Mutation Research 767 (2014) 13–20

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Protective effect of apigenin against *N*-nitrosodiethylamine (NDEA)-induced hepatotoxicity in albino rats

Fahad Ali, Rahul, Falaq Naz, Smita Jyoti, Yasir Hasan Siddique*

Section of Genetics, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, Uttar Pradesh 202002, India

ARTICLE INFO

Article history:
Received 5 August 2013
Received in revised form 26 March 2014
Accepted 5 April 2014
Available online 19 April 2014

Keywords:
Apigenin
N-nitrosodiethylamine
Serum enzymes
Antigenotoxic
Hepatoprotective

ABSTRACT

A number of pharmacological properties have been attributed to apigenin. In the present study the effect of apigenin was investigated with respect to hepatotoxicity induced by *N*-nitrosodiethylamine (NDEA), a compound that is present in many food stuffs and has been reported to be a hepatocarcinogen. Male rats were exposed to NDEA (0.1 mg/ml) dissolved in drinking-water separately, and with 10, 20, or 40 mg/ml of apigenin for 21 days. The activity of glutamic-oxaloacetic transaminase (SGOT), glutamic-pyruvic transaminase (SGPT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) was measured in blood serum. Lipid peroxidation, protein carbonyl content and micronucleus frequency were determined in hepatocytes. To assess the effect on DNA damage, the comet assay was performed on hepatocytes, blood lymphocytes and bone-marrow cells of the exposed rats. The results of the study reveal that the treatment of NDEA together with apigenin showed a significant dose-dependent decrease in the serum concentration of the enzymes SGOT, SGPT, ALP and LDH ($p < 0.05$). Histological sections of the liver also showed a protective effect of apigenin. A significant dose-dependent reduction in lipid peroxidation and protein carbonyl content was observed in rats exposed to NDEA (0.1 mg/ml) together with apigenin ($p < 0.05$). The results obtained for the comet assay in rat hepatocytes, blood lymphocytes and bone-marrow cells showed a significant dose-dependent decrease in the mean tail length ($p < 0.05$). The present study supports the role of apigenin as an anti-genotoxic and hepatoprotective agent.

1. Introduction

Apigenin is ubiquitously distributed in leaves, vegetables, stems and fruits of several vascular plants [1]. It has been shown to possess not only anti-inflammatory, antioxidant, and anticancer properties but it may also be protective in other diseases that are affected by oxidative processes such as cardiovascular and neurological disorders [2]. There are only few reports on the occurrence of adverse metabolic reactions by the consumption of apigenin and for this reason apigenin has gained more interest in recent years [3]. In our earlier studies apigenin showed antigenotoxic effects against anticancer drugs in cultured human lymphocytes [4], and in mouse bone-marrow cells [5]. It has also shown protective effects against the genotoxicity of hydrogen peroxide and ethinylestradiol in cultured human blood lymphocytes [6,7]. In our recent study on a *Drosophila* model for Parkinson's disease, apigenin showed a protective effect against PD symptoms [8].

N-nitroso compounds (NOCs) are present in the human environment and some are well-known carcinogens [9]. *N*-nitrosodiethylamine (NDEA) has been suggested to cause oxidative stress and cellular injury due to the involvement of free radicals [10–12]. NOCs have also been reported to induce hepatic fibrosis. The induction of hepatic fibrosis and the alterations in various biochemical parameters by nitroso compounds have been reviewed by Ahmad and Ahmad [13]. Nitroso compounds are also found predominantly in a variety of food stuffs such as milk products, meat products and preserved juices [14]. NDEA is a well-known hepatocarcinogen present in tobacco smoke, in water containing high concentration of nitrates, fried meals, cosmetics, agricultural chemicals and pharmaceutical agents. In the liver NDEA is activated by cytochrome P450 to form electrophilic and reactive oxygen species [15]. NDEA is metabolized to its active ethyl radical metabolite (CH_3CH^{2+}) [16]. The nitroso compounds are considered as a group of

Abbreviations: NDEA, *N*-nitrosodiethylamine; SGOT, serum glutamic-oxaloacetic transaminase; SGPT, serum glutamic-pyruvic transaminase; ALP, alkaline phosphatase; ALT, alanine transaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; LDH, lactate dehydrogenase; DNA, deoxyribonucleic acid; NOCs, *N*-nitroso compounds; ROS, reactive oxygen species; DMSO, dimethy sulfoxide; BNF, buffered neutral formalin; TCA, trichloroacetic acid.

