Chronic unpredictable stress (CUS) enhances the carcinogenic potential of 7,12-dimethylbenz(a)anthracene (DMBA) and accelerates the onset of tumor development in Swiss albino mice

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Abstract Social stressors evolving from individual and population interactions produce stress reactions in many organisms (including humans), influencing homeostasis, altering the activity of the immunological system, and thus leading to various pathological states including cancer and their progression. The present study sought to validate the effectiveness of chronic unpredictable stress (CUS) in cancer promotion and to assess oxidative stress outcomes in terms of various in vivo biochemical parameters, oxidative stress markers, DNA damage, and the development of skin tumors in Swiss albino mice. Animals were randomized into different groups based on their exposure to CUS alone, 7,12-dimethylbenz(a)anthracene (DMBA) alone (topical), and DMBA-12-*O*-tetradecanoylphorbol-13-acetate (TPA) (topical) and exposure to CUS prior to DMBA or DMBA-TPA treatments and sacrificed after 16 weeks of treatment. Prior

exposure to CUS significantly increased the pro-oxidant effect of carcinogen, depicted by compromised levels of antioxidants in the circulation and skin, accompanied by enhanced lipid peroxidation, plasma corticosterone, and marker enzymes as compared to DMBA-alone or DMBA-TPA treatments. DNA damage results corroborated the above biochemical outcomes. Also, the development of skin tumors (in terms of their incidence, tumor yield, and tumor burden) in mice in the presence and absence of stress further strongly supported our above biochemical measurements. CUS may work as a promoter of carcinogenesis by enhancing the pro-oxidant potential of carcinogens. Further studies may be aimed at the development of interventions for disease prevention by identifying the relations between psychological factors and DNA damage.

Keywords Antioxidants · Corticosterone · Chronic unpredictable stress · 7,12-Dimethylbenz(a)anthracene · DNA damage · Skin tumors

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Introduction

The influence of stress-inducing conditions on cancer development has been the subject of investigation, both at clinical and experimental levels (Pradhan and Prabhati 1974; Sklar and Anisman 1979). Carcinogenesis may either be inhibited (Steplewski et al. 1985, 1987) or be stimulated (Steplewski et al. 1985; Shavit et al. 1983) depending on the time relationships between the activity of the carcinogenic factor and that of the stressor, as well as the stressor type. Physical and psychological stress stimulates numerous pathways leading to increased production of reactive oxygen species (ROS), adding to the oxidant burden associated with normal aerobic

metabolism, and consequently damaging cellular macromolecules (lipids, proteins, and DNA) (Liu and Mori 1999).

The skin is directly and frequently exposed to sunlight and is always in contact with oxygen, resulting in the production of ROS, implying that the skin is always prone to be attacked by ROS (Garmyn and Degreef 1997). Skin carcinogenesis is divisible into two main stages: initiation and promotion. Initiation involves the conversion of some basal epidermal cells into latent neoplastic cells. Promotion involves selective expansion of initiated cells into benign lesions, which is accomplished by repeated applications of promoter for extended periods of time (Kulesz-Martin 1997). ROS including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and singlet oxygen have been shown to be involved in both initiation and promotion stages of carcinogenesis. Indeed, convincing evidences indicate that ROS acts as an endogenous class of carcinogens by triggering the mutation of the cells (Guyton and Kensler 1993; Feig et al. 1994). Moreover, oxidative stress can stimulate the expansion of mutated cell clones by modulating genes related to proliferation and triggering redox-responsive signaling cascades (Droge 2002; Camhi et al. 1998). The effect of ROS on mutation is dose-dependent and in a dynamic equilibrium with antioxidant defenses and cellular repair systems (Dreher and Junod 1996).

Several chemical carcinogens seem to induce oxidative stress either directly or indirectly through modification of cellular antioxidant defense mechanisms. Dimethylbenz(a)anthracene (DMBA), a member of the polycyclic aromatic hydrocarbons, is present in the environment as a product of incomplete combustion of complex hydrocarbons. It is also present in some amount in cigarette smoke. Being a well-known cytotoxic, carcinogenic, mutagenic, and immunosuppressive agent, it is often used as a tumor initiator in animal models (Miyata et al. 2001; Wijnhoven et al. 2001). DMBA exposure results in a pronounced mutagenic response in several *in vivo* and *in vitro* mutation assay systems (Donovan et al. 2004; Wijnhoven et al. 2001; Arciszewska et al. 1982). Being an indirect carcinogen, DMBA requires metabolic activation by cytochrome P450 enzymes to acquire carcinogenicity (Conney 1982). This multistep activation cascade suggests that a number of intervening conditions or pre-dispositions may affect the extent to which environmental carcinogens cause abnormal cell growth. This study examined the effect of one such mediator-chronic unpredictable stress, to mimic the daily stressful situations experienced by humans.

