 0.01
MPV	fL	7.6 ± 0.5	7.6 ± 0.8

Data are expressed as mean ± SD. Significance versus NMU: *P < 0.05.
HGB, hemoglobin; MCV, mean cell volume; MPV, mean platelet volume; PCT, platelecrit; PDW, platelet distribution width; RBC, red blood cells; RDW, red cell distribution width; WBC, white blood cells.

Table 4. The effect of RES on biochemical parameters in NMU-induced mammary carcinogenesis in rats.

Parameters	**SI unit**	**NMU**	**NMU + RES**
Total protein	g/l	47.63 ± 2.41	**62.43 ± 1.96****
Urea	mmol/l	4.53 ± 0.41	**3.46 ± 0.35***
Albumin	g/l	32.60 ± 1.25	35.41 ± 0.93
Creatinine	µmol/l	60.00 ± 5.04	65.50 ± 8.78
Calcium	mmol/l	3.13 ± 0.22	3.32 ± 0.12
Phosphor	mmol/l	1.26 ± 0.46	1.43 ± 0.46
SGOT	(IU/l)	138.61 ± 10.82	123.60 ± 7.82
SGPT	(IU/l)	37.22 ± 4.23	36.03 ± 3.10
ALP	(IU/l)	80.43 ± 9.62	74.43 ± 4.82
Total cholesterol	mmol/l	1.91 ± 0.07	2.02 ± 0.06
HDL-cholesterol	mmol/l	0.82 ± 0.02	**0.51 ± 0.05*****
LDL-cholesterol	mmol/l	1.20 ± 0.75	0.85 ± 0.05
Triglycerides	mmol/l	1.37 ± 0.10	1.16 ± 0.22

Data are expressed as mean ± SD. Significance versus NMU: *P < 0.05, **P < 0.01 and ***P < 0.001, respectively. ALP, alkaline phosphatase; HDL, high density lipoprotein; LDL, low density lipoprotein; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamate-pyruvate transaminase.

Table 5. The effect of RES on cytokine production in NMU-induced mammary carcinogenesis in rats.

Cytokine (pg/ml)	**NMU**	**NMU + RES**
IL1A	20.2 ± 8.01	**34.5** ± **1.0*****
IL1B	234.1 ± 10.7	**297.4** ± **23.2*****
IL2	34.2 ± 2.9	**42.6** ± **3.1** *
IL4	9.2 ± 0.4	9.2 ± 0.2
IL6	227.8 ± 85.8	241.3 ± 23.9
IL10	298.3 ± 29.1	303.3 ± 5.3
IL12	14.2 ± 3.5	11.8 ± 1.3
IL13	90.2 ± 20.0	101.1 ± 28.3
INF-γ	196.2 ± 8.5	187.8 ± 7.2
TNF-α	23.1 ± 2.7	21.2 ± 2.0
GM-CSF	9.0 ± 0.4	9.0 ± 0.5
RANTES	35.2 ± 2.5	31.2 ± 1.7

Data are expressed as mean ± SD. Significance versus NMU: *P < 0.05 and ***P < 0.001, respectively.

Estrogen receptors ERα and ERβ in tumors

In all examined breast intact and tumor tissues, the high levels of immunoreactive ERα were observed. As seen on *Fig. 3A*, staining for ERα was concentrated predominantly on the ductal side of the tumor. Weak intensity of ERα was seen in the stroma. Concerning ERβ, the level of this receptor was statistically unchanged in cancerous tissues of NMU + RES animals in compare to NMU. The expression was evident in the surroundings of mammary ducts rather than in stromal parts of the tissue (*Fig. 3B*).

Total blood analysis and serum biochemical parameters

Daily intake of RES only had a minor impact on cellular blood components. However, the number and percentage of lymphocytes significantly increased by 17.6% and 27.5%, respectively (P < 0.05, *Table 3*). Other blood parameters were only slightly affected. RES also significantly increased total protein by 23.7% (P < 0.01) compared to the NMU group (*Table 4*). Urea was significantly lower (–30.9%, P < 0.05), reaching the value in healthy animals (data not shown). Other biochemical parameters were changed only slightly.

