Journal of Pharmaceutical Analysis 2015;5(1):51–57

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Journal of Pharmaceutical Analysis

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ORIGINAL ARTICLE

Protective effect of Genistein against N-nitrosodiethylamine (NDEA)-induced hepatotoxicity in Swiss albino rats

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Received 13 February 2014; revised 18 June 2014; accepted 7 July 2014
Available online 22 July 2014

KEYWORDS

Genistein;
N-nitrosodiethylamine;
Serum enzymes;
Antigenotoxic;
Hepatoprotective

Abstract In the present study, we studied the effect of Genistein against the hepatotoxicity induced by N-nitrosodiethylamine (NDEA). NDEA is present in almost all kinds of food stuff and has been reported to be a hepatocarcinogen. The male rats were exposed to NDEA (0.1 mg/mL) dissolved in drinking water separately and along with 25, 50, 100 mg/mL of Genistein for 21 days. The activities of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were measured in blood serum. Lipid peroxidation, protein carbonyl content, micronucleus frequency and DNA damage (Comet assay) were performed on rat hepatocytes. The results of the study reveal that the treatment of NDEA along with Genistein showed a significant dose-dependent decrease in the levels of blood serum enzymes *i.e.,* SGOT, SGPT, ALP and LDH ($P<0.05$). The HE staining of histological sections of the liver also revealed a protective effect of Genistein. A significant dose-dependent reduction in the lipid peroxidation and protein carbonyl content was observed in rats exposed to NDEA (0.1 mg/mL) along with Genistein ($P<0.05$). The results obtained for the comet assay in rat hepatocytes showed a significant dose-dependent decrease in the mean tail length ($P<0.05$). Thus the present study supports the hepatoprotective role of Genistein.

1. Introduction

Genistein is an isoflavone and acts as a both phytoestrogen and antioxidant [1]. It inhibits the growth of cancerous cells by binding itself with the receptor in place of estrogen [2,3]. It has also been reported to be an antihelmintic [4]. It shows protective effects against various types of cancers [5–11]. An epidemiological study showed that the consumption of isoflavones is associated with an increased risk for hepatocellular carcinoma (HCC) in women [12]. It inhibits the bone resorption in rats [13]. It does not show teratogenic potential *in vivo* even at a very high dose of 1000 mg/kg/day by oral gavage in the embryo–fetal toxicity [14]. It has been reported to be non-mutagenic in the *Salmonella typhimurium*

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Peer review under responsibility of Xi'an Jiaotong University.

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http://dx.doi.org/10.1016/j.jpha.2014.07.003

assay and non-clastogenic in the mouse and rat micronucleus test [15]. N-nitrosocompounds (NOCs) are present in a variety of food stuff and are well-known carcinogens [16]. One of the NOCs such as N-nitrosodiethylamine (NDEA) has been suggested to cause oxidative stress and cellular injury by generating free radicals [17–19]. NDEA has been reported to generate electrophilic and reactive oxygen species by activation of cytochrome P450 in the liver [20]. This leads to liver cytotoxicity, carcinogenicity and mutagenicity [21]. The high intake of natural products has been reported to be associated with a decrease in the risk of cancer and toxicity in various epidemiological and animal studies [22]. In the present study, the effect of Genistein was studied against NDEA-induced hepatotoxicity in Swiss albino rats.

2. Experimental

2.1. Chemicals and reagents

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

2.2. Animals and treatment

Male rats (Wistar strain), weighing 100–120 g, were used in the study. The animals were divided into nine groups (5 rats/group). The first group was allowed to feed on N-nitrosodiethylamine (NDEA) dissolved in water (0.1 mg/mL); the second, third and fourth groups were allowed to feed on water containing NDEA (0.1 mg/mL) plus 25, 50 and 100 mg/mL of Genistein, respectively; the fifth group served as a control (normal drinking water); the sixth, seventh and eighth groups were allowed to feed on water containing Genistein at final concentration of 25, 50 and 100 mg/mL, respectively. The ninth group was taken as a negative control, and was allowed to feed on water containing a dimethysulfoxide (DMSO) (3 μL/mL). Genistein was dissolved in 0.03% DMSO and in drinking water the final concentrations of 25, 50 and 100 mg/mL were established. The rats were allowed to feed *ad libitum* for 21 days and were sacrificed under mild ether anesthesia.

