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EFFECT OF SILYMARIN ON *N*-NITROSODIETHYLAMINE INDUCED HEPATOCARCINOGENESIS IN RATS

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Aim: **To study the effect of silymarin on the levels of tumor markers and MDA (malondialdehyde) – DNA adduct formation during N-nitrosodiethylamine induced hepatocellular carcinoma in male Wistar albino rats.** ***Methods:*** **The levels of AFP, CEA and activities of liver marker enzymes in serum, MDA-DNA immunohistochemistry were done according to standard procedures in the control and experimental groups of rats.** ***Results:*** **Hepatocellular carcinoma was evidenced from significant ($p < 0.05$) increases of alpha-fetoprotein, carcinoembryonic antigen, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, acid phosphatase, lactate dehydrogenase, gamma-glutamyltransferase and 5′-nucleotidase in serum and increased MDA-DNA adducts were also observed in the tissue sections of hepatocellular carcinoma. Silymarin treatment significantly attenuated the alteration of these markers and decreased the levels of MDA-DNA adduct formation.** ***Conclusion:*** **Silymarin could be developed as a promising chemotherapeutic adjuvant for the treatment of liver cancer.**

Key Words: **silymarin, *N*-nitrosodiethylamine, hepatocellular carcinoma, flavonoids, AFP, MDA-DNA.**

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver and accounts for as many as one million deaths worldwide in a year. In some parts of the world it is the most common form of internal malignancy and the most common cause of death from cancer [1]. Well-known risk factors of hepatocellular carcinoma includes hepatitis B virus (HBV), hepatitis C virus (HCV), aflatoxins, alcohol and oral contraceptives. Smoking, androgenic steroids and diabetes mellitus are also suspected risk factors [2]. One approach to control liver cancer is chemoprevention — when disease is prevented, slowed or reversed substantially by the administration of one or more non-toxic naturally occurring or synthetic agents. In this regard, recently naturally occurring polyphenols are receiving increased attention because of their promising efficacy in several cancer models [3]. Silymarin is one of such naturally occurring compounds isolated from *Silybum marianum*, which has shown to have significant anticancer effect on several cancers both *in vitro* and *in vivo* [4–9].

Classically, a marker is synthesized by the tumor and released into the circulation, but it may be produced by normal tissues in response to invasion by cancer cells [10]. A variety of substances, including enzymes, hormones, antigens, and proteins may be considered as tumor markers. Analysis of tumor markers can be used as an indicator of tumor response to therapy. Sensitive and specific liver cancer marker enzymes are used as indicators of liver injury. Analysis of these marker enzymes reflects mechanisms of cellular damage and subsequent release of proteins and extracellular turnover [11]. Lipid peroxidation generates a complex variety of products, many of which are reactive electrophiles some of these react with protein and DNA and as a result are toxic and mutagenic [12]. Malondialdehyde (MDA) is one of products of lipid peroxidation that reacts with DNA to produce MDA-DNA adducts, which have been implicated in the induction of G→T transversions and A→G transitions [13]. The ability of MDA-DNA adducts to induce frame shift mutations in sequences for genetic instability is emerging as a possible direct link between oxidative stress and human cancers [14, 15]. Thus, the purpose of present study is to evaluate the effect of silymarin on the level of tumor markers, and MDA-DNA adducts formation during *N*-nitrosodiethylamine induced hepatocellular carcinoma in rats.

MATERIALS AND METHODS

Animals. Wistar male rats weighing about 150–180 g were obtained from Tamilnadu Veterinary & Animal Science University (TANUVAS), Madhavaram, Chennai, India. The animals were housed in cages under proper environmental conditions and were fed with a commercial pelletted diet (M/s Hindustan foods Ltd., Bangalore, India). The animals had free access to water.

Chemicals. *N*-nitrosodiethylamine (NDEA) and silymarin were manufactured by Sigma chemical Co., (St. Louis, MO, USA). All other chemicals used were from SRL (Mumbai, India).

