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Modulatory Effect of Chrysin against N-Methyl Nitrosourea (NMU)-Induced Proliferative Lesions in Rat Mammary Glands

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ABSTRACT

This study aims to investigate the role of the phytoestrogen (chrysin) in the treatment or protection of N-methyl-N-nitrosourea (NMU)-induced preneoplastic lesions in mammary glands of post estrous female rats. Rats were divided into seven groups. Group1 is the negative group. Groups 2 &3 are the positive controls where rats were injected intraperitoneally (i.p.) with 4 doses of NMU (75 mg/kg b.w., once/5days) before and after oral administration of glycofurol (vechile of chrysin), respectively. Groups 4 &5 are the treatment groups where rats were received 4 doses of NMU and were administered 3 doses/week of chrysin (125 or 250 mg/kg b.w. per orally; p.o), respectively, as in group 2. Groups 6 & 7 are the protective groups where rats were received 3 doses/week of chrysin (125 or 250 mg/kg b.w., p.o.), respectively, and were injected with 4 doses of NMU as in group3. The NMU injection caused a significant decrease in platelet count, hemoglobin, serum estradiol, and total antioxidant capacity levels as well as serum glutathione-S-transferase and Catalase activities. On the other hand, NMU significantly elevated white blood cell count, red cell distribution width, serum C-reactive protein, carcinoembryonic antigen, malondialdehyde and nitric oxide levels as well as serum arginase activity. Either treatment or protection with chrysin modulated the adverse effects of NMU and ameliorated the biochemical parameters. The biochemical observations were also confirmed by histological studies. In conclusion, phytoestrogens may relieve the severity of post estrous pre-cancerous disorders, especially when consumed in a high dose for a while before the incidence of lesions.

Keywords: N-Methyl-N-nitrosourea; Phytoestrogen; Chrysin; Mammry glands; Rat.

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INTRODUCTION

Globally breast cancer (BC) is the most often diagnosed cancer and the leading cause of cancer death among women accounting for 23% of the total cancer cases and 14% of the cancer deaths [1]. Around 5-10% of all BC cases are caused by an inherited genetic mutation. The remaining 90-95% of BC cases are caused by other risk factors [2].The etiology of BC is multi-factorial and the major risk factors of this kind of cancer include sex, age, childbearing, hormones, high fat diet, tobacco, alcohol intake, obesity and environmental factors such as radiation [3].

NMU is a direct-acting alkylating agent that interacts with DNA. Accumulation of mutations may enhance cancer risk in target organs or cause cell death in susceptible tissues or cells when excessive DNA damage is not repaired. NMU-induced rat mammary tumors have many similarities to those of human BC [4]. NMU does not require metabolic activation to form DNA adducts and has a very short half-life. The NMU induced mammary tumors are more estrogen dependent, locally aggressive and able to metastasize [5]. Estrogen signaling and estrogen receptor (ER) are implicated in BC progression and the majority of the human breast cancers start out as estrogen dependent [6].

Phytoestrogens are secondary polyphenolic plant substances with similarities to 17-beta estradiol (E_2) in chemical structure [7]. Phytoestrogens constitute a group of plant-derived estrogens possessing significant estrogen agonist/antagonist activity. Their effects mediated *via* the interaction with ER subtypes ERα and ERβ. They are characterized by high tissue specificity and dose-dependent activity [8]. Chrysin (5,7dihydroxyflavone), is a flavone occurring in various natural sources such as propolis and honey [9]. It has shown to have cancer chemoprotective activity *via* induction of apoptosis in a diverse range of human and rat cell types [10].

Epidemiologic studies have suggested that high consumption of phytoestrogens, mostly soy and unrefined grain products, may lower the risk of some cancers such as colorectal, prostate, and BC [11]. Phytoestrogens are able to bind to ERs *in vitro* and thereby induce or modulate the estrogen signaling pathway [12]. The aim of this study was to evaluate the effect of two doses of chrysin as a phytoestrogen in modulating benign proliferative (pre-cancerous) lesions induced by NMU in post estrous female rats.

MATERIALS AND METHODS

Chemicals:

N-Methyl-N-nitrosourea (NMU), chrysin (chry) (5,7-dihydroxyflavone) and tetraethylene glycol (glycofurol) were purchased from Sigma Chemical Company (St Louis, MO 63103, USA). N-Methyl-N-nitrosourea was given intra peritoneally (i.p.) at a dose level of 75 mg/kg b.w. [13] and was freshly prepared by dissolving it in physiological saline containing 0.05 % acetic acid [14]. Chrysin was given per os (p.o.) *via* an intragastric tube at doses of 125 mg/kg b.w. or 250 mg/kg b.w. [2] and was freshly prepared by dissolving it in glycofurol [15].