2.3. Histological evaluation of the liver

A portion of the 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 hematoxylin–eosin stain.

2.4. Biochemical analysis

The blood samples were collected directly by cardiac puncture in a vacutainer having a clot activator (AKÜ ret, Medkit). The serum samples were collected for the biochemical analysis of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). The levels of the enzymes were estimated according to the method described in the commercial kits (Crest Biosystem, India).

2.5. Preparation of liver homogenate

The homogenate was prepared according to the procedure described by Singh et al. [23]. The livers were washed thoroughly with chilled 0.9% saline. The final wash was given with chilled homogenizing buffer (pH 7.5) containing 0.024 M EDTA, 0.075 M NaCl and 10% DMSO. After weighing, the liver was cut into pieces, suspended in chilled homogenizing buffer at a concentration of 1 g/mL, and homogenized on ice using homogenizer at 1500 rpm. The homogenate was then centrifuged at 7000 rpm for 10 min at 4 °C. The supernatant was removed, resuspended in a 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 protocol described by Hawkins [24] and Ali et al. [25].

2.7. Estimation of lipid peroxidation

The method described by Siddique et al. [26] was used for the estimation of lipid peroxidation in liver cells. The absorbance was read at 586 nm [25].

2.8. Micronucleus assay

Micronucleus assay was performed according to the method of Igarashi and Shimada [27]. A total of 500 cells were counted per rat for the presence of micronuclei using a light microscope [25,27,28].

2.9. Comet assay

Comet assay was performed according to the method described by Singh et al. [29]; and with modification as suggested by Dhawan et al. [30]. Frosted microscopic slides were dipped in 1% normal melting agarose and the underside was wiped to remove the agarose (dissolved in PBS). About 40 μL of the cell suspension (liver cells) was mixed with 60 μL of 0.5% low melting agarose (dissolved in PBS) and was layered on the prepared base slides. Three slides were prepared per rat and a total of 50 randomly captured comets per slide were analyzed under fluorescence microscope for scoring comet tail length by using comet 1.5 software (TriTek Corporation) [25].

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 estimated at the 5% level.

3. Results and discussion

The HE staining of histological sections of the liver showed the 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 (Fig. 1B). The liver deformities were reduced as the rats were exposed to different doses of Genistein together with NDEA (0.1 mg/mL). Fig. 1C shows the protective effect in rats exposed to 100 mg/mL of Genistein together with 0.1 mg/mL of NDEA. The results obtained for various enzymes activity in the blood serum are shown in Figs. 2–5. The SGOT activity (U/mL) in the group of rats exposed to NDEA (0.1 mg/mL) was associated with the mean value of 80 ± 1.531. The rats exposed to 25, 50 and 100 mg/mL of Genistein together with NDEA (0.1 mg/mL) were associated with the mean SGOT activity of 50 ± 1.032, 41 ± 1.210 and 32 ± 1.013 U/mL, respectively (Fig. 2). The control group was associated with the mean SGOT of 28 ± 1.241 U/mL. The rats exposed to 25, 50 and 100 mg/mL of Genistein were associated with the mean SGOT values of 30 ± 1.043, 31 ± 1.041 and 27 ± 1.032 U/mL, respectively (Fig. 2). A significant decrease in the mean values of SGPT (U/mL) was observed in the rats exposed to NDEA combined with 25 (42 ± 1.43), 50 (39 ± 1.31) and 100 mg/mL (31 ± 1.62) of Genistein as compared to the rats exposed to NDEA alone (55 ± 1.21) (Fig. 3). The group of rats exposed to 25, 50 and 100 mg/mL of Genistein did not show a significant increase in the mean values for SGPT (U/mL) (Fig. 3). A significant decrease in the mean values of ALP (U/L) was observed in the rats exposed to 0.1 mg/mL of NDEA combined with 25 (320 ± 1.13), 50 (290 ± 1.02) and 100 (281 ± 1.24) mg/mL of Genistein compared to the rats exposed to 0.1 mg/mL of NDEA alone (Fig. 4). The rats exposed to 25, 50 and 100 mg/mL of Genistein did not show any significant increase in the mean values of ALP (U/L) (Fig. 4). A significant decrease in the mean values of LDH (U/L) was observed in rats exposed to 25, 50 and 100 mg/mL of Genistein together with 0.1 mg/mL of NDEA, compared to the group of rats exposed to NDEA alone (Fig. 5). The results obtained for lipid peroxidation assay are shown in Fig. 6. A significant decrease in the mean absorbance values for lipid peroxidation was observed in the rats exposed to 25, 50 and 100 mg/mL of Genistein together with 0.1 mg/mL of NDEA compared to the group of rats exposed to 0.1 mg/mL of NDEA alone (Fig. 6). A significant decrease in the mean absorbance values for protein carbonyl content was observed in the group of rats exposed to 25, 50 and 100 mg/mL of Genistein together with 0.1 mg/mL of NDEA, as compared to the group of rats exposed to NDEA (0.1 mg/mL) alone (Fig. 7). The results obtained for micronucleus assay are shown in Fig. 8 (A and B). A significant decrease in the mean values of micronucleated cells was observed in the rats exposed to 25 (0.0168 ± 0.321), 50 (0.014 ± 0.121) and 100 (0.0124 ± 0.321) mg/mL of Genistein together with 0.1 mg/mL of NDEA as compared to the group of rats exposed to 0.1 mg/mL NDEA alone (Fig. 8C). No significant increase in the mean values of micronucleated cells was observed in rats exposed to 25, 50 and 100 mg/mL of Genistein (Fig. 8C), as compared to the control group of rats (Fig. 8C). The results obtained for the comet assay performed on hepatocytes are shown in Fig. 9(A and B). A significant decrease in the mean tail length was observed in the group of rats exposed to 25 (64 ± 0.43), 50 (61 ± 0.67) and