Experimental design. The experimental animals were divided into five groups (as shown in Fig. 1), 6 animals per group. Rats from group 1 (normal control) were fed with standard diet and pure drinking water; in group 2 hepatocellular carcinoma was induced by providing 0.01% NDEA through drinking water for 15 weeks as described in [16]; rats from group 3 were treated with 1000 ppm silymarin alone in diet for 16 weeks; rats from group 4 were pretreated with 1000 ppm silymarin one week before the administration of 0.01% NDEA and received it till the end of the experiment (i.e. 16 weeks); rats from group 5 were post treated with 1000 ppm silymarin for 5 weeks after the administration of NDEA for 10 weeks and received it till

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Abbreviations used: AFP – alpha-fetoprotein; ACP – acid phosphatase; ALP – alkaline phosphatase; ALT – alanine aminotransferase; AST – aspartate aminotransferase; CEA – carcinoembryonic antigen; γ-GT – gamma-glutamyltransferase; HCC – hepatocellular carcinoma, LDH – lactate dehydrogenase; MDA – malondialdehyde, 5′NT – 5′-nucleotidase; NDEA – *N*-nitrosodiethylamine.

the end of experiment. After the stipulated experimental period the rats were anaesthetized with diethyl ether followed by cervical decapitation. The experiments are performed after the approval of the Institutional Animal Ethics Committee IAEC No. 01/009/06.

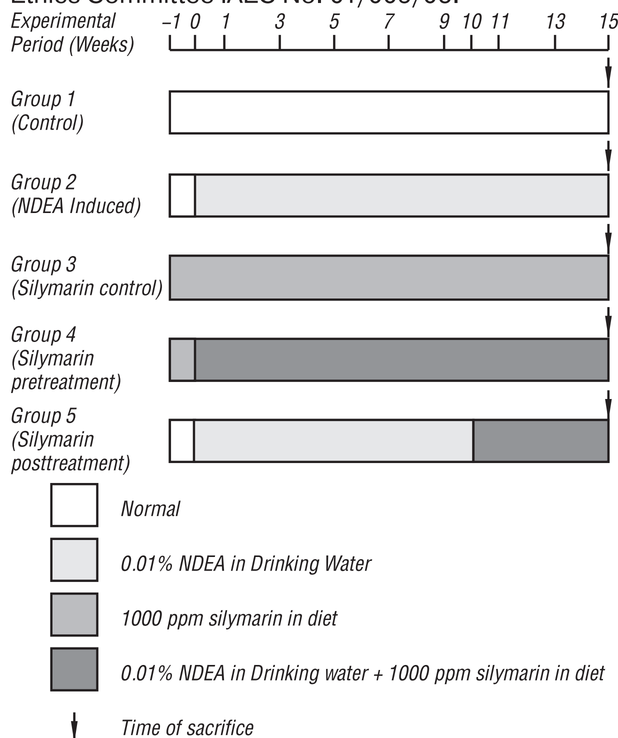

Fig. 1. Experimental protocol

Analysis of alpha-fetoprotein (AFP) & carcinoembryonic antigen (CEA). AFP and CEA were measured in blood serum by chemiluminescent immunoassay (Fully Automated ADVIA Centaur, Bayer U.S.A. chemiluminescence system).

Biochemical studies. The blood samples were collected from the experimental animals, liver tissue was removed and washed in ice-cooled saline, and tissues were chilled in a beaker on cracked ice for 5 min, and then minced with sharp scissors. 10% homogenate was prepared in ice-cooled 0.1 M Tris-HCl buffer (pH 7.4). Standard procedures were used to assay the various biochemical parameters. Protein content was estimated by Lowry et al. [17]; activity of enzymes was valued as described elsewhere (aminotransferases (AST, ALT) — [18], phosphatases (ACP, ALP) — [19], lactate dehydrogenase — [20], gamma-glutamyltransferase — [21], 5′-nucleotidase — [22]).