Animals:

One hundred and five adult female albino rats "Sprague-Dawely" weighing 250-280 g, obtained from the animal house of El- Salam farm, Giza, Egypt were used in this study. After one week of acclimatization, rats were housed individually in an animal care facility with constant environment in controlled stainless steel cages, under a room temperature (25 ±5 °C) and a relative humidity (50%± 10%) with 12 hour light/dark cycles. All animals received human care in compliance with the internationally valid guidelines of the Animal Care and the Use Ethic Committee of Ain Shams University, Cairo, Egypt.

Experimental Design:

Rats were randomly divided into seven groups with 15 rats in each with a similar average weight and were provided with a standard commercial diet [16] and water *ad libitum* for 12 weeks. Rats were weighed weekly to adjust the oral dose of chrysin and the intraperitoneal dose of NMU. The groups were as follows:

Group1 (Negative control): Rats received equivalent volumes of vehicles, glycofurol (p.o.) and saline + 0.05% acetic acid (i.p.), parallel to the other treated groups, throughout the course of the study of 12 weeks.

Group2 (NMU/glycofurol): Rats were injected with 4 i.p. doses of NMU (75 mg/kg b.w., one dose every 5 days) from the starting day of the experiment and left without treatment till the end of the sixth week. Glycofurol were administered (2 ml /kg, p.o., 3 times every other day/week) from the beginning of the seventh week for 6 consecutive weeks till the end of the experiment.

Group3 (glycofurol/NMU): Rats were administered glycofurol (2 ml /kg, p.o., 3 times every other day/week) for 6 consecutive weeks and were injected with 4 i.p. doses of NMU (75 mg/kg b.w., one dose every 5 days) from the beginning of the seventh week and left without treatment till the end of the experiment.

Groups 4 (NMU/chry., LD) & 5 (NMU/chry., HD): Rats were injected with NMU and were administered chrysin instead of glycofurol at doses of 125 or 250 mg/kg b.w., respectively, similar to group2.

Groups 6 (chry., LD/NMU) & 7 (chry., HD/NMU): Rats were administered chrysin at doses of 125 or 250 mg/kg b.w., respectively, and were injected with NMU similar to group3.

Biochemical Studies:

At the end of the experimental period (12 weeks), animals were sacrificed after 12 hours fasting. Blood samples were collected from hepatic portal vein, left for 15 min. at 37°C then centrifuged at 4000 rpm for 20 min. for serum separation and were stored at -20 °C in plastic vials until analysis. Part of the blood from each rat was collected into tubes which contained EDTA used for determination of white blood cell (WBC) and platelet (PLT) count, hemoglobin concentration (Hb) and red cell distribution width (RDW%) by Mindray Vet-BC2800 (Mindray International/ Electronic company, China).

Serum C-reactive protein (CRP) was estimated by means of particle enhanced turbid metric immunoassay kit (Spectrum Company) [17]. Serum carcinoemberyonic antigen (CEA) [18] and estrogen level (as estradiol, E_2) [19] were determined using ELISA kit (Glory Science Company). Serum total antioxidant capacity (TAC) [20], nitric oxide (NO) [21] and lipid preoxidation (as malondialdehyde, MDA) [22] were estimated by colorimetric method kit developed by Biodiagnostic Company, Giza, Egypt. Furthermore, arginase [23], glutathione-s-transferase (GST) [24] and catalase (CAT) [25] activities were determined in serum by colorimetric method kit supplied by Biodiagnostic Company, Giza, Egypt.

Histopathological Examination:

Tissue specimens from the mammary glands of the experimental groups were immediately excised, washed using chilled physiological saline solution, fixed in 10% neutral buffered formalin for 24 hours, dehydrated in ascending ethanol series, cleared in xylene and embedded in paraffin wax [26]. Paraffin blocks were cut in sections of 5–6 µm thickness using a rotatory microtome and stained with hematoxylin and eosin (H&E) stain. Examination of sections from all groups under light microscope and assessment of various groups were performed [27].

Statistical Analysis:

The data were presented as mean ± standard error (SE). One-way analysis of variance followed by *post hoc*-least significant difference analysis was performed using the statistical package for social science (SPSS) version 16 to compare all the studied groups. The values of $P < 0.05$ and $P < 0.001$ were considered significant and very highly significant, respectively.

RESULTS

Biochemical Studies:

Table (1) illustrates that injection with NMU (groups 2&3) led to significant increase in WBC count as compared with group1 (negative control rats). Furthermore, all rats in the treatment and protective groups

showed a significant decrease in the WBC count as compared with their respective positive control groups. On the other hand, treatment and protection by chrysin induced non-significant change in WBC count compared with negative control rats.