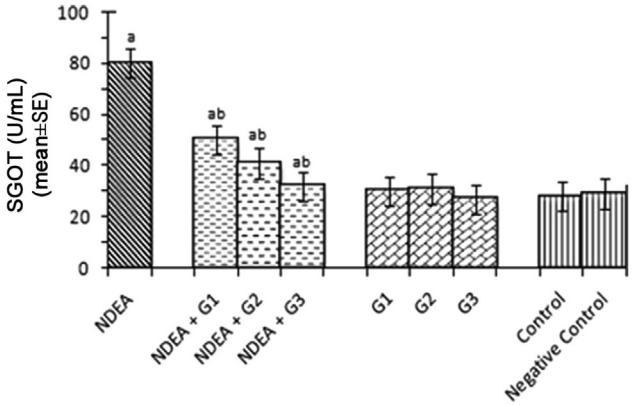

Fig. 2 Serum glutamic oxaloacetic transaminase (SGOT) activity measured after 21 days of treatment of N-nitrosodiethylamine separately and together with different doses of Genistein in rats. [NDEA = N-Nitrosodiethylamine; G1 = Genistein (25 mg/mL); G2 = Genistein (50 mg/mL); G3 = Genistein (100 mg/mL); and negative control = Dimethyl sulfoxide (3 µL/mL). a—Significant with respect to control. b—Significant with respect to NDEA treatment.] ($P < 0.05$).

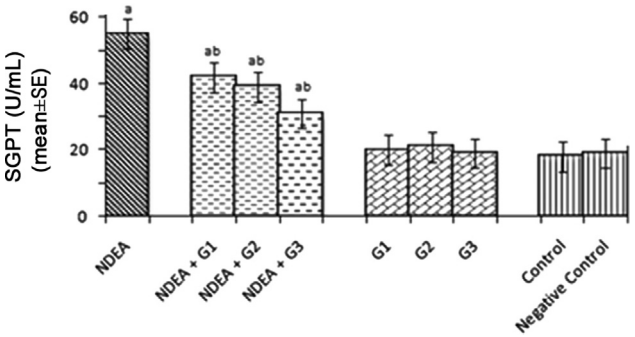

Fig. 3 Serum glutamic pyruvic transaminase (SGPT) activity measured after 21 days of treatment of N-nitrosodiethylamine separately and together with different doses of Genistein in rats. [NDEA = N-Nitrosodiethylamine; G1 = Genistein (25 mg/mL); G2 = Genistein (50 mg/mL); G3 = Genistein (100 mg/mL); and negative control = Dimethyl sulfoxide (3 µL/mL). a—Significant with respect to control. b—Significant with respect to NDEA treatment.] ($P < 0.05$).