Immunohistochemical staining of MDA-DNA adducts. Immunohistochemistry for MDA-DNA adducts was carried out according to Zhang et al. [23]. Briefly, tissue sections were deparraffinized in two changes of xylene at 60 °C and rehydrated through a graded series of alcohols. Then the slides were washed in 1 x PBS, treated with RNase (100 μl/ml) at 37 °C for 1 h, washed with 1 x PBS, treated with proteinase K (10 μg/ml) at room temperature for 10 min and washed. To denature the DNA, the slides were incubated with 4 N HCl for 10 min and then washed with 50 mM Tris base for 5 min, both at room temperature. After washing with 1 x PBS, slides were incubated with 0.3% H_2O_2 in methanol at room temperature for 30 min. Non-specific binding was blocked with 3% BSA and the slides were incubated overnight at 4 °C with anti-MDA monoclonal antiserum number D10A1 (Dr. P. Srinivasan, Korea Atomic Energy Research Institute, Korea). The slides were washed in PBS and then incubated with anti-mouse HRP labelled secondary antibody (Genei, Bangalore, India) for 1 h at room temperature. The peroxidase activity was visualized by treating slides with 3,3′-diaminobenzidine tetrahydrochloride (SRL, Mumbai, India), the slides were slightly counterstained with Meyer's hematoxylin. The labeling index was expressed as number of cells with positive staining per 100 counted cells in five randomly selected fields at the magnification of objective 40 x under light microscope.

Statistical analysis. Data were evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. Statistical significance was defined as P values less than 0.05. All results were expressed as mean ± standard deviation.

RESULTS

The significant increase in the levels of AFP and CEA in the serum was observed in group 2 as compared with group 1 (Fig. 2). Significant decrease of the level of these tumor markers was demonstrated in the silymarin-treated animals from groups 4 and 5 as compared with group 2.

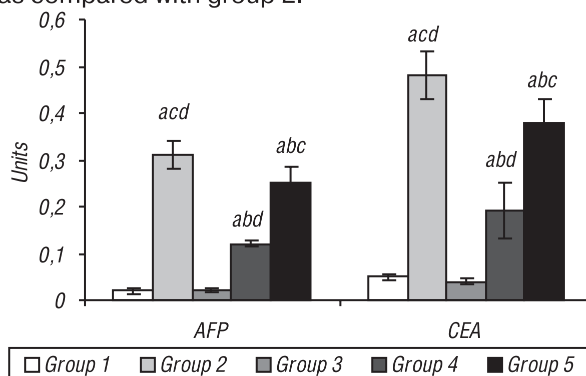

Fig. 2. Effect of silymarin on the levels of AFP and CEA in the serum of control and experimental groups of animals (n = 6 per group). Results are expressed as mean ± SD. $p < 0.05$ compared with [a]group1, [b]group 2, [c]group 4, [d]group 5. Units: IU/mL for AFP, ng/mL for CEA

Table. Effect of silymarin on the levels of AST, ALT, ACP, ALP, LDH, GGT and 5′NT in the serum of control and experimental group of animals

Groups	AST	ALT	ACP	ALP	LDH	GGT	5′NT
Group 1	3.90 ± 0.44	25.3 ± 2.85	27.23 ± 2.37	145.6 ± 16.6	1.42 ± 0.16	1.47 ± 0.17	3.09 ± 0.36
Group 2	7.58 ± 0.85[acd]	47.51 ± 4.99[acd]	48.8 ± 5.56[acd]	283.9 ± 32.37[acd]	2.48 ± 0.28[acd]	2.92 ± 0.29[acd]	6.19 ± 0.71[acd]
Group 3	3.82 ± 0.48	24.91 ± 2.83	26.24 ± 2.25	143 ± 16.3	1.4 ± 0.16	1.45 ± 0.16	3.08 ± 0.35
Group 4	5.3 ± 0.60[abd]	31.6 ± 3.60[abd]	33.42 ± 3.69[abd]	196 ± 22.30[abd]	1.71 ± 0.19[abd]	1.91 ± 0.21[abd]	4.31 ± 0.49[abd]
Group 5	6.42 ± 0.73[abc]	38.9 ± 4.43[abc]	40.7 ± 4.64[abc]	241 ± 28.4[abc]	2.09 ± 0.24[abc]	2.39 ± 0.27[abc]	5.15 ± 0.57[abc]

Note: Results are expressed as mean ± SD, (n = 6); $p < 0.05$ compared with [a]group 1, [b]group 2, [c]group 4, [d]group 5. Units: μmoles of pyruvate liberated mg protein per min for AST, ALT and LDH; μmoles of phenol liberated mg protein per min ACP and ALP; nmoles of p-nitroaniline formed mg protein per min for GGT; nmoles of Pi liberated mg protein per min for 5′NT.