Table (1) indicates that injection with NMU led to decrease in hemoglobin (Hb) concentration. Such decrease was significant in group2 (NMU/glycofurol) and very highly significant in group3 (glycofurol/NMU) as well as significantly increased red cell distribution width (RDW %) as compared with negative control rats. Only the treatment group (NMU/chry., HD) showed normalization of Hb level compared with the treatment group (NMU/chry., LD) and protective groups. Compared to the negative control group, the data also revealed that group5 (NMU/chry., HD) provided a pronounced improvement on the RDW% level. Moreover, injection with NMU led to a significant and non-significant decrease in PLT count in group2 and group3, respectively, compared to negative control. PLT count was increased significantly in rats treated with high dose of chrysin, (NMU/chry., HD) compared to rats of its positive control group (NMU/glycofurol). On the other hand, treatment and protection by chrysin induced non-significant change in PLT count compared to the negative control rats.

Table 1: White blood cell (WBC) and platelet (PLT) count and level of hemoglobin (Hb), and red cell distribution width (RDW) in different experimental groups

Groups		Parameters			
		WBCX10^9/L	PLT X10^9/L	Hb (g/dl)	RDW %
Negative control group	Group1	4.28±0.34	767±73.70	13.88±0.41	11.46±0.30
Positive control groups	Group2	7.42±0.59[1]	581±60.44[a]	12.50±0.24[a]	15.74±0.84[1]
	Group3	6.02±0.71[a]	705±47.57	11.72±0.16[1]	15.78±0.56[1]
Treatment groups	Group4	5.00±0.29[b]	649.±38.91	12.90±0.22[a,c]	13.72±0.44[a,b,c]
	Group5	4.24±0.42[2,c]	774±27.16[b]	13.46±0.38[3,b]	12.44±0.45[2,3]
Protective groups	Group6	4.17±0.81[2,c]	765±37.32[b]	11.96±0.25[1,5,d]	14.59±0.29[1,e]
	Group7	4.30±0.33[2,c]	773±7.62[b]	11.92±0.24[1,5,d]	13.82±0.43[a,b,c]

Values are represented as means ± SE.
Group1, **negative control**; Group2, **NMU/glycofurol**; Group3, **glycofurol/NMU**; Group4, **NMU/chry., LD**; Group5, **NMU/chry., HD**; Group6, **chry., LD/NMU** & Group7, **chry., HD/NMU**.
Numbers 1,2,3& 5 represent statistical differences with groups 1, 2, 3 & 5, respectively, at $P<0.001$.
Letters a, b, c, d & e represent statistical differences with groups 1, 2, 3, 4 & 5, respectively, at $P<0.05$.

The data in table (2) reveals that rats injected with NMU (groups 2&3) had a significant increase in the levels of serum CRP and CEA compared to the negative control group at $P<0.001$. Table (2) also indicates that E_2 concentrations of the positive control groups (groups 2&3) significantly decreased as compared to the negative control group. Treatment with a high dose of chrysin (NMU/chry.HD) significantly decreased the concentration of serum CEA and significantly increased the concentration of E_2 at $P<0.001$ as compared with its respective positive control (NMU/glycofurol). On the other hand, protection by a high dose of chrysin (chry., HD/NMU) significantly decreased levels of CRP and CEA than protection by a low dose of chrysin, (chry.LD/NMU) as compared to its positive control group (glycofurol/NMU).The high dose in group7 (chry.,HD/NMU) of chrysin had a marked significant decrease in serum CRP concentration at $P<0.001$ compared to group4 (NMU/chry., LD). Values of CEA concentration of rats consumed chrysin at a high dose [(NMU/chry., HD) and (chry., HD/NMU)] were very significantly decreased compared to rats which consumed low dose of chrysin as protection (chry., LD/NMU). The protection with a high dose of chrysin in group7 (chry., HD/NMU) nearly restored the CRP and CEA concentration to be near to that of the negative control group. On the other hand, there were no significant differences in the mean values of E_2 concentration among the protective, treatment and the negative control groups.

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Table 2: Concentrations of C-reactive protein (CRP), carcinoembryonic antigen (CEA) and estradiol (E_2) in different experimental groups

Groups		Parameters		
		CRP mg/dL	CEA ng/mL	E_2 ng/mL
Negative control group	Group1	6.52±0.58	0.41±0.03	2.72±0.11
Positive control groups	Group2	17.52±1.43[1]	0.82±0.03[1]	1.98±0.08[1]
	Group3	19.60±0.89[1]	1.06±0.07[1,2]	2.28±0.05[a]
Treatment groups	Group4	16.32±0.78[1,c]	0.50±0.03[2,3]	2.63±0.17[2,c]
	Group5	10.10±0.97[2,3,4,.a]	0.42±0.01[2,3]	2.79±0.13[2,c]
Protective groups	Group6	10.82±0.40[2,3,4,a]	0.84±0.04[1,3,4,5]	2.88±0.07[2,3]
	Group7	7.74±0.60[2,3,4,f]	0.45±0.02[2,3,6]	2.85±0.13[2,3]