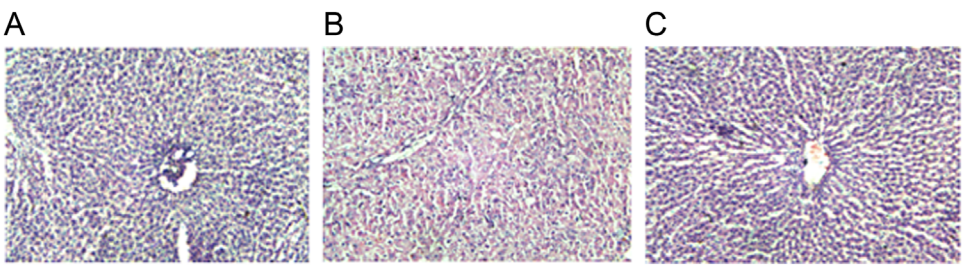

Fig. 1 Microscopic illustrations of the liver of rats stained by hematoxylin–eosin. (A) Control; (B) NDEA (0.1 mg/mL) for 21 days; and (C) rats exposed to 0.1 mg/mL of NDEA together with 100 mg/mL of Genistein.

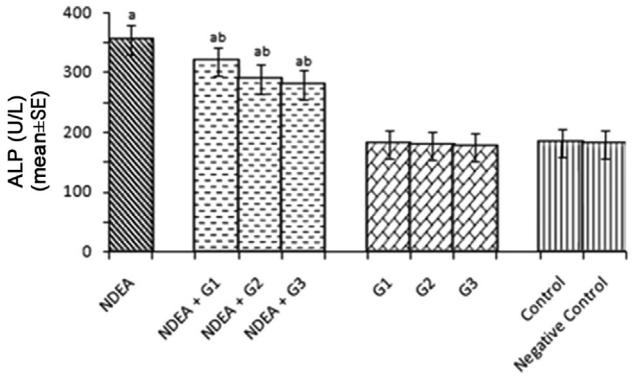

Fig. 4 Serum alkaline phosphatase (ALP) activity measured after 21 days of treatment of N-nitrosodiethylamine separately and together with different doses of Genistein in rats. [NDEA = N-Nitrosodiethylamine; G1 = Genistein (25 mg/mL); G2 = Genistein (50 mg/mL); G3 = Genistein (100 mg/mL); and negative control = Dimethyl sulfoxide (3 μL/mL). a—Significant with respect to control. b—Significant with respect to NDEA treatment.] ($P < 0.05$).

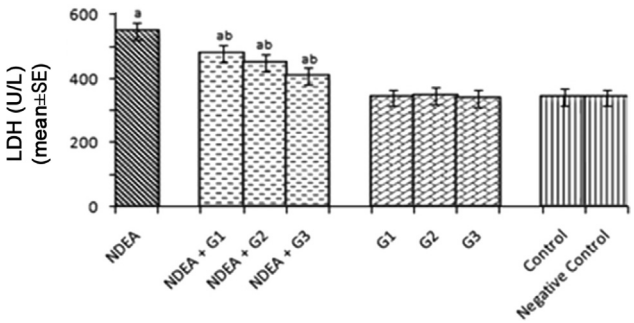

Fig. 5 Lactate dehydrogenase (LDH) activity in serum after 21 days of treatment of N-nitrosodiethylamine separately and together with different doses of Genistein in rats. [NDEA = N-Nitrosodiethylamine; G1 = Genistein (25 mg/mL) ; G2 = Genistein (50 mg/mL) ; G3 = Genistein (100 mg/mL); and negative control = Dimethyl sulfoxide (3 μL/mL). a—Significant with respect to control. b—Significant with respect to NDEA treatment.] ($P < 0.05$).