* Corresponding author.
E-mail address: yasir_hasansiddique@rediffmail.com (Y.H. Siddique).

http://dx.doi.org/10.1016/j.mrgentox.2014.04.006
1383-5718/

carcinogens that may be formed endogenously in the human body [17]. NDEA has been suggested to cause oxidative stress and cellular injury by generating reactive oxygen species [18]. The reactive products and free radicals also result in an increase in the serum indices of liver function such as alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and total bilirubin, and in severe histopathological lesions in the liver, which may lead to neoplastic transformation [19]. The presence of reactive oxygen species (ROS) also results in carbonylation of protein, peroxidation of lipids and DNA damage leading to cytotoxicity, carcinogenicity and mutagenicity [1]. ROS are potentially dangerous by-products of cellular metabolism that can lead to the development of cancer [20]. The supplementation of dietary antioxidants in addition to the cellular defense system has been proposed for the protection against oxidative stress [15]. A number of epidemiological and animal studies have shown that high intake of natural products may be associated with a decreased risk of many cancers [19]. Flavonoids are ubiquitously present in fruits, vegetables and are suggested to have various pharmacological properties without causing significant toxicity [21]. In the present study the effect of apigenin was investigated in respect of the NDEA-induced hepatotoxicity in Swiss albino rats.

2. Materials and methods

2.1. Chemicals

N-nitrosodiethylamine and apigenin were purchased from Sigma Chemicals Co. (USA). Agarose (normal and low-melting), Triton X, ethidium bromide, dimethyl sulfoxide (DMSO), Tris, EDTA and all other chemicals were purchased from SISCO Research Laboratories, India. May-Gruenwald's stain and Giemsa stain were procured from Merck Ltd. (India).

2.2. Animals and treatment

Male Wistar rats weighing 100–120 g were used in the study. The animals were divided over nine groups (5 rats/group). The first group received *N*-nitrosodiethylamine (NDEA) dissolved in drinking-water (0.1 mg/ml), other groups received the same solution of NDEA (0.1 mg/ml) plus apigenin at 10 mg/ml (group 2), apigenin at 20 mg/ml (group 3), or apigenin at 40 mg/ml (group 4). The fifth group served as a control (normal drinking-water), and the sixth, seventh and eighth group received drinking-water with apigenin at final concentrations of 10, 20 and 40 mg/ml, respectively. The ninth group (negative control) received drinking-water containing DMSO (3 μl/ml). Apigenin was first dissolved in 0.03% DMSO and the final concentrations of 10, 20 and 40 mg/ml in drinking-water were established. The rats were allowed to feed *ad libitum* for 21 days and were sacrificed under mild ether anaesthesia. All animal care procedures and animal ethics were taken into consideration while performing the experiments. The study was approved by an ethical committee.

2.3. Histological evaluation of the liver

A portion of liver was removed and washed thoroughly with 0.9% saline. The tissue was kept in 10% buffered neutral formalin (BNF) for 24 h. Then the fixed liver specimens from each group were embedded in paraffin and processed for light microscopy by staining individual sections with haematoxylin–eosin.

2.4. Biochemical analysis

The blood samples were collected directly by cardiac puncture in a vacutainer with a clot activator (AKÜret, Medkit). The serum was collected for biochemical analyses of glutamic-oxaloacetic transaminase (SGOT), glutamic-pyruvic transaminase (SGPT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). The enzyme concentrations were estimated according to the method described in the commercial kits (Crest Biosystems, India).

2.5. Preparation of liver homogenate

The homogenate was prepared according to the procedure described by Singh et al. [22] with minor modifications. The livers were washed thoroughly with chilled 0.9% saline. The final wash was given with cold homogenizing buffer (0.024 M EDTA, 0.075 M NaCl, 10% DMSO; pH 7.5). After weighing, the liver was mixed, suspended in cold homogenizing buffer at a concentration of 1 g/ml, and was homogenized on ice at 300 × *g*. The homogenate was then centrifuged at 5500 × *g* for 10 min at 4 °C. The supernatant was removed, and the pellet re-suspended in homogenization buffer and kept at −20 °C for further analyses.