Values are represented as means ± SE.
Group1, **negative control**; Group2, **NMU/glycofurol**; Group3, **glycofurol/NMU**; Group4, **NMU/chry.**, **LD**; Group5, **NMU/chry.**, HD; Group6, **chry., LD/NMU** & Group7, **chry., HD/NMU**.
Numbers 1,2,3,4,5 & 6 represent statistical differences with groups 1, 2, 3, 4,5 & 6, respectively, at *P*<0.001.
Letters a, c & f represent statistical differences with groups 1, 3 & 6, respectively, at *P*<0.05

Table (3) represents that injection with NMU in (groups 2&3) induced a significant increase in serum arginase activity, and a significant decrease in serum GST and CAT activities as compared to the negative control group at *P*<0.001. All rats receiving chrysin for treatment either by a low (NMU/chry., LD) or a high dose (NMU/chry., HD) exhibited a significant increase in GST and CAT activities at *P*<0.05 as well as a significant decrease in arginase activity at *P*<0.001 when compared to its positive control group (NMU/glycofurol). On the other hand, the activity of serum GST which was recorded in protective groups [(chry., LD/NMU) and (chry.,HD/NMU)] was significantly increased at *P*<0.001, while the activity of CAT was significantly increased at *P*<0.05 and *P*<0.001, respectively, when compared to group3 (glycofurol/NMU). It is clear that there is a very highly significant increase in serum GST and CAT activities and a non-significant difference in the mean values of serum arginase activity in protective groups [(chry., LD/NMU) and (chry., HD/NMU)] in comparison to both treatment groups [(NMU/chry., LD) and (NMU/chry., HD)].

Table 3: Activity of serum arginase, glutathione-S-transferase (GST) and catalase (CAT) in different experimental groups

Groups		Parameters		
		Arginase U/L	GST U/L	CAT U/L
Negative control group	Group1	120.00±10.85	27.55±2.87	48.42±2.61
Positive control groups	Group2	209.67±10.11[1]	11.47±1.68[1]	5.65±0.90[1]
	Group3	172.17±7.69[1]	15.20±0.75[1]	34.44±1.59[1,2]
Treatment groups	Group4	142.17±9.70[2,c]	16.63±1.18[1,b]	12.08±0.58[1,3,b]
	Group5	136.83±8.48[2,c]	18.40±0.74[1,b]	13.93±0.85[1,3,b]
Protective groups	Group6	156.17±15.44[2,a]	25.45±0.89[2,3,4,e]	40.22±2.09[2,4,5,a,c]
	Group7	160.33±4.32[2,a]	30.58±1.65[2,3,4,5,f]	46.20±2.43[2,3,4,5,f]

Values are represented as means ± SE.
Group1, **negative control;** Group2, **NMU/glycofurol;** Group3, **glycofurol/NMU;** Group4, **NMU/chry.,LD;** Group5, **NMU/chry., HD;** Group6, **chry., LD/NMU** & Group7, **chry., HD/NMU.**
Numbers 1,2,3,4 & 5 represent statistical differences with groups 1,2,3,4 & 5, respectively, at *P*<0.001.
Letters a, b, c, e & f represent statistical differences with groups 1, 2,3,5 &6, respectively, at *P*<0.05.

Table (4) shows that injection with NMU (NMU/glycofurol) induced a significant decrease in the TAC level at *P*<0.001 as compared to the negative control group. Investigations had also shown that injection with NMU [(NMU/glycofurol) and (glycofurol/NMU)] resulted in significant increase in the levels of serum MDA and NO as compared to negative control group. The MDA and NO levels were significantly lowered at *P*<0.001 in the treatment and protective groups than those in their positive control groups. However, the values of MDA and NO remained higher than the normal control values. There was a significant elevation *P*<0.001 in the level of serum TAC of protective groups when compared to the treatment groups and the level of TAC recorded in the protective groups approached to that found in the negative control group.