Animals from group 2 exhibited a significant increase in the activity of AST, ALT, ACP, ALP, LDH, GGT and 5′NT in blood serum as compared with group 1 (Table). Silymarin-treated animals from groups 4 and 5 showed a significant decrease in the levels of these enzymes when compared with group 2 (animals with NDEA-induced hepatocarcinoma). The activity of AST and ALT was significantly decreased in the liver tissue of animals from group 2 as compared with group 1 (Fig. 3), silymarin-treated animals (groups 4 and 5) showed a significant increase in the level of transaminases as compared with group 2. The activity of ACP, ALP, LDH, GGT and 5′NT was significantly increased in the liver tissue of animals from group 2 as compared with group 1. There was a significant decrease in the activity of these enzymes in silymarin-treated groups as compared with group 2.

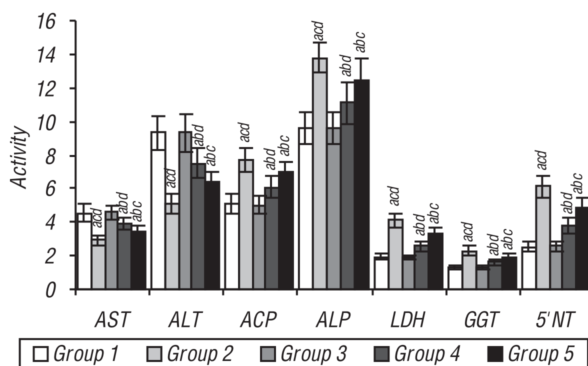

Fig. 3. Effect of silymarin on the activity of AST, ALT, ACP, ALP, LDH, GGT and 5'NT in the liver of control and experimental groups of animals (n = 6 per group). Results are expressed as mean ± SD. $p < 0.05$ compared with [a]group 1, [b]group 2, [c]group 4, [d]group 5. Units: μmoles of pyruvate liberated mg protein per min for AST, ALT and LDH; μmoles of phenol liberated mg protein per min for ACP and ALP; nmoles of p-nitroaniline formed mg protein per min for GGT; nmoles of Pi liberated mg protein per min for 5′NT

As it was shown by immunohistochemical analysis of liver sections, the MDA-DNA adducts were observed in 3% of hepatocytes in normal control animals (Fig. 4, *a*). The rate of hepatocytes which were positive to MDA-DNA adducts was 43%, 2.96%, 18% & 29% in the liver sections from NDEA-induced hepatocarcinoma (Fig. 4, *b*), silymarin alone (Fig. 4, *c*), silymarin pretreated group (Fig. 4, *d*), and silymarin post-treated animals (Fig. 4, *e*) respectively. The graphical representation of % positive cells for MDA-DNA is shown in Fig. 4, *f*.

DISCUSSION

During carcinogenesis, some enzymes can be used as an biochemical indicators of tumor response to therapy [11]. Hepatospecific enzymes were activated when hepatocellular damage gave rise to abnormalities of liver function and these enzymes are remarkably increased in HCC. AST and ALT activities in blood serum are generally accepted as an index of liver damage and this tendency is also known to be distinct in rodents [16]. There was a good correlation between the activities of ALT and AST with tumor volume during therapy. Rocchi et al. [24] reported that there was an increase in the levels of these transaminases activity in serum of HCC patients. In concurrent with the above findings an elevated serum aminotransferase activities were observed in animals bearing HCC with simultaneous decrease in the liver tissue; silymarin treatment significantly attenuated this alteration thereby showing its anticarcinogenic activity.

Elevation of alkaline phosphatase is one of the signs, suggesting space-occupying lesions in the liver. *An increased activity* of ACP and ALP was seen in blood serum and liver of animals with HCC, this may be due to the disturbance in secretory activity or due to altered gene expression in these conditions. Development of tumor results in tissue damage that lead to the release of ALP into circulation [25] and this enzyme level have been elevated in blood serum and liver tissue of the tumor-bearing animals and this elevation is significantly suppressed by the supplementation of silymarin in diet. GGT has been shown to play an important role in the metabolism of foreign substances and also during cell growth and differentiation [26] and is overexpressed in tumor cells resistant to therapeutic drugs [27]. Experimental studies have shown that GGT was strikingly activated during the course of hepatocarcinogenesis induced by several hepatocarcinogens in animals [28]; chemical carcinogens may initiate some systematic effects that induce GGT synthesis [29]. This elevation reflects the progress of carcinogenesis, since its activity correlates with tumor growth rate, differentiation and survival of the host [30]; in concurrent with above findings there was an increase in the levels of GGT in the serum and liver of animals bearing HCC. This elevation indicates the basic tumor burden, and silymarin treatment significantly decreased the elevation of the level of this enzyme.