The present study was undertaken to address the effectiveness of chronic unpredictable stress (CUS) in the induction of tumor promotion and to assess oxidative stress outcomes in terms of various *in vivo* biochemical parameters, oxidative stress markers, and DNA damage in the skin and circulation of Swiss albino mice throughout a two-stage carcinogenesis protocol. Development of skin papillomas by various treatments was also monitored and used as a marker of carcinogenesis.

Materials and methods

Chemicals

DMBA, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and dexamethasone were purchased from Sigma-Aldrich Chemical Company, USA. All the other chemicals were of analytical grade, purchased from Qualigens, Fine Chemicals, and HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Animals

Adult (6–8 weeks) male Swiss albino mice (45 ± 5 g) obtained from Jamia Hamdard University, Delhi, India, were used for the study. They were kept and treated under humane and hygienic conditions (25 ± 5 °C with 12-h day/night cycle). Animals were allowed to acclimatize for 10 days prior to the treatment on standard pellet mice diet (Ashirwad Industries, Chandigarh, India) and fresh drinking water available *ad libitum*. All the experimental protocols adhered to the guidelines of the Animal Welfare Committee of the University as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India) norms.

CUS procedure

The CUS protocol was carried out according to the published procedures (Katz et al. 1981; Willner et al. 1987) with slight modifications as discussed in our earlier studies (Suhail et al. 2011). The CUS sequence consisted of different types of stressors presented randomly, two per day, over a period of 15 days as outlined in Table 1. All procedures were carried out in isolated rooms adjacent to the housing room, requiring minimal handling or transport of the mice. After each stressor, animals were placed in clean cages with fresh bedding and returned to the housing facility. Control animals were left undisturbed until the time of the experiment with the exception of daily cleaning of the cages and weekly weighing.

Experimental design

Mice were randomly divided into seven groups of 12 mice each. The dorsal skin in the interscapular area was shaved with a surgical clipper 2 days before tumor initiation, and animals showing no hair regrowth were used in the experiment.

Group I: animals served as untreated controls.

Group II (stress alone): animals were exposed to CUS for consecutive 15 days just before the termination of the experiment.

Group III (vehicle control): animals received topical application of acetone (200 μ L/mouse) on the shaved back and served as vehicle controls.

Table 1 Chronic unpredictable stress procedure

Days	Stressor	Time
Day 1	1-h restraint stress	08:00 a.m.
	30-min cold room (4 °C)	11: 00 p.m.
Day 2	1-h shaking/crowding	11:00 a.m.
	5-min cold water swim	03:00 p.m.
Day 3	4-h wet bedding	09:00 a.m.
	Lights on overnight	06:00 p.m.
Day 4	3-h high density housing	10:00 a.m.
	5-min warm water swim	06:00 p.m.
Day 5	2-h restraint stress	08:00 a.m.
	30-min cold room (4 °C)	03:00 p.m.
Day 6	6-h isolation	10:00 a.m.
	Lights on overnight	07:00 p.m.
Day 7	5-min cold water swim	09:00 a.m.
	16-h food and water deprivation	03:00 p.m.
Day 8	2-h restraint stress	10:00 a.m.
	1-h shaking/crowding	02:00 p.m.
Day 9	4-h high-density housing	08:00 a.m.
	Lights on overnight	07:00 p.m.
Day 10	4-h wet bedding	09:00 a.m.
	16-h food and water deprivation	04:00 p.m.
Day 11	3-h restraint stress	11:00 a.m.
	2-h isolation	05:00 p.m.
Day 12	5-min cold water swim	08:00 a.m.
	1-h shaking/crowding	11:00 a.m.
Day 13	10-min tail pinch in restrainer	09:00 a.m.
	1-h shaking/crowing	04:00 p.m.
Day 14	2-h restraint stress	11:00 a.m.
	3-h high-density housing	04:00 p.m.
Day 15	24-h food and water deprivation	08:00 a.m.

Group IV (DMBA alone): DMBA at a dose of 300 µg/200 µL acetone/mouse was applied topically to the shaved back once every week for 4 weeks.

Group V (pre-stress DMBA): animals were first exposed to CUS for consecutive 15 days followed by topical DMBA application as in group IV.

Group VI (DMBA-TPA): animals received the same DMBA dose and application as in group IV. One week after the last DMBA application, animals were further exposed to a weekly application of 5 µg TPA/200 µL acetone/mouse to the same site until the end of the experiment.

Group VII (pre-stress DMBA-TPA): animals were first exposed to CUS for consecutive 15 days followed by topical DMBA-TPA application as in group VI.

Papilloma counts in mice of all the above groups were recorded weekly until sacrifice. All the animals were

sacrificed after 16 weeks of treatment. Blood and skin tissue of six mice from each group were collected for biochemical estimations. Remaining six mice from each group were sacrificed, and their blood and skin samples were subjected to DNA damage analysis by alkaline Comet assay.