Table 4: Levels of serum total antioxidant capacity (TAC), malondialdehyde (MDA), and nitric oxide (NO) in different experimental groups

Groups		Parameters		
		TAC mmol/L	MDA nmol/L	NO mmol/L
Negative control group	Group1	2.22±0.059	1.40±0.068	2.05±0.152
positive control groups	Group2	1.86±0.037[1]	6.12±0.767[1]	7.64±0.158[1]
	Group3	2.11±0.033[b]	7.92±0.307[1,b]	4.68±0.166[1,2]
Treatment groups	Group4	1.94±0.084[1,c]	3.40±0.311[1,2,3]	6.52±0.073[1,2,3]
	Group5	1.94±0.055[1,c]	2.97±0.324[2,3,a]	6.30±0.159[1,2,3]
Protective groups	Group6	2.21±0.037[2,4,5]	3.75±0.427[1,2,3]	1.92±0.087[2,3,4,5,]
	Group7	2.23±0.049[2,4,5]	1.65±0.1856[2,3,6,d,e]	1.85±0.102[2,3,4,5]

Values are represented as means ± SE.
Group1, **negative control;** Group2, **NMU/glycofurol**; Group3, **glycofurol/NMU**; Group4, **NMU/chry., LD;** Group5, **NMU/chry., HD**; Group6, **chry., LD/NMU** & Group7, **chry.,HD/NMU**.
Numbers 1,2,3,4,5 & 6 represent statistical differences with groups 1, 2, 3, 4, 5 &6, respectively, at *P*<0.001.
Letters a, b, c, d & e represent statistical differences with groups 1, 2, 3, 4 &5, respectively, at *P*<0.05.

Histopathological Studies:

Histopathological analyses were performed on mammary glands from all experimental animals. The results showed that the mammary tissue of negative control of post estrous female rats showed no morphological alterations. The mammary glands showed a normal lobular architecture with branched ducts, lined by simple cuboidal epithelium and a normal distribution of fat tissue (Fig. 1 A).

In the case of group 2 (NMU/glycofurol), atypical ductal epithelial hyperplasia (epitheliosis) were observed in multiple ducts and some of the epithelial cells have hyperchromatic compressed nuclei (Fig. 1B). In addition, there were cystically dilated ducts with proliferation of the lining epithelium (Fig. 1 C). On the other hand, non-proliferative epithelial abnormalities including exfoliation of clusters of damagd epithelial cells into the duct were also seen (Fig. 1 C). In group 3 (glycofurol/NMU), the majority of ducts had a higher degree of atypical ductal hyperplasia of epithelium (florid hyperplasia)(Fig. 1 D), which can increase the risk of developing breast cancer in the future. The epithelial cells within the atypical hyperplasia exhibited hyperchromatic nuclei and dense eosinophilic cytoplasmic staining. Additionally, intraluminal projections of benign epithelial proliferation were detected (Fig. 1 E).

Oral administration of chrysin moderately reduced the severity of histopathological changes in groups 4 and 5 compared to group2. In group 4, treatment with a low dose of chrysin after injection with NMU (NMU/chry., LD) showed that portions of ducts had a moderate ductal epithelial proliferation in addition to sloughing of epithelial cells into the dilated ducts (Figure 1 F). On the other hand, in group 5, treatment with high dose of chrysin after injection with NMU (NMU/chry., HD) showed focal regions of moderate to mild ductal epithelial proliferation (Fig. 1G).

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Microscopic abnormalities observed in groups 6 & 7 that were protected by a low or a high dose of chrysin, respectively, were strongly inhibited as compared to group 3. In group 6, focal regions of mild ductal epithelial hyperplasia were still detected in some ducts (Fig. 1 H); however, in group7 nearly normal structure of the majority of the mammary ducts was observed except few ducts in which there was a mild epithelial proliferation (Fig. 1I).