Cytokine production in serum

>From 12 cytokines measured, three were stimulated by RES treatment (*Table 5*). The elevated levels of IL-1A (P < 0.001), IL-1B (P < 0.001) and IL-2 (P < 0.05) were observed in NMU + RES group. Other cytokines were not statistically changed.

Levels of reactive oxygen species in blood leukocytes

The levels of ROS in blood leukocytes were measured using flow cytometry. As seen on *Fig. 4*, ROS level in NMU + RES increased significantly (2.36 ± 0.44; P < 0.05) compared to the NMU group (1.80 ± 0.30).

Concentration of resveratrol and its metabolites in plasma and tumor tissue

RES is extensively metabolized into three conjugates, namely resveratrol-3,4'-O-disulfate (RES-DIS), resveratrol-3-O-glucuronide (RES-GLU) and resveratrol-3-O-sulfate (RES-SUL), which can be found in blood and cancerous tissue after oral administration. In blood, the concentrations of metabolites

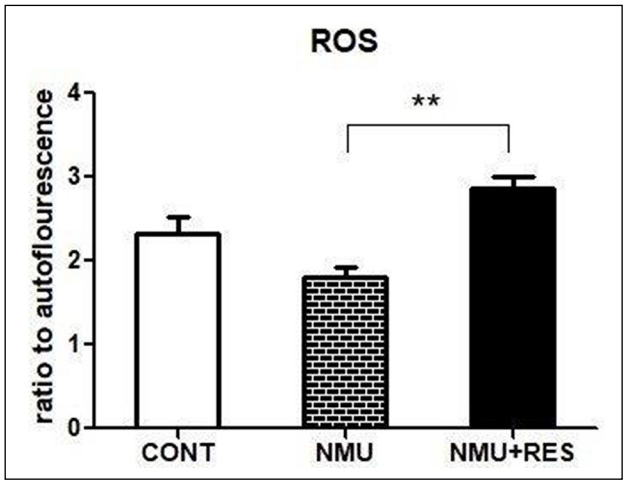

Fig. 4. Level of reactive oxygen species (ROS) in total blood leukocytes. Data are expressed as means ± SD. Significance versus NMU: **P < 0.01.