Preparation of samples and biochemical analysis

The treatment regimen was scheduled in such a way that all the animals were sacrificed on the same day by decapitation, and blood was collected in heparinized tubes. Plasma was obtained by centrifugation at 3000 rpm for 5 min at 4 °C. Level of corticosterone (stress marker); the activities of free radical-metabolizing enzymes viz. superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), and glutathione reductase (GR); levels of malondialdehyde (MDA); reduced glutathione (GSH); and various biochemical markers {glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP), uric acid, and glucose} were examined in the circulation of treated animals and controls.

A weighed portion of skin tissue was homogenized in chilled 0.1 M phosphate buffer pH 7.4 using glass teflon homogenizer and volume adjusted to give 10 % w/v homogenate. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 °C. Clear supernatant thus obtained was subjected to the analysis of SOD, CAT, GST, GR, GSH, and MDA. All the samples were labeled properly and kept at −70 °C until analysis.

Estimation of corticosterone level by HPLC using UV detector

An HPLC/UV system (Waters, USA) was used for quantification of plasma corticosterone according to Woodward and Emery (1987) with minor modifications using dexamethasone as an internal standard. Briefly, 500 µL of plasma containing known quantity of dexamethasone was extracted with 5 mL of dichloromethane. The dichloromethane extract was evaporated to dryness and dissolved in 100 µL of mobile phase. Twenty microliters of extract was injected into HPLC system for quantification. Mobile phase consisted of methanol/water (70:30) at a flow rate of 1.2 mL/min, and corticosterone was detected at 250 nm using UV detector (Model 2487, Waters, USA). The chromatogram was recorded and analyzed with Breeze software (Version 3.2). Results were expressed as nanogram corticosterone per milliliter of plasma.

Estimation of enzymatic and non-enzymatic antioxidants

Determination of SOD activity SOD activity in the plasma and skin homogenate was assayed by the method of Marklund and Marklund based on the ability of SOD to inhibit the auto-

oxidation of pyrogallol at 412 nm (Marklund and Marklund 1974). Results were expressed as enzyme units per milligram protein. One enzyme unit is defined as the amount of enzyme required to cause 50 % inhibition of the rate of pyrogallol auto-oxidation.

Determination of CAT activity CAT activity was determined by following the rate of decomposition of H_2O_2 at 240 nm (Claiborne 1985). Results were expressed as enzyme units per milligram protein. One unit is defined as nanomoles of H_2O_2 consumed per minute per milligram protein.

Determination of GST activity GST activity in the plasma and skin homogenate was assayed by the method of Habig et al. using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig et al. 1974). Calculation was made using millimolar extinction coefficient value of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$, and results were expressed as enzyme units per milligram protein. One enzyme unit is defined as nanomoles CDNB conjugate formed per minute per milligram protein.

Determination of GR activity GR activity was assessed according to the method of Carlberg and Mannervik (1975). This enzyme catalyzes the NADPH-dependent reduction of glutathione disulfide to glutathione. Oxidation of NADPH was followed spectrophotometrically at 340 nm after 30-s intervals for 3 min. Results were expressed as enzyme units per milligram protein. One enzyme unit is defined as nanomoles of NADPH oxidized per minute per milligram protein.

Estimation of total reduced glutathione The level of GSH was determined by the method of Jollow et al. (1974) by using sulfosalicylic acid and 5-5'-dithiobis-2-nitrobenzoic acid (DTNB). Results were expressed as micromoles GSH per milligram protein.

TBARS determination Lipid peroxidation was assessed by determining MDA (a thiobarbituric acid-reactive species (TBARS)) spectrophotometrically following the thiobarbituric acid (TBA) test for the formation of TBARS during an acid-heating reaction (Buege and Aust 1978). The pink chromogen formed by MDA-TBA complex was detected at 535 nm and quantified using an extinction coefficient of $1.56 \times 10^5 \text{ M/cm}$.

Estimation of various other biochemical parameters

Activity of GOT and GPT

Commercial kits (Span Diagnostics Ltd India) were used for the measurement of the transaminases (GOT and GPT) in the plasma.

ALP activity

ALP activity in the plasma was measured using p-nitrophenyl phosphate (pNPP) as the substrate (Shah et al. 1979).

Levels of uric acid and glucose

Levels of uric acid and glucose in the plasma were measured by using commercial kits (Span Diagnostic Ltd India).

Estimation of protein content

The protein content in the plasma and skin samples was estimated according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Preparation of cells for Comet assay

Lymphocyte isolation

Immediately after each sacrifice, the heparinized blood was suitably diluted in PBS (Ca^{++} and Mg^{++} free). Lymphocytes were isolated using Histopaque 1077. The isolated cells were finally suspended in RPMI 1640.

Isolation of skin cells

A small portion of skin was minced thoroughly with a blade, and the tissue mass was suspended in ice-cold solution of HBSS. After centrifugation, cells were suspended in RPMI 1640.