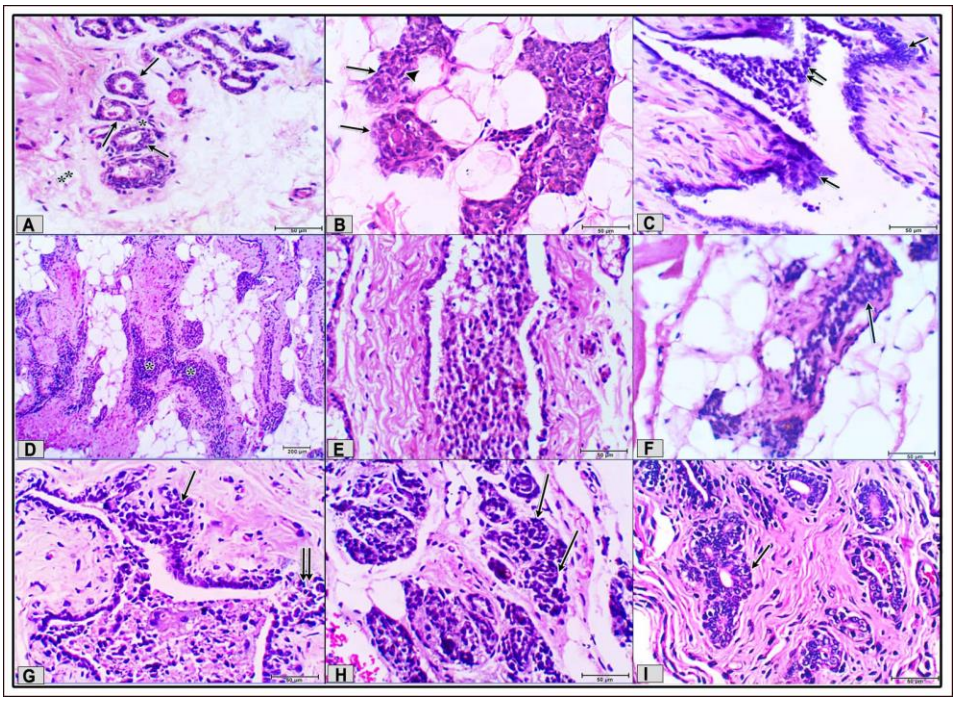

Fig 1: Light micrographs of the mammary glands of female rats. Group1 (negative control) (A) showing normal lobular architecture with intralobular ducts, lined by simple cuboidal epithelium (arrows).Note intralobular (*) and interlobular connective tissue (**). Group 2 (NMU/glycofurol, B&C) (B) showing atypical ductal epithelial hyperplasias (epitheliosis) (arrows). Note that some of the cells have hyperchromatic compressed nuclei (arrowhead). (C) showing cystically dilated duct with proliferation of the lining epithelium (arrows) and non-proliferative lesions including exfoliation of clusters of damaged epithelial cells into the duct (double arrows). Group3 (glycofurol/NMU, D&E) (D) showing severe atypical intraductal epithelial hyperplasia (florid hyperplasia) (*). Note that the duct lumens are distended with proliferated epithelial cells with hyperchromatic nuclei. (E) showing intraluminal projection of benign epithelial proliferation. Group 4 (NMU/chry., LD) (F) showing that portions of the duct contain moderate proliferation of epithelial cells (arrow). Group5 (NMU/chry.,HD) (G) showing portions of moderate (arrow) to mild (double arrows) ductal epithelial proliferation. Group 6 (chry., LD/NMU) (H) showing mild ductal epithelial hyperplasia (arrows) in some ducts. Group7(chry., HD/NMU) (I) showing nearly normal structure of the mammary ducts. However, mild proliferation of lining epithelial cells of few ducts (arrow) still be seen (H&E; A-C X400; D X100; E-I X400).

DISCUSSION

Results obtained in the present study indicated that preneoplastic disorders induced by NMU injection (groups2 & 3) caused a significant increase in WBC count. Previous findings illustrated significant elevation in the number of WBCs in NMU-treated rats [28, 29]. It was also reported that the inflammatory process that takes place during cancer development and progression are, in part, reflected in the abnormalities of the WBC count [30].

The chrysin protected groups 6 &7 brought back the WBC count to near normal levels. Chrysin which is a phytoestrogen has been reported to show estrogenic effects. Estrogen exerts an anti-inflammatory effect by down regulation of the expression of adhesion and chemokine molecules in response to inflammation in many animals [31]. The study of Sridhar et al. [32] showed that WBC count was significantly increased in Dalton's lymphoma and treatment with chrysin brought back the WBC count more or less to normal levels.

Results in table (1) also shows that benign proliferative lesions induced by NMU injection (groups2 &3) caused a significant reduction in Hb level and PLT count as well as a significant elevation in RDW% as compared with the negative control group. A similar effect of NMU on Hb concentration and PLT presently detected had been previously illustrated [28, 33], respectively. On the other hand, a decrease in Hb and an increase in PLT count and a marginal elevation of the red cell size parameter RDW% in the NMU-injected rats were observed [29].

Sridhar et al. [32] attributed the decrease in Hb to iron deficiency or to hemolytic or myelopathic conditions. The cause behind the increase in RDW% may be the increased immature RBCs or reticulocyte number [34]. Free radicals can directly damage red blood cell membranes by peroxidation of membrane polyunsaturated fatty acids [35]. It had been shown that thrombocytopenia in cancer patients were accompanied by anemia [36]. In the same concern, Akinbami et al. [37] reported that BC patients had higher RDW% than healthy patients.

As mentioned in the present study, treatment and protection by chrysin induced a non-significant elevation in Hb concentration and a significant reduction and increase in RDW% and PLT, respectively, as compared to NMU groups (groups 2&3). This indicates that chrysin possesses a protective action on the hemopoietic system. These observations might be due to the fact that phytochemicals stimulate the formation or secretion of erythropoietin in the stem cells of animals in the bone marrow to produce RBCs [38].