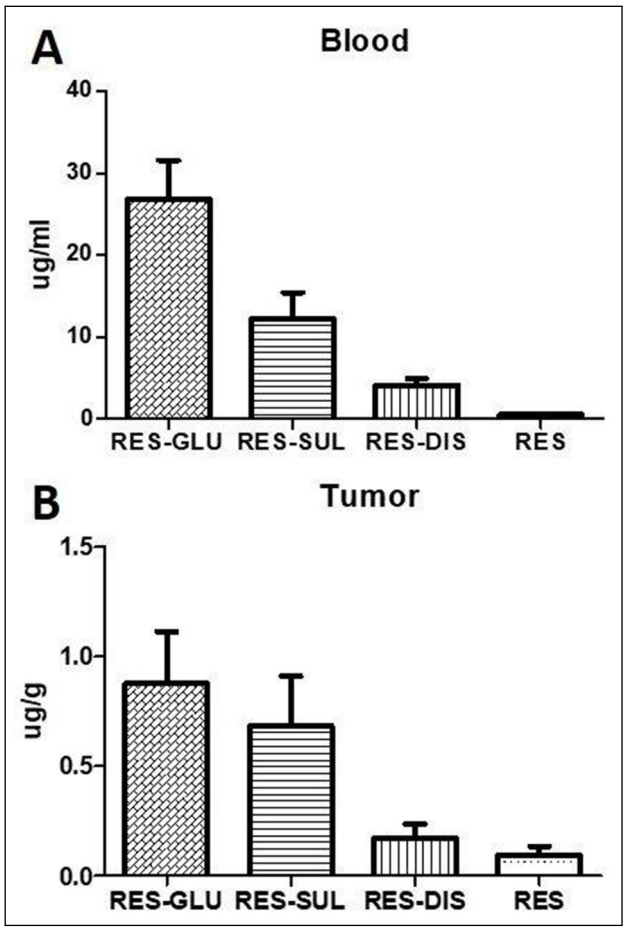

Fig. 5. The concentrations of resveratrol (RES) and its metabolites in blood plasma (A, n = 8) and breast cancer tissue (B, n = 7). RES, parental resveratrol; RES-SUL, resveratrol-3-O-sulfate; RES-GLU, resveratrol-3-O-glucuronide; RES-DIS, resveratrol-3,4'-O-disulfate. Data are expressed as means ± SD.

varied in the order of RES-GLU > RES-SUL > RES-DIS > RES (26.86 ± 13.35, 12.26 ± 9.06, 4.15 ± 2.32 and 0.57 ± 0.29 μg/ml plasma, respectively) (*Fig. 5A*). In the tumor samples, metabolite concentration correlated with plasma demonstrating metabolite

levels of 0.36 – 1.90, 0.26 – 1.73, 0.17 ± 0.17 and 0.095 ± 0.10 μg/g tumor tissue for RES-GLU, RES-SUL, RES-DIS, and RES, respectively (*Fig. 5B*). The levels of parental RES or its metabolites in blood plasma or cancerous tissue did not correlate with the number of tumors in individual animals.

DISCUSSION

Chemically induced breast cancer in rats remains a key model for basic and preclinical studies of early breast cancer underlining its importance in human studies (14, 15). All NMU induced mammary tumors in our experiment were ER-positive reflecting the previous characterization of these tumors for the luminal A subtype (ER and progesterone positive and Her2/neu negative) (12). In breast cancer, ERα promotes tumor growth, but ERβ activation inhibits proliferation of cancer cells (3). ER expression is the main indicator of potential responses in endocrine therapy and its predominantly nuclear localization indicates presence of the activated ERα in the tumor cells. ERβ is localized in luminal epithelium, myoepithelium, and in fibroblasts and lymphocytes in the stroma in the same manner in NMU and NMU + RES group and may counteract ERα.

Present study showed that nocturnal administration of RES strongly decreased tumor frequency up to 40% and lowered tumor incidence especially from 7th to 14th week of the experiment. RES also significantly increased tumor latency by approximately 31% and decreased tumor volume by more than 4-fold. Recent data showed that day time administration of RES in the same NMU rat model was significantly less effective demonstrating a tumor frequency lowered only up to 28%, no change in tumor incidence, very minor increase of tumor latency of 7.6% and an increase of tumor volume of 22% indicating the importance of RES application time (16).

All mammals are controlled by a circadian time system. Circadian rhythms show universally a 24 h oscillation pattern in metabolic, physiological, behavioral, and immune functions (17). The suprachiasmatic nucleus circadian pacemaker is a self-sustained oscillator where clock genes *Per1*, *Per2*, *Cry1*, *Cry2*, *Clock* and *Bmal1* play crucial regulatory roles (18). For example, a decrease in the expression of *Per2* clock gene has been associated with familiar and sporadic breast tumors (19). Vice-versa, *Per2* overexpression in breast cancer cells leads to growth inhibition, loss of clonogenic ability and apoptosis (20). Indeed, *Per2* mRNA levels were increased in all analyzed 'night' samples when compared to 'day' NMU* control.

It has been observed that *Per2* expression is the highest during the dark span (10). Importantly, the expression of *Per2* mRNA in peripheral cell types directly modulates hormones, particularly corticosterone, the main principal glucocorticoid hormone in rodents, serving as a clock gene entraining factor in organs (21).