DNA damage studies by single-cell gel electrophoresis (Comet assay)

Comet assay was performed under alkaline conditions essentially according to the procedure of Singh et al. (1988) with slight modifications as discussed in our earlier studies (Muqbil et al. 2006). The parameter taken to assess cellular DNA damage was tail length (migration of DNA from the nucleus, μm) and was automatically generated by Comet 5.5 image analysis system.

Statistical analysis

All the data are expressed as mean $\pm$ standard error of mean (SEM) and analyzed by one-way ANOVA for differences among controls and treatment groups. *P* values less than 0.05 are considered statistically significant.

Results

Effect of CUS and topical treatments of DMBA and DMBA-TPA on the levels of plasma corticosterone (stress marker) of mice

Plasma corticosterone levels were found to be increased significantly ($P<0.01$) in the mice exposed to CUS alone, DMBA alone, and DMBA-TPA as compared to untreated and vehicle control groups. Exposure of stressed animals to DMBA or DMBA-TPA caused a further significant ($P<0.001$) elevation in these levels, with almost comparable enhancements in both pre-stress DMBA- and DMBA-TPA-treated groups (Fig. 1).

Effect of CUS and topical treatments of DMBA and DMBA-TPA on the in vivo antioxidant status and various other biochemical parameters in the skin and circulation of mice

Exposure of mice to CUS-alone, DMBA-alone, and DMBA-TPA treatments caused significantly compromised activities of all the antioxidant enzymes (SOD, CAT, GST, and GR) in the plasma ($P<0.01$) and skin ($P<0.05$) of animals (Tables 2 and 3) as compared to untreated and vehicle controls. Diminished levels of GSH (Figs. 2 and 3) accompanied by a significant increase in MDA levels (Figs. 4 and 5) were observed in the plasma ($P<0.01$) and skin ($P<0.05$) of mice exposed to CUS-alone, DMBA-alone, and DMBA-TPA treatments as compared to untreated and vehicle controls. Circulatory levels of uric acid and

glucose were also found to be decreased in the stress-alone-, DMBA-alone-, and DMBA-TPA-treated mice accompanied by a significant increase ($P<0.01$) in the levels of marker enzymes GOT, GPT, and ALP when compared to untreated and vehicle controls (Table 4). Exposure to CUS prior to DMBA or DMBA-TPA application caused a similar but more pronounced pattern in all the above biochemical parameters. However, the levels of all the above biochemical parameters in the pre-stress DMBA group were found almost comparable to the DMBA-TPA-treated group.

Briefly, the observed alterations in the biochemical parameters during various treatments in comparison to controls were as follows:

Pre-stress DMBA-TPA > DMBA-TPA > pre-stress DMBA > DMBA alone > CUS alone

Effect of CUS and topical treatments of DMBA and DMBA-TPA on the DNA of lymphocytes and skin cells of mice

Significant DNA damage ($P<0.05$) was observed in the lymphocytes (9.6 ± 0.05 vs. 2.2 ± 0.06 ; 9.6 ± 0.05 vs. 2.1 ± 0.04) and skin cells (9.41 ± 0.07 vs. 2.3 ± 0.07 ; 9.41 ± 0.07 vs. 2.4 ± 0.06) of CUS-exposed mice as compared to untreated and vehicle control groups. Treatment with DMBA alone (28.4 ± 0.1 in lymphocytes; 30.1 ± 0.32 in skin cells) and DMBA-TPA (40.24 ± 0.15 in lymphocytes; 43.2 ± 0.26 in skin cells) increased the extent of damage. Pre-exposure

Fig. 1 Effect of CUS, DMBA-alone (topical), and DMBA-TPA (topical) treatments on the plasma corticosterone levels. Each value represents mean $\pm$ SEM of six animals. * $P<0.01$ when compared to untreated and vehicle control groups. ** $P<0.001$ when compared to CUS-alone- and DMBA-alone-treated groups. # $P<0.001$ when compared to DMBA-TPA-treated group

Table 2 Circulatory levels of various free radical-metabolizing enzymes in mice exposed to CUS alone, DMBA alone (topical), and DMBA-TPA (topical) and exposure to CUS prior to DMBA or DMBA-TPA application

Groups	SOD (units/mg protein)	CAT (units/mg protein)	GST (units/mg protein)	GR (units/mg protein)
Untreated control	27.32±0.41	0.59±0.01	0.48±0.008	3.98±0.06
CUS alone	20.51*±0.33	0.43*±0.01	0.41*±0.008	2.54*±0.09
Vehicle control	26.52±0.91	0.57±0.01	0.47±0.008	3.99±0.13
DMBA alone	15.84*±0.32	0.38*±0.007	0.34*±0.005	1.69*±0.05
Pre-stress DMBA	11.47**±0.11	0.23**±0.009	0.25**±0.006	0.98**±0.006
DMBA-TPA	10.34**±0.12	0.16**±0.003	0.17**±0.002	0.85**±0.006
Pre-stress DMBA-TPA	8.85#±0.15	0.12#±0.002	0.14#±0.003	0.48#±0.05

