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Original research article

The effects of silibin administration for different time periods on mouse liver with Ehrlich ascites carcinoma

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ABSTRACT

Background: Ehrlich ascites carcinoma is the one of the animal cancer models having high malignancy and rapid growth resistance. Silibin has reported to be an antioxidant in previous studies. We aimed to investigate the effects of silibin on mouse liver with Ehrlich ascites tumor (EAT) cells in different time periods.

Methods: Balb/c mice were divided into five groups. Group I (Control): The saline buffer (*sb*) was injected intraperitoneally (*ip*) to the mice for 15 days. Group II (Silibin): 150 mg/kg silibin was injected *ip* for 15 days. Group III (Ehrlich): 2×10^5 cells were transferred from the donor mouse to healthy mice on first day. Group IV (Ehrlich + Silibin): Silibin was given between 5th and 15th days to mice inoculated with EAT. Group V (Silibin + Ehrlich): Silibin was injected for 15 days after EAT cells. The liver sections were stained with matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9), caspase 3, caspase 8, and proliferating cell nuclear antigen (PCNA) antibodies by the Streptavidin–biotin–peroxidase technique. Biochemical analysis and Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) method were performed in the liver.

Results: Superoxide dismutase levels of liver increased in Ehrlich + Silibin group compared with Ehrlich group. Malondialdehyde levels significantly decreased in Silibin + Ehrlich group compared with Ehrlich + Silibin. Matrix metalloproteinase-2 and -9 immunopositive cells increased in Silibin + Ehrlich compared with Ehrlich group. Caspase 3 and TUNEL signals significantly increased in Silibin + Ehrlich group compared with Ehrlich group. PCNA positive signals significantly increased in Ehrlich + Silibin group compared with Ehrlich group.

Conclusion: According to our findings, we suggest that silibin treatment after EAT cells inoculation has more effective than concurrently EAT and silibin treatment.

Introduction

Silymarin includes silibin and other flavonolingans such as isosilybin, dehydrosilybin, silychristin, silydianin, taxifolin and quercetin [1]. Silibin is a component of silymarin isolated from seeds of Milk Thistle named as *Silybum marianum* from *asteraceae* family [2]. Silibin has been suggested about for its protective effects on the liver as inhibiting protein synthesis and tumor necrosis factor (TNF)-α expression in injured hepatocytes [3]. These effects of silibin have been promising as an adjuvant to protect the tissue against damage from chemotherapeutic agents. Silibin also has been reported as an antioxidant by protecting tissues against oxidative stress [4]. In addition, anticancer effects of silibin have been reported *in vitro* studies on the cancers of the liver, pancreas, lung, colon, and bladder [5–7].

Ehrlich ascites tumor model was found as a mouse mammary carcinoma and carried to mice from mice by intraperitoneal passages [8]. EAT model has been used for chemotherapeutic cancer studies on two types as solid and ascitic forms. Solid Ehrlich tumors can develop by subcutaneous injection and ascites tumors can form by intraperitoneal injection [9]. Hepatocellular carcinoma (HCC) is the third cause of cancer-related deaths of the world, having very high mortality and morbidity [10]. Human hepatocellular cancer cell line (HepG2) and Ehrlich carcinoma cell line were used for therapeutic study of hepatocellular carcinoma and found similar antitumor activities [11]. Similar results obtained *in vitro* were shown in Ehrlich ascites carcinoma model *in vivo* [12].

From the past to the future, many HCC treatments have been used such as chemotherapy, radiotherapy, thermotherapy, liver transplantation, and tumor ablation [13,14]. Nowadays, many different antioxidative therapies including silibin have been

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reported for gastrointestinal tumors. Therefore, we aimed to examine effects of silibin on mice liver with Ehrlich ascites carcinoma for different time periods.

Materials and methods

Animals

All animal experimental procedures were approved by the Istanbul University Local Ethics Committee on Animal Research. Male Balb/c mice, weighing 20–30 g, were used in all experiments and were given standard laboratory chow and water *ad libitum*. Animals were maintained on a 12–12 h dark/light cycle. They were kept in 40 cages with one mouse each. They had standard mouse pellet food and allowed to reach water freely. Ehrlich ascites carcinoma cells were transferred to healthy mice from the donor mouse. The donor animal was obtained from Istanbul Medical Faculty, department of Experimental Medicine Research Center.

Experimental design

Ehrlich ascites tumor cells were taken from the donor animal and counted. At the end of the pre-studies for 15 days of survival, 2×10^5 EAT cells were transferred to healthy animals by intraperitoneal injection. Then, the weights of the animals were noticed and growth of EAT cells was detected by taking photos at 1st, 5th, 10th and 15th days.