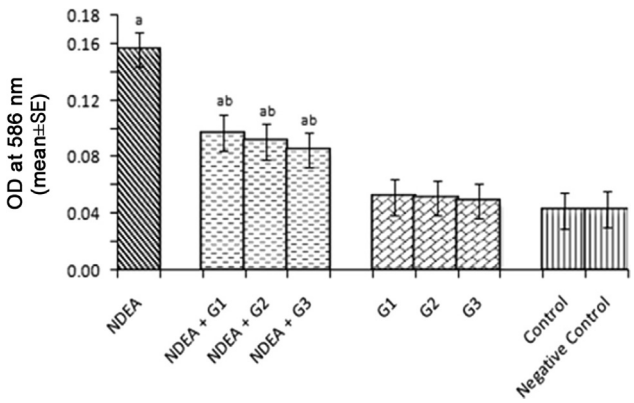

Fig. 6 Lipid peroxidation measured in rat liver after 21 days of treatment of N-nitrosodiethylamine separately and together with different doses of Genistein in rats. [NDEA = N-Nitrosodiethylamine; G1 = Genistein (25 mg/mL); G2 = Genistein (50 mg/mL); G3 = Genistein (100 mg/mL); and negative control = Dimethyl sulfoxide (3 μL/mL). a—Significant with respect to control. b—Significant with respect to NDEA treatment.] ($P < 0.05$).

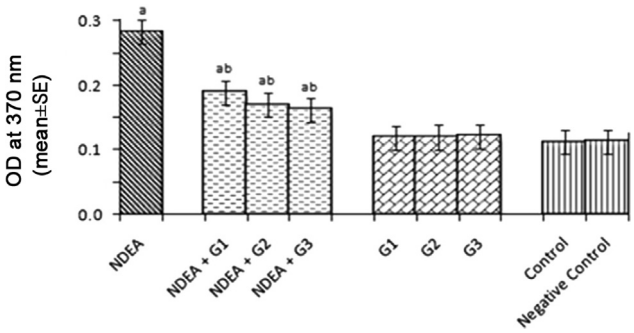

Fig. 7 Protein carbonyl content measured in rat liver after 21 days of treatment of N-nitrosodiethylamine separately and together with different doses of Genistein. [NDEA = N-Nitrosodiethylamine; G1 = Genistein (25 mg/mL); G2 = Genistein (50 mg/mL); G3 = Genistein (100 mg/mL); and negative control = Dimethyl sulfoxide (3 μL/mL). a—Significant with respect to control. b—Significant with respect to NDEA treatment.] ($P < 0.05$).

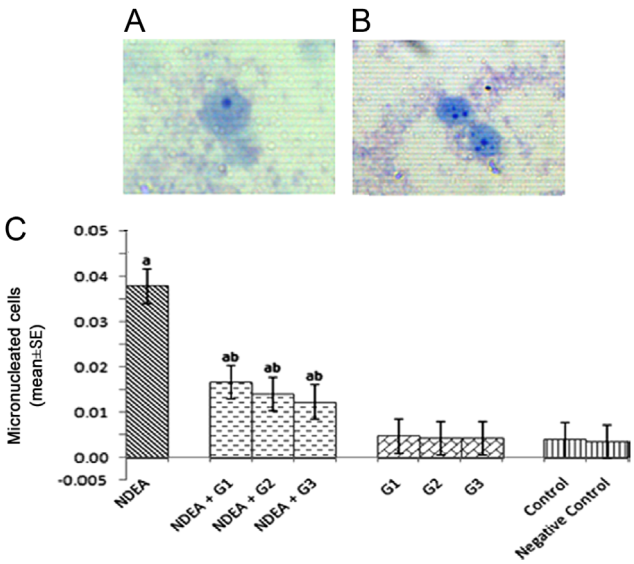

Fig. 8 Hepatocytes show micronuclei: (A) control and (B) treated with NDEA (0.1 mg/mL). (C) Micronucleated cells in the hepatocytes after 21 days of treatment of N-nitrosodiethylamine separately and together with different doses of Genistein in rats. [NDEA = N-Nitrosodiethylamine; G1 = Genistein (25 mg/mL); G2 = Genistein (50 mg/mL); G3 = Genistein (100 mg/mL); and negative control = Dimethyl sulfoxide (3 μL/mL). a—Significant with respect to control. b—Significant with respect to NDEA treatment.] ($P < 0.05$).