The data in table (2) demonstrates that serum CRP and CEA levels were significantly elevated in both positive controls (groups 2 &3) as compared with the negative control (group1). The values of CRP and CEA in group3 were elevated more than group2; this might be due to the fact that the duration between NMU administration and termination of the experiment was shorter in group3 than in group2 so the toxic effect was more evident in this group. Also, it may be due to the beginning appearance of preneoplastic cells generation leading to the liberation of CRP and CEA from recent active inflamed affected cells.

The elevated CRP may be attributed to the inflammatory cytokine interleukin-6 that triggers the hepatic production of CRP. Il-6 has been found to play an important role in various tumor behaviors, including proliferation and differentiation of tumor cells [39], invasion and growth of malignancies [40],

Based on the previous studies, a positive association between elevated levels of CRP and BC prognosis had been reported [41]. Similarly, the results of Ahmed et al. [42] regarding the CRP level in Egyptian females, revealed a significant increase in its level in females with BC as compared to the healthy control.

It was found that serum from individuals with various carcinomas including breast carcinoma had higher levels of CEA than healthy individuals [43]. The reduction of tumor mass in female patients developed a ductal carcinoma was evidenced by a continuing decline of the CEA tumor marker serum level [44]. Moreover, the study of Abdel-Moein et al. [28] showed that injection of NMU for BC induction caused a significant elevation in CEA and estrogen hormone levels compared to the control group.

Results obtained in the present study showed that injection with NMU caused a significant decrease in the level of serum E_2 as compared to the negative control. Meanwhile, rats bearing BC induced by DMBA showed an elevated level of E_2 as compared to the control group [45]. The discrepancy with our results may be related to the difference in the age of the injected rats and the duration of the experiment.

The present work revealed that CRP and CEA levels were significantly decreased in treatment (groups 4 &5) and protective (groups 6 &7) groups compared to their positive controls (groups 2 &3). It's clear that CRP and CEA values of rats which received high dose of chrysin as protection approached nearly to those of the negative control group and subsequently suppressed pre-cancerous lesions during NMU administration. Meanwhile, E_2 level in treatment and protective groups were significantly elevated mostly closer to that of the negative control.

The apparent protective effect of chrysin, i.e. pretreatment with phytoestogen might be through the reduction in ERα than ERβ and then reduction in ERα/ERβ which initiates less nuclear receptor sites for estrogen binding resulting in fewer proliferated mammary tumor [46]. Furthermore, flavonoids have protective effects in estrogen-dependent breast cancer by binding to estrogen receptor and modulating estrogen metabolism [47]. In accordance with these findings, previous results indicated that chrysin may exert an anti-inflammatory activity through down regulation the expression of cyclooxygenase-2 [48].

It is well known that normal blood serum contains only a little activity of arginase. In the present findings, there was a significant increase in the activity of serum arginase enzyme in rats treated with NMU (groups 2 & 3). The increase in the arginase enzyme activity appeared to participate in increased polyamine formation. All these molecules are relevant to the process of tumorigenesis [49].

The present results are in well accordance with the previous findings according to which the arginase activity elevated in the serum of women with BC in comparison to healthy women [50]. It seemed also in the present study that in the treatment groups (groups 4 & 5) chrysin brought back the arginase activity to normal levels in comparison to the protective groups (groups 6 & 7). The inhibitory action exhibited by chrysin might be due to the phenolic hydroxyl groups on its molecular structure at positions 5 and 7, which can neutralize the effect of free radicals generated during DMBA-induced mammary carcinogenesis [51] or directly involved in decreasing ornithine decarboxylase activity by affecting genes encoding ornithine decarboxylase leading to decreased polyamine synthesis [52].

The results of the present study suggest that the activity of GST and CAT were significantly decreased in the serum of rats after injection with NMU (groups 2 & 3). This might be due to the exhaustion of these enzymes in the removal of hydrogen peroxide induced by the abnormal proliferative cells. The present results are in line with the findings of Mallikarjuna et al. [53] who observed a significant depletion in GST and CAT activities in NMU-treated rats compared to normal control animals.

It seems from the current study that oral administration of chrysin for treatment (groups 4 & 5) or protection (groups 6 & 7) significantly reversed the status of the CAT and GST enzymes. The current results suggest that chrysin has potent free radical scavenging property especially at the high protective dose (250 mg/kg b.w.) which brought back these enzymes to normal levels. Chrysin might have either inactivated the metabolic activation of NMU or stimulated the activities of detoxification agents. It is well known that flavonoids are able to induce phase II detoxifying enzymes by preferably activating phase II over phase I [54].