Each value represents mean±SEM of six animals

* $P<0.01$ when compared to untreated and vehicle control groups; ** $P<0.001$ when compared to CUS-alone and DMBA-alone groups; # $P<0.01$ when compared to DMBA-TPA group

of mice to CUS further enhanced the DNA damaging potential of DMBA significantly ($P<0.01$) as is evident by increased tail lengths in the pre-stress DMBA (38.81 ± 0.29 in lymphocytes; 41.56 ± 0.34 in skin cells) and pre-stress DMBA-TPA (49.4 ± 0.22 in lymphocytes; 52.8 ± 0.33 in skin cells) groups as compared to CUS-alone-, DMBA-alone-, or DMBA-TPA-treated groups. The extent of DNA damage observed in the lymphocytes (38.81 ± 0.29 vs. 40.24 ± 0.15) and skin cells (41.56 ± 0.34 vs. 43.2 ± 0.26) of pre-stress DMBA group was found almost comparable to the DMBA-TPA-treated group (Figs. 6 and 7).

A comparison of tail lengths of the lymphocytes and skin cells revealed that skin cells, being the target organ of the carcinogen, exhibited the highest level of DNA damage on DMBA-alone, pre-stress DMBA, DMBA-TPA, and pre-stress DMBA-TPA treatments. The comparative DNA damage observed in the lymphocytes and skin cells by various treatments with respect to controls was comparable as observed for biochemical parameters and can be summarized as follows:

Pre-stress DMBA-TPA > DMBA-TPA > pre-stress DMBA > DMBA alone > CUS alone

Effect of CUS and topical treatments of DMBA and DMBA-TPA on the development of skin papillomas in Swiss albino mice

The untreated, vehicle control and CUS-alone-treated groups did not show any papillomas during the entire 16 weeks of observation (Fig. 8). Papillomas started to appear in mice receiving topical application of DMBA from 12th week and DMBA-TPA from week 7 onward, and the papillomas continued to increase until 16 weeks. The tumor incidence in the DMBA-alone-treated mice reached 58.3 % by the end of the experiment (16 weeks). Pre-exposure to CUS along with DMBA application increased the tumor incidence to 75 % and reduced the time of appearance of these papillomas. The cumulative number of papillomas in these mice was recorded as 19 which was almost comparable to DMBA-TPA group

Table 3 Levels of free radical scavenging enzymes in the skin of mice exposed to CUS alone, DMBA alone (topical), and DMBA-TPA (topical) and exposure to CUS prior to DMBA or DMBA-TPA application

Groups	SOD (units/mg protein)	CAT (units/mg protein)	GST (units/mg protein)	GR (units/mg protein)
Untreated control	107.4±2.3	10.7±0.3	68.6±1.6	18.0±0.1
CUS alone	98.1*±3.0	9.1*±0.1	63.7*±1.0	16.9*±0.2
Vehicle control	106.6±1.6	10.8±0.2	68.2±1.2	18.1±0.1
DMBA alone	88.0*±1.5	7.7*±0.1	59.2*±0.7	13.2*±0.2
Pre-stress DMBA	75.1**±1.6	6.4**±0.1	50.4**±1.0	11.1**±0.2
DMBA-TPA	68.3**±1.4	5.4**±0.1	47.1**±0.8	9.9**±0.1
Pre-stress DMBA-TPA	54.4#±0.5	3.4#±0.1	33.2#±1.0	7.2#±0.04

Each value represents mean±SEM of six animals

* $P<0.05$ when compared to untreated and vehicle control groups; ** $P<0.01$ when compared to CUS-alone and DMBA-alone groups; # $P<0.01$ when compared to DMBA-TPA group

Fig. 2 Effect of CUS, DMBA-alone (topical), and DMBA-TPA (topical) treatments on the circulatory levels of GSH. Each value represents mean±SEM of six animals. * $P<0.01$ when compared to untreated and vehicle control groups. ** $P<0.001$ when compared to CUS-alone and DMBA-alone-treated groups. # $P<0.001$ when compared to DMBA-TPA-treated group

(22). Pre-stress DMBA- and DMBA-TPA-treated groups exhibited almost comparable values of tumor yield (1.58 vs. 1.83) and tumor burden (2.1 vs. 2.2). Pre-exposure to CUS along with DMBA-TPA treatment increased the tumor incidence to 100 % by the end of the experiment, and the cumulative number of papillomas was found to be 34. Tumor yield and tumor burden were also increased to 2.83 (Table 5).

The development of papillomas (in terms of their incidence, tumor yield, and tumor burden) in mice during various treatments can be summarized as follows:

Pre-stress DMBA-TPA > DMBA-TPA > pre-stress DMBA > DMBA alone > CUS alone

Discussion

Oxidative stress, an imbalance in oxidant and antioxidant status that has been repeatedly addressed as an indicator of indirect genotoxicity, is considerably increased in certain pathological conditions including cancer.