The animals were divided into five groups:

Group I (Control): The 2% (w/w) saline buffer prepared in dimethylsulfoxide (DMSO) was injected intraperitoneally (*ip*) to the mice for 15 days.
Group II (Silibin): 150 mg/kg silibin in saline with DMSO as solvent was injected *ip* for 15 days.
Group III (Ehrlich): 2×10^5 EAT cells in saline buffer were transferred on the first day to the mice, and then saline was injected between 5th and 15th days.
Group IV (Ehrlich + Silibin): EAT cells were injected on the first day and 150 mg/kg silibin in *sb* was injected between 5th and 15th days.
Group V (Silibin + Ehrlich): EAT cells were injected on the first day, and then 150 mg/kg silibin in *sb* was injected for 15 days.

At the end of the 15th day, animals were sacrificed with ketamine hydrochloride and xylazine hydrochloride solutions and liver tissues were obtained. Tissues were divided into two groups for immunohistochemical and biochemical searches. Tissues for immunohistochemical search were fixed with formalin and the tissues for biochemical search were kept at −80 °C.

Immunohistochemistry

The liver tissues were fixed with 10% neutral formalin for 18–20 h at +4 °C. Then they were immersed in increased alcohol series from 70% to 100%. After embedding in paraffin, 5-μm tissue sections were cut with a microtome and prepared for immunohistochemical studies. Streptavidin–biotin–peroxidase method using histostain-plus bulk kit (Zymed 85-9043) was carried out as described by the manufacturer. In addition, antigen retrieval process was applied to all sections for demonstration of antigens. The sections were kept 3 times 5 min citrate buffer (pH 6) in a microwave oven. These sections were stained with PCNA (Proliferating Cell Nuclear Antigen, Thermo MS-106-p, 1/400 dilution), caspase 3 (Millipore AB3623, 1/50 dilution), caspase 8 (Lab vision RB-1200-PABX, 1/25 dilution) antibodies for overnight incubation at +4 °C, matrix metalloproteinase-9 and matrix metalloprotease-2 (Santa Cruz sc-21733 and sc-13595, 1/50 dilution, respectively) antibodies for overnight incubation at room temperature. The enzyme activity was developed using 3-amino-9-ethyl-carbazole (AEC) substrate kit (Zymed 00-2007). Mayer's hematoxylin was used for counterstaining. At last, slides were mounted in gelatine and stored at +4 °C.

TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay (Apoptag plus peroxidase S7101 Millipore kit) was used on formalin-fixed, paraffin-embedded sections of the liver. The liver sections were counterstained with Mayer's hematoxylin.

Biochemistry

The liver tissues from mice were homogenized in cold 0.9% NaCl to prepare a 10% homogenate. They were centrifuged, and after centrifuge, the clear supernatants of liver tissue were used for determining protein, superoxide dismutase (SOD), glutathione (GSH), and antioxidant enzyme levels. The glutathione (GSH) levels were determined according to Beutler's method [15]. The superoxide dismutase (SOD) activity was determined by using the method suggested by Sun [16]. The catalase (CAT) activity was assayed by using the method of Aebi [17]. The protein content in liver supernatants was detected by Lowry's method using bovine serum albumin as standard [18]. The malondialdehyde (MDA) levels were determined by using Ledwozyw's method [19]. Absorbance of the liver tissue samples was read in the spectrophotometer by using quartz tubes.

Statistical analysis

Immunopositive and TUNEL positive (apoptotic) cells were calculated by counting 10 areas randomly for the liver section of each mouse. Immunopositive cells were evaluated using a Nikon microscope (Eclipse 80i, USA) equipped with a digital camera using NIS-Elements-D 3.1 microscope imaging software program (USA).

All of the values were analyzed by statistical software named GraphPad Prism (version 5.0 computer package). All data were expressed as Means ± SEM. A comparison between the two groups was performed using Mann–Whitney U nonparametric test, and a comparison among multiple groups was performed using the one-way analysis of variance followed by a Kruskal–Wallis test. A value of $p < 0.05$ was considered statistically significant.

Results

Macroscopic findings

During the sacrifice of the experimental animals, some differences on tissues, including the liver tissue, were observed. A lipoidosis was seen on organ surfaces in abdominal cavity of Silibin control and Silibin treatment groups especially Silibin control group. Weight gain of Silibin group is shown in Fig. 1A and B. To compare the weight gain between Control and Silibin groups and observe increasing of Ehrlich ascites fluid between treatment groups, all animals were weighed on 1st, 5th, 10th and 15th days, which are shown in Table 1. Interestingly, a subcutaneous adiposity was observed only in the Silibin group, yet there was not any in Silibin + Ehrlich or Ehrlich + Silibin treatment groups. Animals of Ehrlich control group had given up their eating habits on 13th, 14th and 15th days of experimental design. In addition, animals had begun to lose their mobility on 9th and 10th days.

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