100 (54 ± 0.53) mg/mL of Genistein together with 0.1 mg/mL of NDEA, compared to the group of rats exposed to NDEA (0.1 mg/mL) alone (Fig. 9C). No significant increase in the mean tail length was observed in the group of rats exposed to 25, 50 and 100 mg/mL of Genistein (Fig. 9C).

The results of the present study suggest the protective role of Genistein against NDEA-induced hepatotoxicity in rats. NDEA has been suggested to cause oxidative stress by generating reactive oxygen species [31]. NDEA is metabolized to its active ethyl radical metabolite ($CH_3CH_2^+$) by the cytochrome P-450 dependent monooxygenase [32]. This results in an increase in the serum indices of liver function (SGOT, SGPT, ALP and LDH) as well as

DNA damage in the liver cells in the present study. The DNA damage is evident by a significant increase in the MN frequency and DNA tail length group as compared to control. It also results in the formation of various histopathological lessions. Phytochemicals provide essential health value to our diet and are emerging as key nutraceuticals. The poorly understood mechanism of action of the vast majority of phytochemicals has led to the regulation of dietary supplements and the understanding of how nutraceuticals provide health value [33]. In our present study, the supplementation of Genistein together with NDEA results in a significant dose-dependent decrease in the toxic effects induced by the treatment of NDEA alone.

After ingestion Genistein is metabolized to dihydroGenistein and 6-hydroxy-o-desmethylangolensin. However, it is still unclear whether the active ingredient *in vivo* is the parent compound or a metabolite [34]. The two features of flavonoids have been suggested for their biological functions *i.e.* their antioxidant properties and their ability to act as signal molecules by interacting with proteins [35]. Although isoflavones have been reported to be antioxidants by scavenging free radicals [36,37], they also induce the expression of antioxidant genes [7]. The administration of Genistein to cancer patients showed the plasma concentration of the parent compound corresponding to those achieved in anti-neoplastic *in vitro* studies [38]. The exposure of rats to 0.1 mg/mL of NDEA for 21 days showed liver damage as evidenced by the increase in the level of aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT), ALP and LDH. Any damage to the liver cells leads to the rise in the level of these enzymes in the blood stream serving as a potential marker of the liver damage [39]. The exposure of rats to NDEA combined with various doses of Genistein showed a dose-dependent decrease in the levels of these enzymes, thus confirming a protective role of Genistein against NDEA-induced liver damage. The metabolic activation of NDEA leads to the formation of ethyl radical ($CH_3CH_2^+$), causing the damage of plasma membrane [32]. The resulting reactive oxygen species may cause the oxidation of protein [32]. The rats exposed to NDEA showed a significant increase in the lipid peroxidation and protein carbonyl content as compared to untreated ones. The rats exposed to NDEA together with different doses of Genistein showed a significant dose-dependent decrease in the lipid peroxidation and protein carbonyl content, thus confirming the antioxidant role of Genistein in the treated rats. It may be due to the scavenging of free radicals [36,37] or by enhancing expression of antioxidant genes [7]. The selected doses of Genistein in our present study successfully reduced the toxic effects of NDEA in rats. However, some *in vitro* studies have demonstrated Genistein as a clastogenic and also a mutagenic agent [40,41]. In a more refined study, Genistein was found to be negative for bacterial mutations and genotoxicity in the *in vivo* micronucleus test even at 2000 mg/kg [15]. In Asian males and females, the plasma concentrations of total Genistein are approximately 500 nM. The study performed on rats for micronucleus assay showed 80% and 49% increase in the average peak plasma level for total Genistein for male and female rats respectively, with no increase in the micronuclei [15]. There is a wide margin of safety with respect to Genistein intake in humans. The selected doses of Genistein in our present study did not show any toxic effects on rats. Once absorbed, a small fraction of the isoflavones circulates systemically in their unconjugated form [42]. The blood serum concentration of Genistein may vary among individuals due to differences in metabolic handling of the isoflavones [42]. In *in vitro* study, the pre-incubation of cultured cancerous prostate cells with Genistein reduced H_2O_2-induced DNA damage [7]. Similar properties of Genistein were reported in human lymphocytes [43,44]. Considering the results obtained in our study and other studies carried out *in vivo* and *in vitro*, the possible mechanism for protection by Genistein against NDEA is shown in Fig. 10. Patel et al. [36] have shown that the polysaturated fatty acids reduce the phenoxyl