Stress has a significant impact on some of the major drug-metabolizing enzyme systems. It alters both constitutive and induced cytochrome P450 expression. There is growing evidence that stress and the resulting changes in behavior, immune status, and stress hormone profiles (particularly catecholamines) significantly influence tumor initiation, progression, and metastasis (Powell et al. 2013). Following stress,

Fig. 3 Effect of CUS, DMBA-alone (topical), and DMBA-TPA (topical) treatments on the levels of GSH in the skin of mice. Each value represents mean±SEM of six animals. * $P<0.05$ when compared to untreated and vehicle control groups. ** $P<0.001$ when compared to CUS-alone and DMBA-alone-treated groups. # $P<0.001$ when compared to DMBA-TPA-treated group

Fig. 4 Effect of CUS, DMBA-alone (topical), and DMBA-TPA (topical) treatments on the circulatory levels of MDA. Each value represents mean $\pm$ SEM of six animals. * P <0.01 when compared to untreated and vehicle control groups. ** P <0.001 when compared to CUS-alone- and DMBA-alone-treated groups. # P <0.001 when compared to DMBA-TPA-treated group

various events appear to hold determinant roles in CYP regulation. These include activation of the hypothalamic–pituitary–adrenal (HPA) axis with the consequent release of glucocorticoids and epinephrine from adrenal glands, oxidative stress, increased release of cytokines/NF- κ B, and the altered secretion of hormones, such as GH, thyroid hormones, and insulin (Dvorak and Pavek 2010).

Body's neuroendocrine response has been shown to directly alter important cellular processes that help protect against the formation of cancer, such as DNA repair and the regulation of cell growth (Antoni et al. 2006). However, exposure to stress results in enhanced release of catecholamines and glucocorticoids due to the activation of sympathoadrenal and HPA axes (Joels et al. 2007), and chronic exposure to these mediators of the stress response has damaging effects, in which oxidative stress plays a major role (Simmons et al.

1991; Perez-Nievas et al. 2007). The present study showed a significant rise of the plasma corticosterone level in the animals treated with DMBA alone or DMBA-TPA as compared to controls or CUS alone, and pre-exposure to CUS caused a further elevation in these levels suggesting the possible role of stress in the promotion of carcinogenesis. Neurodegeneration caused by glucocorticoid stress hormones under stress conditions may be linked to an increase in the generation of ROS, which can directly damage cellular proteins, DNA, and lipids (McIntosh and Sapolsky 1996). Acute stress has immunoenhancing effects, while chronic stress suppresses or dysregulates innate and adaptive immune responses through mechanisms involving suppression of leukocyte numbers, trafficking, or changing the type 1–type 2 cytokine balance (Dhabhar and McEwen 2006; Glaser and Kiecolt-Glaser 2005; Ader 2006).

Fig. 5 Effect of CUS, DMBA-alone (topical), and DMBA-TPA (topical) treatments on the levels of MDA in the skin of mice. Each value represents mean $\pm$ SEM of six animals. * P <0.05 when compared to untreated and vehicle control groups. ** P <0.001 when compared to CUS-alone- and DMBA-alone-treated groups. # P <0.001 when compared to DMBA-TPA-treated group

Table 4 Circulatory levels of various biochemical parameters in mice exposed to CUS alone, DMBA alone (topical), and DMBA-TPA (topical) and exposure to CUS prior to DMBA or DMBA-TPA application

Groups	SGPT (IU/L)	SGOT (IU/L)	ALP (mg/mL)	Glucose (mg/dL)	Uric acid (mg/dL)
Untreated control	27.6±1.71	28.8±1.03	0.96±0.04	126.8±3.65	4.71±0.09
CUS alone	59.2*±1.03	63.4*±1.02	1.65*±0.02	106.8*±1.42	3.12*±0.04
Vehicle control	28.0±1.74	28.4±1.0	0.95±0.02	126.0±2.63	4.79±0.07
DMBA alone	74.4*±2.17	79.4*±1.34	2.71*±0.09	84.0*±2.85	2.04*±0.02
Pre-stress DMBA	88.4**±1.23	91.8**±1.52	3.80**±0.07	65.2**±1.31	1.05**±0.008
DMBA-TPA	94.2**±2.34	97.6**±1.57	4.01**±0.06	57.8**±1.84	0.93**±0.006
Pre-stress DMBA-TPA	107.2#±1.80	112.6#±1.73	4.71#±0.04	37.2#±0.79	0.39#±0.009

Each value represents mean±SEM of six animals

* $P<0.01$ when compared to untreated and vehicle control groups; ** $P<0.01$ when compared to CUS-alone and DMBA-alone groups; # $P<0.01$ when compared to DMBA-TPA group

Chronic stress increases the susceptibility to skin cancer by suppressing type 1 cytokines and protective T cells with an increase in suppressor T cell function (Saul et al. 2005). Stress-induced changes in rodent blood leukocyte numbers are characterized by a significant decrease in the numbers and percentages of lymphocytes and monocytes and an increase in the numbers and percentages of neutrophils (Dhabhar et al. 1994, 1995).