2.6. Estimation of protein carbonyl content

The protein carbonyl content was estimated according to the method described by Hawkins et al. [23]. The liver homogenate was diluted to a protein concentration of approximately 1 mg/ml. About 250 μl of each diluted homogenate was taken in separate Eppendorf centrifuge tubes. Then 250 μl of 10 mM 2,4-dinitrophenyl hydrazine (dissolved in 2.5 M HCl) was added, and the mixture was vortexed and kept in the dark for 20 min. About 125 μl of 50% (w/v) trichloroacetic acid (TCA) was added, mixed thoroughly and incubated at −20 °C for 15 min. The tubes were then centrifuged at 4 °C for 10 min at 9000 × *g*. The supernatant was discarded and the pellet was washed twice in ice-cold ethanol:ethylacetate (1:1). Finally the pellets were re-dissolved in 1 ml 6 M guanidine hydrochloride, and the absorbance was read at 370 nm.

2.7. Estimation of lipid peroxidation

The method described by Siddique et al. [24] was used for the estimation of lipid peroxidation in liver cells. Reagent 1 (R1) was prepared by dissolving 0.064 g of 1-methyl-2-phenylindole in 30 ml of acetonitrile. The preparation of 37% HCl served as the reagent R2. About 200 μl of diluted liver homogenate (protein concentration approximately 1 mg/ml) was mixed with 300 μl of R1. Then 300 μl of R2 was added, and the mixture was vortexed, incubated at 45 °C for 40 min, cooled on ice, and centrifuged at 11,000 × *g* at 4 °C. The absorbance was read at 586 nm.

2.8. Micronucleus assay

The micronucleus assay was performed according to the method of Igarashi and Shimada [25]. The supernatant was removed and fresh homogenizing buffer was used to re-suspend the pellet. A drop of suspension was placed at one end of a pre-cleaned, grease-free microscope slide and spread-out with a cover slip held at an angle of 45° to obtain a smooth layer of cells. Before staining, the slides were allowed to air-dry in a dust-free environment for at least 12 h. The slides were then stained for 2 min in May-Gruenwald stain (0.25% in methanol) followed by staining with 10% Giemsa for 10 min. The slides were rinsed twice in distilled water, dried, rinsed with methanol, cleared in xylene, and mounted in DPX. A total of 500 cells were counted per animal for the presence of micronuclei by use of a light microscope at 40× magnification [26].

2.9. Comet assay

The comet assay was performed according to the method described by Singh et al. [27], with modifications as suggested by Dhawan et al. [28]. Frosted microscope slides were dipped in 1% normal-melting agarose and the underside was wiped to remove the agarose (dissolved in PBS). The slides were allowed to dry for 24 h. For the liver cells, 40 μl of the cell suspension were mixed with 60 μl of 0.5% low-melting agarose (dissolved in PBS) and layered on the prepared slides. For the blood lymphocytes, 20 μl of whole blood in 1 ml of RPMI 1640 was mixed with 100 μl of Ficoll histopaque and centrifuged at 1500 rpm 300 g for 15 min, and the pellet was re-suspended in 70 μl of LMPA. For bone-marrow cells, the femurs were perfused with 1 ml of cold homogenization buffer and 10 μl of the cell suspension was mixed with 70 μl of LMPA. A cover slip was placed on top to spread the suspension evenly. The agarose was allowed to solidify at 4 °C for 10 min. The cover slip was removed and a third layer of 1% low-melting agarose was added and allowed to solidify on ice for 5 min. The slides were then immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% triton X-100, pH 10) at 4 °C, overnight. Next day the slides were kept for 30 min in alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA; pH > 13) for unwinding of the DNA. Electrophoresis was performed at 4 °C at 24 V for 45 min. The slides were washed with neutralization buffer (0.4 M Tris) and stained with ethidium bromide (20 μg/ml). Three slides were prepared per rat, and a total of 50 randomly captured comets per slide were analysed under a fluorescence microscope for scoring comet-tail length by use of Comet 1.5 software (TriTek Corporation).

2.10. Statistical analysis

All data were expressed as the mean ± standard error and Student's *t*-test was used for the analysis. Statistical significance was considered at the 5% level.

3. Results

The histomorphological study of the liver sections revealed a normal structure of the hepatocytes in the control group (Fig. 1a). The rats exposed to NDEA (0.1 mg/ml) showed sinusoidal dilution, swollen and empty hepatocytes. The nuclei were also enlarged and the cytoplasm showed several tiny vacuoles, indicative of vacuolar