5′nucleotidase was found to be elevated in the animals with solid tumors [31]. The increased activity of this enzyme seems to have originated from the proliferating tumor cells [32]. Elevated activities of 5′nucleotidase in carcinoma of liver and leukemia were reported [11, 33]. In our study correlatively increased activities of 5′nucleotidase were observed in blood serum and liver of the carcinogen administered animals, and this elevation is significantly inhibited in the animals treated with silymarin.

LDH is a fairly sensitive marker of solid neoplasm [34] and very high LDH levels correlate with treatment failure [35]; numerous reports revealed increased LDH activity in various types of tumors [10, 36]. The elevated levels of LDH may be due to its overproduction by tumor cells. Proliferating malignant cells exhibit very high rates of glycolysis, which subsequently lead to elevated LDH activity [37]. The results of the present study are in agreement with literature data and show elevated levels of LDH in blood serum and liver of the NDEA administered rats, and this elevation was attenuated in silymarin-treated rats.

Elevation of serum AFP levels has been reported in several diseases including HCC [38]. AFP along with CEA is most extensively used in the diagnosis of HCC [39, 40]. In our study also there was an increased level of AFP and CEA in the carcinogen administered animals confirming the presence of HCC, and silymarin treatment significantly reduced the elevation of both AFP and CEA.