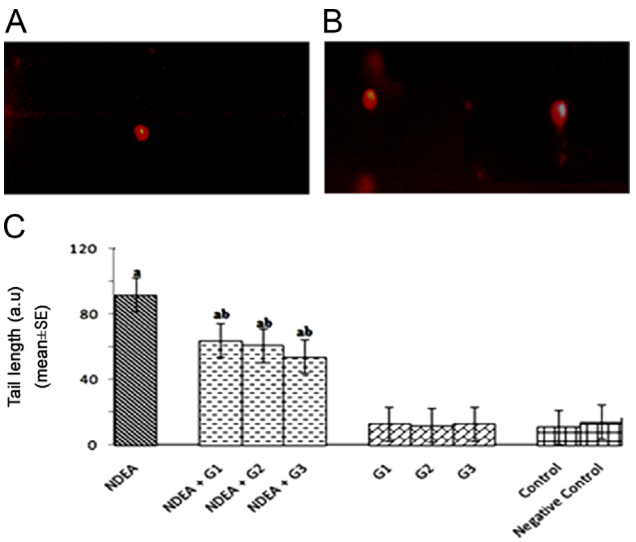

Fig. 9 Comet assay performed in rat hepatocytes [(A) control and (B) treated with NDEA (0.1 mg/mL)]. Comet assay in the hepatocytes after 21 days of treatment of N-nitrosodiethylamine separately and together with different doses of Genistein in rats (C). [NDEA = N-Nitrosodiethylamine; G1 = Genistein (25 mg/mL); G2 = Genistein (50 mg/mL); G3 = Genistein (100 mg/mL); and negative control = Dimethyl sulfoxide (3 µL/mL). a—Significant with respect to control. b—Significant with respect to NDEA treatment] ($P < 0.05$).

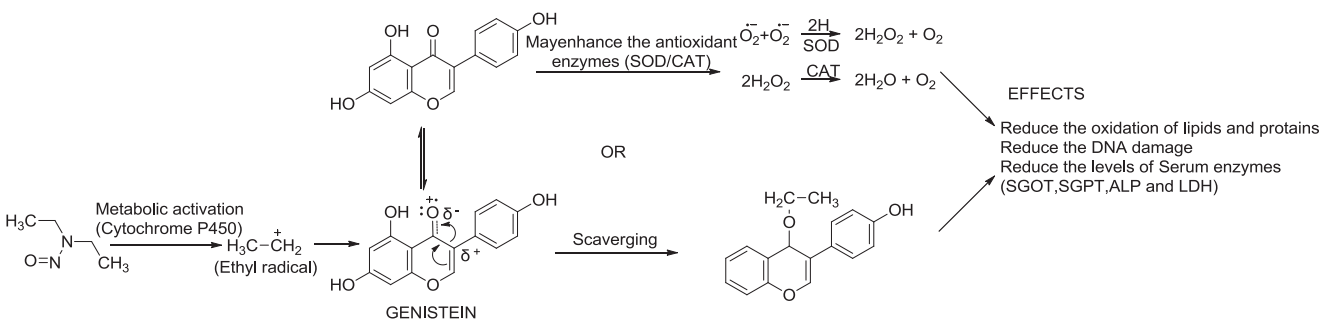

Fig. 10 Possible mechanism of the metabolic activation of N-nitrosodiethyl amine (NDEA) and the protective role of Genistein.

radical formed as a result of reaction polyphenols with lipid peroxyl radicals, thereby regenerating Genistein and peroxyl radical, thus delaying the effect of peroxyl radical [6].

4. Conclusion

The results of the present study reveal that Genistein reduces not only the levels of blood serum enzymes but also the genotoxic damage as well as oxidative stress induced by NDEA. Hence, it is concluded that Genistein is potent in reducing the toxic effects of NDEA in Swiss albino rats.

Acknowledgments

We are gratefully thankful to Science and Engineering Research Board (SERB), Department of Science & Technology, Technology Bhavan, New Delhi, India, for the sanction of the research project (No. SR/FT/LS-60/2011) to Dr. Yasir Hasan Siddique. We are also thankful to the Chairman, Department of Zoology, AMU, Aligarh for providing laboratory facilities.

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