We further found an increase in the number of lymphocytes and a higher level of ROS in leukocytes. This corresponds to data from Feng *et al.* showing immunomodulatory activities of RES in a low dose (0.75–6 micromol/L) resulting in a concentration-dependently promoted lymphocyte proliferation and IL-2 production (22). In line with the findings of Feng *et al.*, we found significantly elevated proinflammatory cytokines IL-1 and IL-2 levels suggesting that RES may stimulate the immune system against breast cancer progression. Based on the low concentration of specially IL-1A and IL-2, immunomodulatory effects of RES may not contribute significantly to the observed reduction in tumor development. On the other hand, RES is also known to reduce oxidative stress as previously shown in a rat model of chronic mild stress-induced depression (23). Reduction of ROS was also observed for other natural compounds like curcumin and grape-seed extract thereby acting as chemopreventive agents (24). *In vitro* experiments indeed showed that antioxidant activity of RES was only observed at low whereas a prooxidant action at higher concentrations (25).

In our experiment we also found a reduction in food intake in the NMU + RES group, concomitant with a reduction of body weight. In adipose rats, similar effects of RES on the body weight together with the reduction of adipokine secretion were described (26). These authors therefore concluded that RES acts as a cancer preventive agent against ER + breast cancer independent on the activation of immune system markers (27). Whether adipose secretory function in our NMU induced breast cancer model may also contribute to the observed chemopreventive effects of RES is not known. What we observed was that food but not water intake was significantly reduced by 17% between weeks 4 and 12 in the NMU + RES group leading to a pronounced reduction in the body mass of about 25% as compared to untreated controls. Importantly, we showed that RES significantly increased the amount of total protein in blood and reduced urea concentration. Hypoproteinemia is an accompanying phenomenon of malignant diseases, while urea levels are high during carcinogenesis (28). RES restored normal values of these markers reflecting the potency of RES to restore the metabolic balance in breast cancer bearing rats.

As expected, control experiments with the vehicle EtOH in the absence of RES did not significantly affect tumor frequency, latency period and incidence compared with NMU animals. However, EtOH non-significantly increased tumor volume by approximately 45%. A tumor promoting effects of EtOH has been previously reviewed by (29).

In our experiment, RES was extensively metabolized into three main conjugates: RES-GLU is the main biotransformation product followed by RES-SUL and RES-DIS; RES concentrations were very low. Currently, limited information is available regarding the possible benefits of RES metabolites. Based on *in vitro* studies, RES sulfates have been found to have comparable or greater potency than RES against specific molecular targets, namely, COX 1 and 2, quinone reductase 1, nuclear factor κB as well as similar ability to scavenge free radicals (30-32). Furthermore, sulfates were also very recently shown to attenuate the *E. coli*-LPS induced IL-6 and TNF-α release (33). TNF-α further stimulates immune cells to release tissue factors which stimulate nociceptors triggering neuropathic cancer pain (34). In contrast to RES sulfates, the few published studies have shown that RES glucuronides are ineffective in various human cell lines, macrophages and HIV-1 infection (33, 35-38). However, the *in vitro* activity of RES metabolites may not necessarily reflect their *in vivo* function given that intracellular sulfatases or β-glucuronidases could easily convert the conjugates back to RES (39).

Our results also indicate that RES is metabolized with high interindividual differences between animals and is accumulated in breast cancer tissue. Although we found an effective uptake of RES into cancer tissue, levels of parental RES and its metabolites in blood plasma or cancerous tissue did not correlate with the number of tumors in individual animals. Besides direct effects of RES in tumor tissues it might also act as a systemic agent influencing biochemical parameters as observed for total proteins and urea. A systemic effect of RES might also be supported by the late onset of tumors before the malignant transformation of cells.

In conclusion, our results indicate for the first time that night administration of RES significantly affected tumor parameter leading to an increased latency and a reduced tumor volume which might also be relevant to breast cancer patients receiving RES as a chemopreventive agent.

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Author's address: Dr. Terézia Kisková, University of Pavol Jozef Šafárik in Košice, 2 Šrobárova, 040 01 Košice, Slovakia.
E-mail: terezia.kiskova@gmail.com