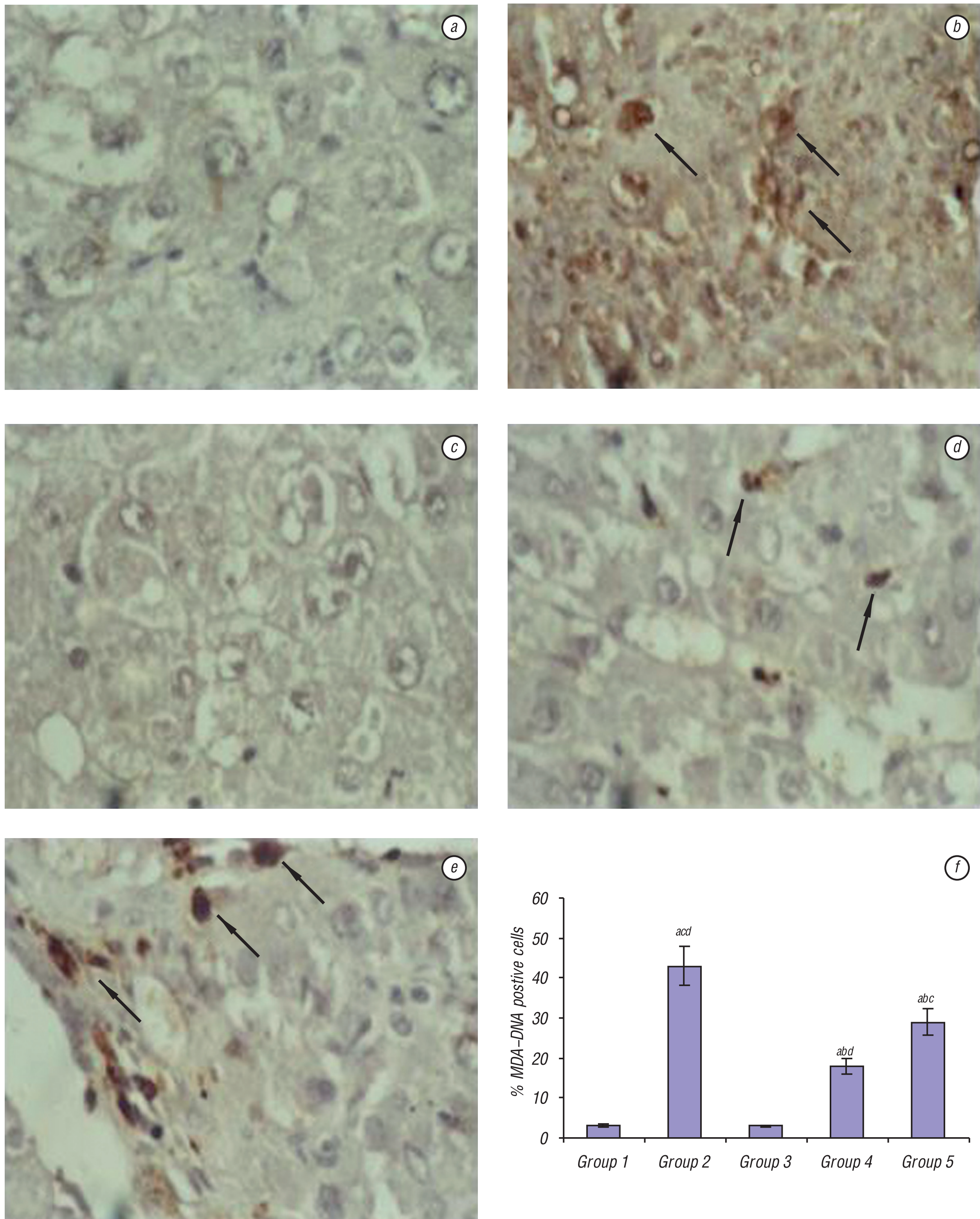

Fig. 4. Immunohistochemical staining of liver sections for MDA-DNA adducts: *a* — group 1; *b* — group 2; *c* — group 3; *d* — group 4; *e* — group 5; (*f*) representative graph of % MDA-DNA positive cells. Magnification 40 x

MDA is a highly reactive electrophile, capable of interacting with DNA to form MDA-DNA adducts [41], that induce frame shift and base-pair substitution mutations [13]. The level of MDA-DNA adducts is found to be increased in several cancers [42–45]. Recent evidence suggests that oxidative stress may contribute to genetic instability and promote tumor progression [14, 15]. In the present study the levels of MDA-DNA adducts are increased in HCC-bearing animals, and pre- and post-silymarin treatment significantly reduced the formation of MDA-DNA adduct. So, we can conclude that silymarin could be developed as a promising chemotherapeutic adjuvant for the treatment of liver cancer.

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ВЛИЯНИЕ СИЛИМАРИНА НА ГЕПАТОКАНЦЕРОГЕНЕЗ, ИНДУЦИРОВАННЫЙ У КРЫС *N*-НИТРОЗОДИЭТИЛАМИНОМ

Цель: **изучить влияние силимарина на уровень экспрессии опухолевых и биохимических маркеров и формирование аддуктов малонового диальдегида с ДНК (MDA-DNA) при развитии гепатокарциномы у крыс линии Вистар.** ***Методы:*** **стандартными биохимическими методами определяли активность ферментов в сыворотке крови и проводили иммуногистохимическое определение MDA-DNA в ткани печени крыс.** ***Результаты:*** **показано, что при развитии злокачественной гепатокарциномы в сыворотке крови животных значительно увеличивается количество альфа-фетопротеина, раковоэмбрионального антигена, активность аспартат- и аланинаминотрансферазы, щелочной и кислой фосфатазы, лактатдегидрогеназы, гамма-глутамилтрансферазы и 5′-нуклеотидазы. При проведении иммуногистохимического исследования отмечали повышенное образование аддуктов MDA-DNA в ткани печени крыс со злокачественной гепатокарциномой. При введении силимарина значительно снижался уровень указанных ферментов в сыворотке крови и формирование аддуктов MDA-DNA в ткани печени.** ***Заключение:*** **применение силимарина может быть эффективно для предупреждения развития злокачественной гепатокарциномы, индуцированной *N*-нитрозодиэтиламином у крыс, и этот препарат может быть многообещающим химиотерапевтическим адъювантом для лечения рака печени.**

Ключевые слова: **MDA-DNA, альфафетопроеин, злокачественная гепатокарцинома, *N*-нитрозодиэтиламин, силимарин, флавоноиды.**

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