Heat shock proteins (Hsp) play a critical role in the body's self-defense under a variety of stresses, including heat shock, oxidative stress, radiation, and wounds, through the regulation of folding and functions of relevant cellular proteins (Feder and Hofmann 1999; Morano and Thiele 1999). Exercise is associated with transient elevations of Hsp expression, body temperature, hormones, and oxidative stress, which may reduce inflammatory mediators (Locke et al. 1990). Hsps not

Fig. 6 Single-cell gel electrophoresis of mice lymphocytes showing Comets ($\times 100$) after treatment with CUS, DMBA-alone (topical), and DMBA-TPA (topical). **a** Untreated control. **b** CUS treated. **c** Vehicle control. **d** DMBA alone. **e** Pre-stress DMBA. **f** DMBA-TPA. **g** Pre-stress DMBA-TPA

only protect cells and organisms against proteotoxic stresses, but also are also critical in normal functioning of several cellular processes (Noble et al. 2008). Several signaling pathways involving Hsps are implicated in the regulation of immune and inflammatory systems (Jones et al. 2011; Shields et al. 2012). Though there is some controversy regarding their exact role (Eden et al. 2012), Hsps may not only activate the immune response (Radons and Multhoff 2005) but also dampen the inflammatory pathways (Jones et al. 2011). Chronic stress inhibits HSP70 activity in rat intestinal epithelial layer that is associated with intestinal epithelial barrier dysfunction (Yang et al. 2009).

During the multistage carcinogenesis process, the activities of antioxidant enzymes tend to decrease which leads to a pro-oxidant state of the cell, facilitating tumor promotion and progression (Oberley and Oberley 1993). To cope with the generation of ROS, GSH acts as an endogenous non-enzymatic biological antioxidant. In addition to its well-known antioxidant properties, GSH is emerging as a regulator of the expression of proteins (or molecules) involved in apoptosis (Friesen et al. 2004; Armstrong et al. 2002). A reduction in GSH level due to its utilization in the conjugation reaction is quite likely to affect several pathways and may further lead to a state of

oxidative stress. This is evident in the present study from the diminished levels of circulatory GSH in the DMBA-alone- and DMBA-TPA-treated groups. Furthermore, because oxidative stress plays a major role in the induced adaptive expression of antioxidants, malignant cells are often characterized by differential expression in the levels of a number of antioxidant enzymes (Teoh et al. 2009; Pompella et al. 2007). In the present study, it was noted that the treatment to the initiator and promoter decreased the enzyme activities of not only SOD, but also other key antioxidant enzymes such as CAT, GST, and GR and the plasma levels of uric acid and glucose in stress-alone-, DMBA-alone-, and DMBA-TPA-treated groups which further confirms the state of oxidative stress. GSH in conjunction with GST detoxifies reactive intermediate species generated during DMBA metabolism, thereby enhancing resistance against oxidative stress (Locigno and Castronovo 2001). The decreased levels of GSH along with decreased activity of GST might contribute significantly in enhancing DMBA-TPA-induced carcinogenesis due to its reduced clearance from the system. Recent studies from our lab have documented oxidative damage to the liver and kidney of mice by prior exposure to CUS. In these organs, it was shown that CUS decreased the activities of SOD, CAT, GST, and GR

Fig. 7 Single-cell gel electrophoresis of mice skin cells showing Comets ($\times 100$) after treatment with CUS, DMBA alone (topical), and DMBA-TPA (topical). **a** Untreated control. **b** CUS treated. **c** Vehicle control. **d** DMBA alone. **e** Pre-stress DMBA. **f** DMBA-TPA. **g** Pre-stress DMBA-TPA

Fig. 8 Effect of CUS and topical treatments of DMBA and DMBA-TPA on the development of skin papillomas in Swiss albino mice. All the mice are approximately the same size (45 ± 5 g). **a** Untreated control. **b** CUS treated. **c** Vehicle control. **d** DMBA alone. **e** Pre-stress DMBA. **f** DMBA-TPA. **g** Pre-stress DMBA-TPA

and markedly reduced the degree of protection offered by the GSH system, thus aggravating oxidative damage (Suhail et al. 2011). The present study also demonstrates that pre-exposure to CUS further decreased the levels of all the above antioxidants by deranging the oxidative status of animals either by enhanced production or by decreased clearance of free radicals. Under such compromised state, the infusion of DMBA or DMBA-TPA further aggravated the situation which is observed in terms of significantly decreased activities of free radical scavenging enzymes SOD, CAT, GR, and GST in

comparison to CUS-alone-, DMBA-alone-, and DMBA-TPA-treated groups.