Results of the present study showed that the level of TAC was significantly decreased by the injection of NMU (groups 2 & 3) and the more severe reduction was observed in group2 in which rats received NMU at the beginning of the experiment. The depletion of TAC level was normalized upon the pre-treatment of NMU-injected rats with chrysin (groups 6 & 7).The previous study demonstrated that TAC values were significantly decreased in patients with breast cancer [55]. Also, Hoshyar et al. [33] found that TAC values were significantly decreased in rats after NMU injection than in controls.

It is clear from the present study that the NMU injection (groups 2 & 3) caused significant increase in MDA and NO levels compared to the control group. The increase in lipid peroxidation (LPO) levels might be the result of reduction in antioxidant status or increased production of reactive oxygen species (ROS). This result is in agreement with the previous observation illustrating that there was a significant increase in NO concentration in rats injected with NMU when compared to controls [56]. Also, another study showed that the increased production of NO has a critical role in the development of cancer cells by the stimulation of angiogenesis and increased mutation [57]. Additionally, Yeh et al. [58] showed that the level of MDA in the blood of patients with BC was significantly higher than healthy individuals. The increased levels of LPO products and chronic induction of NO play a role in the early phases of tumor growth [59].

It was observed that chrysin significantly inhibited the lipid membrane damage and elevated the level of antioxidants as evidenced in the present study from the decreased levels of serum MDA and NO in the NMU-injected rats (groups 4, 5, 6 and 7). The levels of MDA and NO were significantly restored to normal levels in the protective high dose group (group7). The current results were in accordance with other

investigators who had demonstrated that chrysin as a chemoprotective agent clearly normalized and significantly decreased oxidative stress indices such as MDA and NO [60].

In the present study, histopathological examination of the mammary glands of post estrous female rats treated with NMU showed a number of lesions ranging from sloughing of damaged epithelial cells into the duct, simple ductal hyperplasia to benign atypical intraductal hyperplasia. The relevance of these findings is that intraductal hyperplasias are considered to be the precursors of carcinomas both in rodents and humans [61].

The results presented here are consistent with many literature review data. Earlier studies demonstrated that in the experimental model of mammary carcinogenesis in female Sprague-Dawley rats induced by two intrajugular injection of MNU (50 mg/kg), beginning at 44-49 days of age, the accumulation of p53 protein (tumor supressor gene) in cell was demonstrated in 22 from 37 rat mammary tumors. These results indicated that an elevated cellular content of p53 is a common event in invasive palpable mammary tumors induced by NMU in this model system [62]. Also, treatment of rats with chemical carcinogens such as NMU resulted in the development of intraductal hyperplasias, intraductal carcinomas *in situ* (CIS) and adenocarcinomas. Intraductal hyperplasias are believed to be the precursor lesion for both CIS and adenocarcinomas [63]. Additionally, it has been reported that, in NMU-induced mammary tumors in rats, the majority of tumors were identified as *in situ* ductal carcinomas with papillary and/or cribriform characteristics and they added that mutation of the H-ras gene in codon 12 has been implicated in the pathogenesis of NMU-induced tumors [64]. Recent study showed that the histopathological features of tumor developed in rats injected by MNU were anaplastic activity in the lining epithelium associated with cystic dilatation in some of them as well as inflammatory cells infiltration in stromal connective tissue and categorized under adenocarcinoma [28].

In this study, oral administration of phytoestrogen, chrysin, as treatment induced a partial reduction of ductal epithelial cell proliferation. Meanwhile, using chrysin as protection resulted in a marked suppression of this benign epithelial cell proliferation. Using the high dose of chrysin was more effective than using low dose. In support of this, previous study showed chrysin significantly suppressed the abnormal cell proliferation and inhibited tumor formation in DMBA-treated rats [2]. The authors attributed these features to the antioxidant efficacy of chrysin allowing it to neutralize the increase in free radicals caused by DMBA. Also, a recent investigation demonstrated that inhibiting the Akt signal pathway might play a central role in chrysin-induced antimetastatic activities in triple-negative breast cancer (TNBC) cells by regulating matrix metalloproteinase (MMP) and epithelial-mesenchymal transition [65]. More recently, *in vitro* and *in vivo* models have shown that chrysin inhibits cancer growth through various mechanisms including induction of apoptosis, alteration of cell cycle and inhibition of angiogenesis, invasion and metastasis without causing any toxicity and undesirable side effects to normal cells. This broad spectrum of antitumor activity in conjunction with low toxicity underscores the translational value of chrysin in treating breast cancer [66].

CONCLUSION

These results indicated that chrysin was found to be a potent antioxidant which ameliorated the severity of pre-malignant disorders in mammary glands of post estrous female rats, especially when given as protective agent with a high dose (250 mg/kg b.w.). Histopathological observations also correlated with the biochemical parameters and further supported the protective effects of chrysin against NMU-induced benign proliferative lesions in breast tissue.

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