Insufficiency in the antioxidant systems and consequent rise in free radical-mediated processes enhance the rate of lipid peroxidation as evidenced by the increased levels of MDA in the plasma and skin tissue of DMBA- and DMBA-TPA-treated animals in the present study. The levels were further significantly increased when the animals were subjected to CUS prior to DMBA or DMBA-TPA treatment as compared to stress-alone-, DMBA-alone-, and DMBA-TPA-treated

Table 5 Different parameters observed during CUS, DMBA-alone (topical), and DMBA-TPA (topical) treatments on the development of skin papillomas in Swiss albino mice

Groups (12 mice/group)	Cumulative number of tumors	Tumor incidence (%)	Tumor yield	Tumor burden
Untreated control	0	0	0	0
CUS alone	0	0	0	0
Vehicle control	0	0	0	0
DMBA alone	11	58.33	0.91	1.57
Pre-stress DMBA	19	75.0	1.58	2.1
DMBA-TPA	22	83.33	1.83	2.20
Pre-stress DMBA-TPA	34	100	2.83	2.83

groups, suggesting that already existing oxidative stress facilitates the effect of DMBA on lipid peroxidation and a cumulative effect is observed. This may be due to the generation of OH $\cdot$ radical through the metal-catalyzed Haber–Weiss reaction during the course of stress which plays a direct role in stress-induced oxidative damage (Das 2002). MDA is one of the most intensively studied lipid hydroperoxide-derived bi-functional electrophiles that forms adducts with nucleic acid bases at physiological pH, causing damage to the genetic material (Stone et al. 1990). Increased lipid peroxidation accompanied with compromised host antioxidant status may result in accumulation of free radicals, and as a consequence, the levels of marker enzymes, GOT, GPT, and ALP, in circulation were increased significantly following DMBA or DMBA-TPA treatments depicting an effect on liver cell integrity.

Topical treatment with DMBA alone leads to the formation of skin papillomas in mice as observed in the present study; however, application of TPA following DMBA treatment decreased the time of appearance (from 12 to 7 weeks) of the papillomas accompanied by their increased incidence. TPA-induced tumor promotion is accompanied by alterations in various cellular processes including cell growth, cell differentiation, and cell death (Kim et al. 2005; La et al. 1999). However, pre-exposure to CUS followed by DMBA or DMBA-TPA infusion resulted in significantly increased oxidative stress and enhanced the incidence of skin papillomas relative to their non-stressed counterparts, with comparable effects being observed in the pre-stress DMBA- and DMBA-TPA-treated groups, suggesting that the role of stress in enhancing the carcinogenic potential of DMBA is comparable to TPA. Further, a concordance exists between endocrine changes induced by large tumor burdens, consisting of increased corticosterone and growth hormone (Besedovsky et al. 2000). Thus, the present study further confirms that stress accelerates the onset of tumor formation produced in response to DMBA and significantly influences the type and location of tumors as reported earlier (Flint et al. 2011).

DNA damage has been implicated in the pathogenesis of various diseases including cancer. Stress affects DNA integrity by production of ROS resulting in oxidative stress and increased lipid peroxidation (Adachi et al. 1993; Guerin 1978). Previous studies from our laboratory have already reported that chronic restraint stress not only causes increased lipid peroxidation and compromised antioxidant status but also enhances the pro-oxidant effect of DMBA in rats (Muqbil et al. 2006; Muqbil and Banu 2006). The results of the present study showed that CUS caused significant damage to the DNA of lymphocytes and skin cells of mice as compared to controls by incurring DNA damage within the cell either by altering the ability of cells to repair DNA or by causing oxidative stress as enhanced lipid peroxidation is observed in the skin and plasma of stressed animals. The DNA damage was further enhanced in mice treated with DMBA or DMBA-TPA

following exposure to stress as compared to those treated with DMBA alone or DMBA-TPA. ROS formed during DMBA metabolism produce deleterious effects by initiating lipid peroxidation directly or indirectly by acting as second messengers for the primary free radicals, and the toxic metabolites of DMBA bind to adenine residues of DNA causing damage (Guerin 1978). The DNA breakage may be the result of the generation of hydroxyl radicals and other ROS in situ. These concurrent interactions of initiating (adducts) and promoting (oxidized bases) processes may be key to complete carcinogenesis, and exposure to stress plays an important role from the very early stages of carcinogenesis right at the basic cellular level by causing oxidative stress and/or increasing the damaging potential of a carcinogen.

Alterations of various in vivo biochemical parameters and the extent of DNA damage in the pre-stress DMBA-treated group were found almost comparable to the DMBA-TPA-treated group, suggesting that exposure to stress prior to the carcinogen treatment may work as a promoter of carcinogenesis. Thus, in the absence of any promoter, stress alone can enhance the carcinogenic potential of a carcinogen.

In conclusion, the results of our study clearly indicate the influence of stress events, produced by various unpredictable psycho-social problems on carcinogenesis. Further studies may be aimed at the development of interventions for disease prevention by identifying the relations between psychological factors and DNA damage.

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